THE EFFECT OF REPEATED EXPOSURE TO BRIGHT LIGHT AT THE SAME TIME OF THE NIGHT ON SALIVARY MELATONIN IN HUMANS

by

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Abstract

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Conditioning of salivary melatonin suppression by light was attempted in 15 healthy male and female undergraduates. Enhancement of melatonin suppression (CR) after repeated exposure to bright light at the same time of night was expected to occur due to the learned association between the time of exposure (CS) and light (US). On 10 training nights, 45 min of bright light (experimental group, n=8) or 45 min of dim light (control group, n=7) were presented 2 hr past bedtime. Before and after training, all subjects collected multiple melatonin samples under conditions of 45 min of bright light and, separately, 4.5 min of bright light presented 2 hr past bedtime. Melatonin was suppressed by 45 min of bright light, $F(2, 32) = 18.3$, $p < .0005$, but there was no difference between groups on either pretest or posttest. Neither group evidenced melatonin suppression by 4.5 min of bright light on pretest or posttest. Thus, the effect of conditioning on the posttest melatonin values was absent in the experimental group. While future studies measuring circadian phase shift may help clarify the possibility of conditioning of the human circadian system's response to light, the present results suggest that the usefulness of conditioning in the bright light treatment of circadian rhythm sleep disorders may be limited at best.

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Glossary

Advance (or phase-advance); **to advance** – a shift of the circadian cycle to an earlier environmental time, so that the onset of the next phase of the cycle occurs earlier according to the external clock; to effect such a shift.

Circadian rhythm (or circadian cycle) – endogenous rhythmic variability in any biological or behavioral variable that follows an approximately 24-hour cycle.

Circadian rhythm sleep disorders – a category of sleep disorders in which the patient's endogenous sleep-wake cycle does not entrain to the environmental time by a normal bedtime and uptime routine.

Circadian time – subjective time; a specified time point in the internally generated near-24-hour circadian cycle.

Delay (or phase-delay); to delay – a shift of the circadian cycle to a later environmental time, so that the onset of the next phase of the cycle occurs later according to the external clock; to effect such a shift.

Endogenous – originating from the organism.

Entrainment; to entrain – an adjustment of internal circadian rhythms so as to synchronize them with the external schedule; to effect such an adjustment.

Exogenous – originating from the environment.

Free run; to free-run – a condition, lasting for any number of days, in which all exogenous time cues are absent and the endogenous circadian rhythms are not synchronized with the external time; to live in such a condition.

Phase (or circadian phase) – the relative position of the highest and the lowest points within the circadian cycle.

Phase-dependent – a qualifier that indicates that the effect of a zeitgeber on the endogenous circadian cycle varies as a function of the endogenous circadian phase.

Phase response curve – a curve that describes the effect of a zeitgeber on the endogenous circadian cycle as a function of the endogenous circadian phase.

Phase shift; to phase-shift – a shift of the circadian cycle to either an earlier (advance) or a later (delay) environmental time; to effect such a shift.

tau – the length of a circadian cycle, in hours.

T-cycle – the length of the exogenous daily cycle, in hours, to which the organism is entrained and which may be varied experimentally, e.g., a 25-hour day.

Zeitgeber – an environmental cue that serves to synchronize internal circadian rhythms with the external schedule.

List of Abbreviations

12:12 LD – an exogenous daily light cycle which has 12 hours of light and 12 hours of darkness.

ANOVA – analysis of variance

BT – bedtime

CR – conditioned response

CS – conditioned stimulus

CT – circadian time

LD – light-dark

IGL – intergeniculate leaflet

PRC – the phase response curve

preNBL – pretest nocturnal melatonin baseline

postNBL – posttest nocturnal melatonin baseline

PVT – paraventricular nucleus

SCN – suprachiasmatic nucleus

sDLMO – salivary dim light melatonin onset

T24 or T25 – T-cycles of 24 hours or 25 hours, respectively

UR – unconditioned response

US – unconditioned stimulus

UT – uptime

The Effect of Repeated Exposure to Bright Light at the Same Time of the Night on Salivary Melatonin in Humans

Light is the most powerful zeitgeber in mammals, including humans (Boivin, Duffy, Kronauer, & Czeisler, 1996; Czeisler et al., 1986). For instance, the circadian cycle of wheel-running activity in rodents is shifted by light pulse properly timed to the endogenous phase (Takahashi & Zatz, 1982). Similarly, exposure to bright light at the appropriate time shifts the sleep-wake cycle in humans and is used to treat circadian rhythm sleep disorders (Campbell, Dawson & Anderson, 1993; Duffy, Kronauer, Czeisler, 1996; Yang & Spielman, 1999). However, patients often find bright light exposure as a treatment to be too time consuming, which creates a compliance problem (Yang & Spielman, 1999). The results of several animal studies suggest that the effect of light on the mammalian circadian system may be modulated by associative learning (Amir & Stewart, 1998a; 1998b; 1996; Arvanitogiannis & Amir, 2001; 1999). Using rats as subjects in a classical conditioning procedure, Amir and colleagues (Amir & Stewart, 1998a; 1998b; 1996; Arvanitogiannis & Amir, 2001; 1999) found that a short light pulse or an arbitrary non-photic stimulus can act similarly to prolonged light exposure on such indices of the mammalian circadian system as rest-activity, melatonin and body temperature cycles as well as protein Fos expression. Modulating the circadian system's response to light using associative learning procedures may have significant practical implications for the treatment of circadian rhythm sleep disorders in humans. If associative learning can be used to reduce the length of light exposure while maintaining the therapeutic effect, it may lead to the modification of the treatment protocol that could substantially improve compliance. The possibility of using classical conditioning to modify the circadian system's response to light has not been tested in humans. Therefore, the aim of the present study is to utilize a procedure conceptually similar to the paradigms used by Amir and associates in order to test whether in human subjects associative learning can produce an effect of short bright light exposure on the circadian system that is comparable to the effect of prolonged light exposure.

The Effect of Light on the Mammalian Circadian System

The effect of light on the mammalian circadian system is exerted through its influence on the suprachiasmatic nucleus (SCN) of the hypothalamus (Meijer et al., 1996; Murphy & Campbell, 1996). The SCN receives information about ambient light from the retina directly through the retinohypothalamic tract and indirectly through the geniculo-hypothalamic tract (Gillette & Tischkau, 1999; Meijer et al., 1996; Moore, Weis, & Moga, 2000; Reuss, 1996). The environmental light-dark cycle entrains circadian rhythms of the SCN and its efferent structures (Gillette & Tischkau, 1999; Meijer et al., 1996; Reuss, 1996).

The effect of light on the circadian system depends on the circadian time (CT, which is the subjective time) when the light exposure takes place within the circadian cycle. Exposure to light during the subjective evening and the first half of the night delays the onset of the cycle, whereas exposure to light during the second half of the subjective night and the morning advances the onset of the cycle in both nocturnal (rodents; Takahashi & Zatz, 1982) and diurnal (humans; Czeisler, 1995) organisms. Light exposure during the subjective day has little effect on the phase of circadian rhythms (Meijer at al., 1996; Murphy & Campbell, 1996; Czeisler, 1995; Takahashi & Zatz, 1982). This relationship between the CT of light exposure and the degree and direction of the resultant phase shift has been termed a phase-response curve (PRC; Czeisler et al., 1981; Meijer at al., 1996; Murphy & Campbell, 1996).

The phase-shifting effect of light on the circadian system is evident on both behavioral and physiological measures. Rest-activity cycle, as seen in the locomotor activity pattern in rodents (Amir & Stewart, 1996; Laakso, Leinonen, Joutsiniemi, Porkka-Heiskanen, & Stenberg, 1992; Takahashi & Zatz, 1982) and in the sleep-wake pattern in humans (Czeisler et al., 1981; Honma, Honma, & Wada, 1987), has been the primary behavioral measure. The rhythms of body temperature and melatonin secretion have been frequently used in human and animal studies as physiological measures (Amir & Stewart, 1996; Arendt & Broadway, 1987; Czeisler et al., 1989; Honma et al., 1987; Laakso et al., 1992; Maeda & Lincoln, 1990; Shanahan, Kronauer, Duffy, Williams, & Czeisler, 1999; Shanahan, Zeitzer & Czeisler, 1997).

In the absence of environmental light cues, mammalian rest-activity cycle, melatonin secretion and body temperature begin to free-run, exhibiting circadian rhythmicity (humans: Honma et al., 1987; Krauchi & Wirz-Justice, 1994; Murphy & Campbell, 1996; Lewy et al., 1995; Middleton, Arendt, & Stone 1996; Wever, Polasek, & Wildbruber, 1983; rodents: de Groot & Rusak, 2000; Fukuhara, Aguzzi, Bullock, & Tosini, 2005; Liu & Borjigin, 2005; Scheer, Pirovano, Van Someren, & Buijs, 2005). As during free run the SCN regulates circadian rhythms of rest-activity, melatonin secretion and body temperature, entrainment and phase-shifting of these variables by environmental light are thought to occur via the SCN (Arendt & Broadway, 1987; Fukuhara et al., 2005; Gillette & Tischkau, 1999; Lewy et al., 1995; Maeda & Lincoln, 1990; Murhpy & Campbell, 1996; Reuss, 1996; Scheer et al., 2005; Shanahan et al., 1997, 1999).

In addition to entrainment and phase-shifting, light exposure exerts acute effects on melatonin secretion and body temperature. Exposure to bright light during the sensitive portion of the PRC suppresses melatonin secretion in animals and humans (Arendt, 1995; Arendt & Broadway, 1987; Brainard, Richardson, Petterborg, & Reiter, 1982; Lewy et al., 1995) and affects body temperature by increasing it in diurnal organisms, e.g., humans (Dijk, Cajochen, & Borbely, 1991), and decreasing it in nocturnal organisms, e.g., rats (Sheer et al., 2005). Administration of exogenous melatonin, on the other hand, has been shown to shift circadian rhythms in animals and humans according to the PRC in the direction opposite of light (Arendt & Broadway, 1987; Cassone, Chesworth, Armstrong, & Stuart, 1986; Lewy et al., 1998; Middleton, Arendt, & Stone 1997; Steinlechner, 1996). Further, the SCN, which has melatonin receptors (Reppert, Weaver, Rivkees, & Stopa, 1988; Vanecek & Watanabe, 1999), mediates the phase-shifting properties of exogenous melatonin (Cassone et al., 1986; Steinlechner, 1996). Therefore, the acute effect of light on melatonin production may be part of the mechanism by which light regulates circadian rhythms via the SCN (Arendt, 1995).

Light exposure also has an acute effect on the activity of the SCN and closely related neural structures. Animal studies have shown that light exposure increases expression of an immediate-early gene c-fos and, as a result, immunoreactivity of Fos protein in the SCN cells (Amir & Stewart, 1996; 1998a; Kornhauser, Mayo & Takahashi, 1996; Rusak, Robertson, Wisden & Hunt, 1990; Schwartz, Takeuchi, Shannon, Davis, & Aronin, 1994). Similar light-induced increases in c-fos expression and Fos reactivity occur in two thalamic nuclei, the intergeniculate leaflet (IGL) and the paraventricular nucleus (PVT), which have reciprocal connections with the SCN and with each other (Amir & Stewart, 1996; 1998a; Moga, Weis, & Moore, 1995; Moore et al., 2000; Morin Goodless-Sanchez, Smale & Moore, 1994). Increases in c-fos expression and Fos reactivity in the SCN, IGL, and PVT depend on the timing of light exposure within the endogenous phase, because they occur only when light is presented during the CT appropriate for a phase shift (Amir & Stewart, 1996; 1998a; Kornhauser et al., 1996; Rusak et al., 1990; Schwartz et al.,1994). As the effect of light on c-fos expression and Fos reactivity is both acute and phase-dependent, this effect of light exposure has been implicated in the entraining and phase-shifting properties of light (Kornhauser et al., 1996; Murphy & Campbell, 1996; Rusak et al., 1990). Thus, the response of the mammalian circadian system and its physiological and behavioral indices to the properly timed bright light is thought to be based on physiological properties of the SCN and related neural structures.

Melatonin and Light Exposure in Rodents and Humans

Melatonin is a hormone produced mainly by the pineal gland (Arendt, 1995). Secretion of melatonin into the bloodstream follows a clear pattern, with high levels at night and low levels during the day found in both diurnal (humans; Arendt & Broadway, 1987) and nocturnal (rodents; Laakso, Alila, Hatonen, & Mustanoja, 1996) organisms. The circadian rhythm of melatonin secretion is present during free-running under the experimentally induced conditions of constant darkness or very dim illumination (rodents: Lui & Borjigin, 2005; humans: Middleton et al., 1996). Exposure to light between subjective dusk and dawn both directly suppresses melatonin production and shifts the circadian melatonin cycle according to the PRC in rodents as well as humans (Arendt, 1995; Arendt & Broadway, 1987; Laakso et al., 1992).

The circadian rhythm of melatonin production by the pineal gland is regulated by the SCN (Arendt, 1995). The pathway from the SCN reaches the PVT and the sympathetic cells of the superior cervical ganglia that have norepinephrine projections to the pineal gland (Arendt, 1995). In addition, the pineal gland also receives afferent projections from the IGL, and the IGL appears to play a role in the entrainment of the melatonin cycle (Arendt, 1995; Edelstein & Amir, 1999; Moore et al., 2000). Although mammalian neural pathways involved in the endogenous melatonin regulation by the SCN and the exogenous regulation by light have been described almost exclusively in animals (Arendt, 1995), at least two post-mortem human studies provide limited evidence for a similar organization, including retinofugal inputs to the SCN and the PVT (Sadun, Schaechter, & Smith, 1984; Schaechter, & Sadun, 1985). During free run, the SCN maintains circadian rhythmicity of melatonin secretion (Arendt, 1995; Arendt & Broadway, 1987). Environmental light acutely suppresses and entrains melatonin secretion via the SCN (Arendt, 1995; Arendt & Broadway, 1987; Moore et al., 2000).

Melatonin output is well-suited for the analysis of circadian rhythms and effects of light in animals and humans due to the close relationship between melatonin secretion, the SCN activity, and environmental light, as well as due to the stability of the melatonin cycle under similar environmental conditions (Arendt, 1995; Lewy & Sack, 1989; Wehr, 1997). In animal research, melatonin levels in serum, urine and pineal extracts are frequently used to measure the response of the circadian system to various manipulations of environmental lighting conditions (Arendt, 1995; Amir & Stewart, 1996; Fukuhara et al., 2005; Laakso et al., 1992, 1996). In humans, plasma, salivary and urinary melatonin concentration levels are typical measures of circadian rhythmicity and effects of light (Arendt, 1995; Aoki, Yamada, Ozeki, Yamane, & Kato, 1998; Laakso, Hatonen, Stenberg, Alila, & Smith, 1993; Lewy & Sack, 1989; Paakkonen et al., 2006; Wehr, 1997).

Acute suppression and phase-shifting effects of light on melatonin require substantially lower light intensity and shorter duration of exposure in rodents in comparison to intensity and duration needed to produce a measurable effect in humans (Arendt, 1995). In rats, light intensity not exceeding 200 lx has been used to produce large and reliable phase-shifting and suppressing effects on melatonin secretion (Arendt, 1995; Laakso, 1992, 1996). In terms of duration, light pulses of 1 to 60 min have been successfully used in rat studies (Arendt, 1995; Laakso, 1992, 1996). For the same CT of light presentation, a lower intensity requires a longer duration of exposure to produce a consistent effect in the rat (Arendt, 1995). In human studies, light intensities of approximately 100-9,000 lx and durations of 30 min to 6.5 hr have been used to produce reliable effects on melatonin (Aoki et al., 1998; Bojkowski et al., 1987; Hatonen, Alila-Johansson, Mustanoja, & Laakso, 1999; Laakso et al., 1993; Lack & Wright, 1993; Lewy, Wehr, Goodwin, Newsome, & Markey, 1980; Zeitzer, Dijk, Kronauer, Brown, & Czeisler, 2000). Similar to animal data, lower light intensities require longer exposure in human subjects. Curve estimations based on varying intensities and durations of light have shown that the low end of the effective intensity range in humans lies approximately between 100 lx and 400 lx for the exposure durations of 6.5 hr (Zeitzer et al., 2000) and 30 min (Aoki et al., 1998), respectively.

Principles of Classical Conditioning

As the present experiment examines the possibility of using classical conditioning to modify the human circadian system's response to light, it is necessary to briefly present the basic principles of classical conditioning. Much of the information related here is part of general knowledge routinely found in any textbook chapter on classical conditioning, and as such is not attributed to any particular source.

Classical conditioning as first described by Pavlov (1927) involves two types of environmental stimuli and two types of behavioral responses. An event called unconditioned stimulus (US) has the capacity to trigger a reflexive response based on inborn properties of the responding organism's nervous system which is termed unconditioned response (UR). During conditioning, another event, called conditioned stimulus (CS), that does not trigger any specific response is repeatedly paired with the US until the organism begins to produce the behavior previously elicited by the US in response to the CS alone. This learned response to the CS is called the conditioned response (CR). In Pavlov's traditional example, a tone (CS) was presented shortly before food powder (US) was placed in a dog's mouth, and the dog would initially salivate in response to the US presentation (UR), but after several pairing trials it would start to salivate as soon as the tone was presented (CR). The development of the CR during pairing trials is referred to as acquisition, and the gradual disappearance of the CR with repeated presentations of the CS alone is referred to as extinction.

The existence of pseudoconditioning, or responding in a UR-like manner to any salient stimulus presented after the exposure to the US alone, underscores the need for a control procedure during conditioning. A control procedure that is routinely used involves the presentation of a stimulus similar to the CS either always in the absence of the US or with random time intervals between that stimulus and the US.

The differential CR to the proper CS that is consistently paired with the US, but not to the control CS that is not paired at all, or haphazardly paired with the US, has been first experimentally shown by Rescorla in dogs, using an electric shock as a US and a tone as a CS (Rescorla, 1967; Rescorla & Wagner, 1972). This control procedure establishes the role of contingency between the CS and the US in classical conditioning, which implies that during conditioning the organism develops a way to predict the US based on the information obtained from its environment.

The information about the appearance of the US can also be provided by a passage of time. In temporal conditioning, as described by Pavlov (1927), the US is presented to the organism at equidistant time intervals, and with repeated US presentations the organism begins to produce the CR shortly before the next presentation of the US. Examples of temporal conditioning have been studied in animals (e.g., rats; Kirkpatrick & Church, 2000; Williams, Frame, & LoLordo, 1992; Williams & LoLordo, 1995) as well as humans (Harley, 1973; Lockhart, 1966). It is thought that during temporal conditioning the time interval between US presentations becomes the CS, and this time interval is “measured” by the organism based on an internal biological process (Pavlov, 1927; Kirkpatrck & Church, 2000).

In several experiments on conditioning of the circadian system’s response to light in rats, the effects of light were mimicked or modulated by an arbitrary non-photic CS that had been made predictive of light onset or offset during pairing trials (Amir & Stewart, 1996; 1998a; 1998b; Arvanitogiannis & Amir, 1999; Golombek, Chuluyan, Kanterewicz & Cardinali, 1994). Two other animal studies suggest that the CT of light presentation, if kept constant across learning trials, may itself become associated with light exposure and therefore serve as a CS (Arvanitogiannis & Amir, 2001; Lerchl, 1995), which is consistent with the concept of temporal conditioning. The ability of the CT of light exposure to serve as a CS will be considered in detail in the section summarizing data on conditioning of the circadian system in rats.

Applications of Classical Conditioning in Humans

The principles of classical conditioning have been successfully applied in several clinical areas, including systematic desensitization treatment for different anxiety disorders, extinction of conditioned withdrawal in treatment of drug addiction, and conditioned taste aversion in treatment of alcohol abuse.

In systematic desensitization, which is based on conditioning of emotional states, the feared or anxiety-provoking stimulus is used as the CS and the previously learned relaxation response as the US (Wolpe, 1982). With repeated pairing of the phobic stimulus with the relaxed state, during which the intensity of the CS is gradually increased, the client learns to be exposed to the CS without experiencing fear or anxiety. In addition, systematic desensitization has been used to address conditioned nausea and vomiting as frequent side effects of chemotherapy in cancer patients (Burish & Redd, 1983).

During extinction of conditioned drug withdrawal symptoms, the client is exposed to stimuli previously associated with drug use in a controlled environment (Childress, Ehrman, McLellan, & O'Brien, 1986). Due to repeated pairing of these stimuli (CSs) with the drug consumption (US) in the past, conditioned withdrawal symptoms (CR) are elicited by the presentation of these stimuli in the absence of the drug. However, with repeated exposure to the CS alone extinction takes place, and the client becomes capable of being exposed to the stimuli previously associated with drug use without experiencing conditioned withdrawal symptoms, which reduces the possibility of relapse.

In conditioned taste aversion, the smell and the taste of alcohol (CSs) are paired with experimentally induced nausea and vomiting (US). The US is typically induced pharmacologically (Howard, 2001), although the use of motion sickness has also been reported (Mellor & White, 1978). After several pairings, the smell and the taste of alcohol start triggering an aversive response in the client.

Conditioning of other physiological responses encompasses endocrine and immune systems and may play a role in clinical practice. Conditioned allergic reactions to stimuli associated with allergen exposure have been found in eczema (Ikemi & Nakagawa, 1962) and rhinitis (Gauci, Husband, Saxarra, & King, 1994). For a patient with an autoimmune disorder, the pairing of a neutral stimulus (CS) with an immunosuppressing agent cyclophosphamide (US) was used to produce conditioned immunosuppression and thus to reduce the number of doses of the toxic medication (Olness & Ader, 1992).

In experimental studies, the human immune system has been observed to produce a conditioned increase in the number and activity of natural killer cells in response to a previously neutral taste (CS) after the taste was repeatedly paired with the epinephrine injection (US) that unconditionally increases the immune system's response (Buske-Kirschbaum, Kirschbaum, Stierle, Jabaij, Hellhammer, 1994; Buske-Kirschbaum, Kirschbaum, Stierle, Lehnert, Hellhammer, 1992). Conditioned increases in endogenous insulin, cortisol and noradrenaline, and a conditioned decrease in blood glucose level in response to a smell (CS) were also produced experimentally in human subjects by pairing the CS with insulin and glucose injections (Stockhort et al., 2004; Stockhorst, Steingrüber, & Scherbaum, 2000).

Classical Conditioning of the Circadian System in the Rat

Empirical data on classical conditioning of the circadian system in rats are directly relevant to the current project aimed to conceptually replicate results of animal studies in human subjects. The following detailed review of animal experiments identifies several methodological points that are important for the procedures utilized in the present research.

Acute effects.

Several animal studies to date have successfully shown acute effects of classical conditioning of the circadian system. In an experiment by Amir and Stewart (1996, 1998b), the CS presented alone was shown to acutely increase Fos immunoreactivity in the SCN, PVT, and IGL. In this experiment, four conditions were created, all of which involved a 10-day training period with a 15 min pulse of light (US) delivered at the third hour of the dark phase of the 12:12 LD cycle. Also during training, 20 min of air disturbance (CS) started 15 min prior to the light pulse in Condition 1 (pairing present), while in Condition 2 the CS started some time after the termination of the light pulse (pairing absent). Conditions 3 and 4 did not include either the CS or the US during “training”, which, in these two conditions, did not differ from simply following the 12:12 LD schedule. On the 11th day, at the third hour of the dark phase, the rats in the conditions 1, 2 and 3 received only the air disturbance (CS), and the rats in Condition 4 received only the light pulse (US). Shortly after the termination of the air disturbance or light pulse on the testing night, all 4 groups of rats were sacrificed and their SCN were assayed for the presence of the protein Fos. Fos reactivity was significantly and similarly elevated in the conditions 1 and 4, compared to the conditions 2 and 3, in which Fos expression levels were minimal and virtually the same. These results indicated that the air disturbance alone (CS) was able to increase Fos expression in the SCN to the level similar to the one triggered by the light pulse, but that the CR was evident only after the CS had become predictive of the onset of light through proper conditioning procedures. The absence of such predictive value, as in Condition 2, or the absence of prior experience with the CS, as in Condition 3, precluded the elevated Fos expression in response to the CS alone.

In a series of experiments, Golombeck et al. (1994) were able to increase pineal melatonin content under light exposure at the beginning of subjective night by presenting an arbitrary stimulus which had been used to predict the offset of light. The offset of light at the light-dark (LD) transition of a 12:12 LD cycle was defined as the unconditioned stimulus (US) and the rise of the pineal melatonin content 4 hr after the LD transition, as the unconditioned response (UR). The arbitrary, non-photicCS was a single 20 -min period during which drinking water was available to the animals within a 24 hr cycle; this CS was paired with, and made predictive of, the light offset US. The CR was the increase in the pineal melatonin content 4 hr after the usual time for the LD transition on the test trial when the water availability (CS) was given at the usual time for the LD transition, but the light was never turned off (no US).

In the first experiment, Golombeck et al. (1994) created 6 independent groups of rats. After all animals were entrained to a 12:12 LD photoperiod, they were subjected to a 7-day training phase followed by a single trial on the 8th day. During the training phase, the 12:12 LD schedule was maintained, so that the exposure to the US (lights off) was identical for all 6 groups.

The animals from Groups 1 and 2 were used as controls and did not receive any additional treatment and their water availability was not restricted during the training phase (Golombeck et al., 1994). The experimental animals from Groups 3, 4, 5, and 6, were given water only for 20 min a day, beginning 10 min prior to the LD transition, so that the availability of water (CS) was predictive of the offset of light (US). For the test trial, Group 1 stayed under continuous light for 4 hr past the time when the LD transition would normally occur (no US controls), and Group 2 was kept in the dark as usual for 4 hr after the LD transition (US controls). Among the four experimental groups, darkness versus no darkness for 4 hr after the usual time for LD transition (US vs. no US) and the presence versus absence of the 20 min water availability period starting 10 min prior to the usual time for LD transition (CS vs. no CS) were varied orthogonally between the groups on the day of the trial. All animals were sacrificed on the day 8, 4 hr past the usual time for the LD transition, and their pineal melatonin content was assayed.

To summarize, the six groups were as follows: (a) Group 1, control rats who had the US only during training, and the US only for the test trial; (b) Group 2, control rats who had the US only during training, no US for the test trial; (c) Group 3, experimental rats who had the CS-US pairing during training, and the CS only for the trial; (d) Groups 4, experimental rats who had the CS-US paring during training, and neither CS nor US for the test trial; (e) Group 5, experimental rats who had the CS-US pairing during training, and the CS-US for the test trial; (f) Group 6, experimental rats who had the CS-US pairing during training, and the US only for the test trial.

On the test trial, 4 hr after the usual time for the LD transition, Group 3 showed that even under continuous light melatonin production was triggered by a single presentation of the CS, as evidenced by a significantly higher mean level of melatonin in this group in comparison to both Group 2 and Group 4. The latter two groups, who did not receive the CS, showed similar and significant suppression of the pineal melatonin content by continuous light exposure, when compared with Group 1 in which the animals simply remained on the same LD schedule (Golombeck et al., 1994). The level of melatonin in Group 3 was still significantly lower than in Group 1, indicating that the CS alone was not potent enough to cue the normal melatonin release in the presence of its biological suppressor, light.

To rule out the possibility that during training water availability might have become a secondary time cue without any connection with the US, simply due to being always presented at the same time, Golombeck et al. (1994) conducted a control experiment. In this experiment, when the restricted water availability (CS) had preceded the light offset (US) during training, rats showed a significant increase in melatonin (CR) under continuous light (no US) after the presentation of water (CS) on the test trial night. However, when the restricted water availability had followed the light offset during training, rats did not show any increase in melatonin under light on the trial night regardless of the presentation of water. These data indicate that the water availability can trigger melatonin release under continuous light only by acquiring the predictive value for the light offset, as through conditioning, and not by becoming an independent time cue.

In another control experiment, the rats for whom restricted water availability was not paired with the light offset had the same pineal melatonin content as the rats with free access to water in both light and dark conditions (Golombeck et al., 1994). This finding ruled out the possibility that simply restricting water availability might increase melatonin levels. Based on the data from these two control experiments, the elevated level of melatonin found in the first experiment in Group 3, but not in the other two light-exposed groups, 2 and 4, has been interpreted as showing that due to classical conditioning the presence of the CS alone can have an effect on the pineal melatonin content similar to the effect of the light offset US.

Further evidence for the effect of conditioning on melatonin content was provided by the results of Group 5 and Group 6 of the first experiment (Golombeck et al., 1994). In Group 6 the presentation of the US alone after the CS-US pairing resulted in significantly lower melatonin content in comparison to both Group 5, in which the US was preceded by the CS on the test trial as it had been during training, and Group 1, in which no CS-US pairing had occurred before the test trial presentation of the US alone. The groups 5 and 1 showed similar levels of melatonin content. Thus, Golombeck et al. (1994) found that after CS-US pairings the response to the US alone became weaker in comparison to the response to the CS-signaled US.

Overall, by using a non-photic CS to predict the offset of light during subjective evening, Golombeck et al. (1994) trained experimental rats to acutely produce melatonin in response to a single CS presented at the same time as during training even under conditions of continuous light exposure. In addition, following CS-US pairings, rats produced more melatonin when the offset of light was signaled by the CS than when it was not.

The finding of a stronger acute response to the CS-signaled US was conceptually replicated by Amir and Stewart (1998a). These authors analyzed the number of cells immunostained for the protein Fos in the SCN, PVT, and IGL of rats exposed either to light alone or to light signaled by a CS previously paired with light. In this experiment, 4 groups of rats, after entrainment to 12:12 LD photoperiod, were maintained for 10 days on a skeleton photoperiod with only two 1 hr light pulses given daily 11 hr apart, marking the beginning of the *Light* (subjective day) and the *Dark* (subjective night) phases of the cycle, respectively. For two experimental groups, a 20 min period of mild air disturbance produced by a low noise fan was delivered twice a day starting 15 min before each light pulse, whereas the two control groups did not experience such a manipulation. The light pulse was defined as the US, and the air disturbance, as the CS. After the full 10 days of training, starting 15 min prior to the beginning of the *Dark* phase on the 11th day, one experimental group and one control group received the CS (20 min of air disturbance), and all four groups received a 15 min test light at the beginning of the *Dark* phase. The number of Fos-stained cells in the CSN, PVT, and IGL 1 hr after the beginning of the *Dark* phase was significantly lower in the experimental group that did not experience the CS prior to the test light, in comparison with the experimental group that was given the CS prior to the test light. The latter group was not different from the two control groups. Thus, the acute effect of light on the immunoreactivity of Fos was modulated by the CS that had been made predictive of the light onset, with a stronger response to the CS-signaled light than to the light presented alone.

The results of the three aforementioned experiments by Golombeck et al. (1994) showed an acute effect of conditioning, indicating that a CS predictive of the LD transition could trigger the release of melatonin under continuous light. Similarly, the results of Amir and Stewart (1996, 1998a, 1998b) evidenced acute effects of conditioning by showing that a CS predictive of light onset could trigger Fos expression when presented alone or modulate the reactivity of Fos in response to light. Even though Fos expression triggered by the CS suggests the activation of the mechanism underlying the phase-shifting or entrainment of the circadian system, this finding does not yet directly show whether a phase shift or entrainment can be induced by the CS alone.

Phase-shifting and entrainment.

Golombeck et al. (1994) reported that in another experiment the CS previously paired with the offset of light at the LD transition triggered melatonin release when presented alone under continuous light 4 hours prior to the LD transition. This finding bears weight on the question of phase-shifting properties of the CS.

In this experiment, the offset of light at the LD transition was used as the US, and 20 min of water availability was used as the CS (Golombeck et al., 1994). Four groups of rats went through 7 days of training so that Groups 1 and 2 (non-conditioned groups) received only the US at the LD transition of a 12:12 LD cycle, and Groups 3 and 4 (conditioned groups) received the CS-US pairings at the LD transition. On the testing day, starting 4 hr prior to the time of the LD transition, one conditioned group and one non-conditioned group received 4 hr of darkness (US) while the other two groups – one conditioned and one non-conditioned – remained under light (no US). Also, both conditioned groups were given 20 min of water availability (CS) starting 4 hr and 10 min prior to the time for the LD transition. The pineal melatonin content was measured 4 hr after the experimental manipulation, at the time for the LD transition. The two conditioned groups, one of which received the CS alone and the other, the CS together with the US on the trial day, showed similar and significantly increased levels of melatonin, in comparison with the two non-conditioned groups which weren't different from each other despite that only one of them received the US for the test trial. These data showed that the pineal melatonin content at the LD transition time was not affected by the light offset 4 hr earlier in the non-conditioned rats, but was increased by the introduction of the CS 4 hr prior to the LD transition in the conditioned rats, regardless of whether or not the CS was accompanied by the light offset.

Based on the results of this experiment, it appears that the presentation of the CS predictive of the light offset during the late part of the subjective day may phase-advance the melatonin rhythm (Golombeck et al., 1994). The same experiment also shows that the light offset itself given at the same CT to non-conditioned rats does not advance the melatonin secretion. Therefore, these results may indicate that water availability is conditioned to signal not the darkness per se but the time of the LD transition, so that the early presentation of this CS phase-advances melatonin release. Alternatively, water availability during training may have become a trigger for acute melatonin release regardless of the time of presentation. Since both of these interpretations can be applied to the data reported by Golombeck et al. (1994), the conclusion as to which effect has been exerted by the CS in their experiments cannot be made.

Several studies conducted by Amir and associates (Amir & Stewart, 1996; 1998b; Arvanitogiannis & Amir, 1999) successfully tested the phase-shifting properties of an arbitrary CS by employing measures of circadian phase over the course of several cycles. In their experiments, light was used as the US known to have a strong phase-shifting effect, a mild air disturbance produced by a low-noise fan was used as a CS to signal the onset of light, and a number of physiological and behavioral variables served to evaluate the ability of the CS to affect the circadian system.

Amir and Stewart (1996; 1998b) trained rats with a 15 min pulse of light (US) delivered at the third hour of the dark phase of the 12:12 LD cycle. For rats in Group 1, the light pulse was preceded by 20 min of air disturbance (CS) which started 15 min prior to the light pulse (conditioning). Rats in Group 2 were given the CS some time after the termination of the light pulse (no conditioning), and rats in Groups 3 and 4 simply followed the 12:12 LD schedule. These manipulations were performed for 10 days, after which all animals were allowed to free-run for 5 full days in constant darkness. On the testing day that followed, Groups 1, 2, and 3 were given the CS only, and Group 4, the US only at the third hour into the subjective night. After the testing day, the animals were observed for 5 more days in constant darkness, and their circadian rest-activity and body temperature cycles were analyzed during the period of 5 days before to 5 days after the testing day. Groups 1 and 4 experienced significant and similar phase delays on both behavioral and physiological measures following the testing day, whereas Groups 2 and 3 did not show any effect of the air disturbance on their free-running rest-activity and body-temperature rhythms. Thus, only the rats for whom the CS predicted the US during training (Group 1) evidenced the phase-shifting effect of the CS alone similar to that of the US alone (Group 4).

More evidence for phase shifting by the CS was reported by Arvanitogiannis and Amir (1999). In this experiment, rats in one group were entrained to a 25 hr T-cycle (T25) for 40 days using a single 30 min pulse of light (US) always preceded by a 30 min period of mild air disturbance (CS), with the stimulus onset asynchrony of 5 min. After the training, the rats were allowed to free-run for ten 25 hr days in total darkness, on the 11th day they were presented with the CS alone at the third hour into the subjective night, and then were allowed to free-run for 10 more days. The entire experimental procedure was repeated with the same subjects two more times, the second time using the entrainment cycle of 24 hr (T24), and the third time using the T25 again. The analysis of the rest-activity cycle 10 days before and 10 days after the CS-only presentation revealed a phase delay after the presentation of the CS, but only when the T25 entrainment cycle was used.

Arvanitogiannis and Amir (1999) observed that under the T24 condition the single pulse of light given to the rats had not been producing a phase-delay, because the endogenous rhythms of these rats were slightly longer than 24 hours as measured in constant darkness prior to the beginning of the study. A light pulse entraining these rats to the T24 was producing a slight phase-advance rather than phase-delay. Therefore, since the phase-delay UR was absent during conditioning under the T24 cycle, the learning of a phase-delaying CR could not occur.

However, if the exogenous cycle of 24 hr is maintained by appropriately timed light exposure, one unconditioned circadian response is still present, namely, the entrainment of the endogenous rhythms. The possibility of using a CS to produce entrainment as a CR under the T24 conditions has been tested experimentally.

Robinson, Stewart, and Amir (1996) reported that entrainment to a 24 hr cycle could be maintained by a CS that had been paired with light previously used for entrainment to the same T-cycle. In this experiment, rats were trained under the skeleton photoperiod protocol, with the 20 min of air disturbance (CS) preceding the onset of each of the two 1 hr long daily light pulses (US) by 15 min. After 10 days of training, rats were given the CS alone twice daily in constant darkness at the same time as during training, while their body temperature was monitored continuously. The animals were able to maintain entrainment with the CS alone for 5 days before they started to free-run. When subsequently the same animals underwent a 10-day extinction period in which the air disturbance (CS) was unpaired with the two light pulses (US) of the skeleton photoperiod, the CS alone failed to maintain entrainment altogether.

To summarize, studies by Amir and associates (Amir & Stuart, 1996, 1998a, 1998b; Arvanitogiannis & Amir, 1999; Robinson, Stewart, & Amir, 1996) have shown that a CS alone can phase-shift and entrain the circadian system after it has been paired with light. From Arvanitogiannis and Amir's (1999) study it appears, however, that the nature of the CR may depend on the effect that light exposure has on the circadian rhythms during conditioning, which is defined by the CT when conditioning takes place.

Circadian time and conditioning.

The role of CT in the process of conditioning a circadian response was first suggested by Golombeck et al. (1994), who pointed out that bright light as the circadian system's US is not a typical US. As a circadian US, bright light has an effect only during the time between subjective evening and morning (the sensitive portion of the PRC), and has virtually no effect during the subjective day. In addition, the phase-shifting effects of light are the opposite if light is delivered during subjective morning or evening (two opposite segments of the PRC). Thus, the timing of light exposure within the circadian cycle defines the presence and the direction of the effect of light on the circadian system, which effect constitutes the UR. Therefore, the CT is likely to play an essential role in the process of conditioning of the circadian system's response to light.

As the process of conditioning can only occur at the time when the US triggers the UR, conditioning must involve the CS-US pairing during the sensitive portion of the PRC as determined by the CT of the organism. With repeated CS-US pairing, the CS becomes associated not only with the US but also with the respective CT, which makes the CS a time cue, that is, a mild zeitgeber. In addition, during the process of conditioning the CS and the US presented together become a compound zeitgeber, as now their conjoint appearance is associated with the CT used for training. The studies showing that after conditioning the CS-signaled US produces a stronger response than the US alone (Fos reactivity CR: Amir & Stewart, 1998a; melatonin secretion CR: Golombeck et al., 1994) support these observations. Further, the ability of the CS alone to phase-shift and entrain endogenous rhythms (Amir & Stuart, 1996, 1998b; Arvanitogiannis & Amir, 1999; Robinson, Stewart, & Amir, 1996) confirms that the CS can serve as a zeitgeber, and emphasizes the importance of the CT during which conditioning takes place.

The role of the CT used for conditioning as a determinant of the UR and, therefore, the CR, is directly supported by the results presented by Arvanitogiannis and Amir (1999). In their experiment, rats were entrained with a single 30 min light pulse

(US) predicted by a 30 min period of air disturbance (CS) the onset of which preceded the onset of light by 5 min. When entrainment was done to the T25 cycle during acquisition, on the test trial the rats showed a phase delay in response to the CS presented alone during the early subjective night. However, when the T24 cycle was used during conditioning, the CS alone did not produce a phase delay.

Arvanitogiannis and Amir (1999) interpreted these results as evidence that the CS could exhibit the phase-shifting effect only if both the CS-US training and the CS-only testing occur within the same part of the endogenous PRC. Indeed, a phase delay was triggered by the CS given at the appropriate CT after the conditioning trials under the T25 cycle, in which the light exposure drove the endogenous cycle to phase-delay. The same subjects, however, showed no effect of the CS presented at the CT appropriate for phase-delay after they had been trained under the T24 cycle, because under the T24 cycle the light exposure entrained the rest-activity cycle to a period slightly shorter than the endogenous cycle (endogenous cycle in these rats ranged from 24.16 to 24.33 hr as measured in constant darkness prior to the beginning of the study), causing a small phase advance rather than a delay. Thus, the phase-delaying effect of the CS delivered during the delay-sensitive part of the PRC was present in the T25 condition when the US had a phase-delaying effect, and absent in the T24 condition when the US had a mild phase-advancing effect. This finding shows that for the successful demonstration of conditioning there has to be a match between the training and testing sessions with respect to the endogenous part of the PRC, delay-sensitive or advance-sensitive. This conclusion lends support to the assumption that during conditioning the CS becomes a zeitgeber whose effect is linked to the CT at which the CS-US pairing occurred, and further underscores the role of the CT in the process of conditioning.

The results of two other experiments further suggest that circadian time itself may serve as an associative cue. In one study, hamsters showed a greater suppression of melatonin in response to a 1 min light pulse in the middle of the night if they had been exposed to the same light stimulus at the same time on the previous night, in comparison with control animals that had not had a previous exposure to the light stimulus (Lerchl, 1995). The CT of light exposure on the previous night may have served as a cue for the light exposure on the subsequent night. However, the results of this study may be easily interpreted on the bases of sensitization, a non-associative single-event learning process, as the observed effect occurred already on the second presentation of the stimulus, and the study lacked a control procedure in which the time of light presentation would be varied between the nights.

Arvanitogiannis and Amir (2001) were able to show more conclusively that the CT can play the role of a CS. In this experiment, three groups of rats were entrained to 12:12 LD photoperiod and maintained on this schedule during the training part of the experiment. During training, one group of rats was given a 30 min training light once a night for 10 consecutive nights at the same time appropriate for phase-delay, namely, 3 hours into the dark phase. Arvanitogiannis and Amir (2001) named this group *Paired*, referring to the hypothesized association between the CT of light exposure and the light itself. The group named *Unpaired* was also given the training light once a night for 10 consecutive nights, but the time of exposure varied between the nights by several hours. The *Control* rats were simply maintained on 12:12 LD schedule and not exposed to the training light at all. Thus, Arvanitogiannis and Amir (2001) did not use any non-photic stimulus to predict the onset of the training light. Instead, in the *Paired* group, the constant time of exposure to the training light was considered an associative cue predictive of the onset of light.

After the training was complete, Arvanitogiannis and Amir (2001) allowed all three groups of rats to free-run in constant darkness for 10 days. On the following day, the 3 min testing light, which was of the same intensity but 10 times shorter than the training light, was given to all rats at the same CT as the training light in the *Paired* training condition. After this manipulation, the rats were allowed to free-run for another 10 days. The phase-shift in the rest-activity cycle induced by the testing light was compared between the groups. It was found that the *Paired* rats had a significantly greater phase-delay in response to the testing light in comparison to the *Unpaired* rats and the *Control* rats. This finding strongly suggests that the *Paired* rats learned the association between the CT at which the light stimulus was repeatedly presented and the appearance of the 30 min light stimulus itself, which enhanced their response to a brief 3 min testing light (Arvanitogiannis & Amir, 2001).

It therefore appears that the CT of light exposure may serve as a cue for future light presentations, thereby enhancing the effect of light on melatonin secretion (Lerchl, 1995) and on the rest-activity cycle (Arvanitogiannis & Amir, 2001). It should be noted, however, that the CT of light exposure is only one part of a compound CS, with the other part being either a non-photic stimulus made predictive of the light onset during training (Arvanitogiannis & Amir, 1999) or the onset of light exposure (Arvanitogiannis & Amir, 2001; Lerchl, 1995).

Attempted replications and reconceptualization.

De Groot and Rusak (2000) conducted three experiments with rats in attempt to find circadian CRs in response to a non-photic CS, and, based on their negative results, questioned whether the circadian system can be conditioned.

In the first experiment, de Groot and Rusak (2000) entrained rats to a 12:12 LD cycle and subsequently subjected them to a 10-day training period during which the 12:12 LD cycle was maintained and the rats were separated into 4 groups as follows. Group 1 received daily a 15 min light pulse (US only) starting at 3 hr 15 min into the Dark phase. Group 2 received daily a 20 min air disturbance period (CS only) starting exactly at 3 hr into the Dark phase. Group 3 received daily both the CS and the US starting at, respectively, 3 hr and 3 hr 15 min into the Dark phase, so that the onset of the CS preceded the US by 15 min and overlapped by 5 min (CS and US are paired). And finally, Group 4 received the US starting at 3 hr 15 min, and the CS starting at 9 hr into the Dark phase, so that the onset of the CS followed the termination of the US by 5.5 hr (CS and US are not paired).

After training, all rats were allowed to free-run in constant darkness for 9 days and on the 10th day the test of conditioning was administered (de Groot & Rusak, 2000). Group 1 was given the US alone at 3 hr 15 min into the subjective night, and Groups 2, 3, and 4 were given the CS alone at 3 hr into the subjective night. The rats were allowed to free-run in constant darkness for 10 more days and on the 10th day of the second free-running period, the identical testing procedure was performed and the rats were sacrificed shortly thereafter to analyze the expression of Fos in their SCN, the IGL and the PVT. The analysis of the free-running rest-activity cycle from 9 days before to 9 days after the first testing indicated that only Group 1 had a significant phase delay in response to the light pulse (US alone), while the rest-activity cycle in the other three groups was unaffected by the air disturbance (CS alone). Similarly, after the second testing, the SCN and the IGL had significantly more Fos-stained cells in Group 1 (US only) compared to the other three groups (CS only) which were similar to each other. No significant intra-group differences were found in the PVT. Thus, this experiment showed no effect of the CS alone on the behavioral and immunocytochemical measures of the circadian system's response.

The second experiment performed by de Groot and Rusak (2000) involved training under the skeleton photoperiod after the rats were entrained to a 12:12 LD schedule. The experimental rats were given 20 min of air disturbance (CS) starting 15 min prior to each of the two 60 min daily light pulses (US), and the two control groups of rats were either given the CS 3 hr after the termination of each light pulse or not exposed to the air disturbance at all. The training continued for 10 ½ days, after which the animals were placed in constant darkness for 7 days. On the 8th day of constant darkness, the rats who had been exposed to the CS during training, whether predictive of light pulses (the experimental group) or not (one of the control groups), underwent a period of air disturbance at 3 hr into the subjective night. The control rats who had not experienced the air disturbance during training were given a 15 min light pulse at 3 hr into the subjective night. All rats remained in constant darkness for 8 more days. The analysis of the free-running rest-activity cycle revealed no phase shift subsequent to the delivery of the CS during early subjective night in both the experimental and the CS-exposed control rats, whereas a significant phase delay was observed in the control rats tested with the light pulse.

Further, to evaluate the effect of the CS on Fos expression, the experimental group and the CS-exposed control group underwent another 4½-day training phase consisting of exactly the same manipulations as during the first training phase (de Groot & Rusak, 2000). Following the second training phase, the animals were placed in total darkness for 1 full day at the end of which the CS was delivered at 3 hr into the subjective night. Additional rats who had been maintained on the skeleton photoperiod schedule without any other manipulations were also placed in total darkness for 1 day and then half of them were given a 15 min light pulse at 3 hr into the subjective night while the others remained in the dark. All animals were sacrificed shortly after the experimental manipulation and their SCN, IGL and PVT areas were assayed for the presence of Fos. As in the first experiment, rats exposed to the light pulse on the testing night had significantly more immunoreactive cells in their SCN and the IGL in comparison to the other three groups which were not different from each other. The number of immunoreactive cells in the PVT did not differ between the groups. Overall, the results of the second experiment presented no evidence for the phase-shifting or Fos-inducing effects of the CS.

The third experiment was designed to test the ability of the CS to entrain rats to a previously established 24 hr cycle in the absence of environmental light (de Groot & Rusak, 2000). A control group of rats, after having been entrained to a 12:12 LD cycle, was placed in total darkness and subjected to a single daily 20 min period of air disturbance for several weeks. No effect of the air disturbance exposure on the usual free-running activity pattern was evident in this group. The experimental group of rats was also initially entrained to a 12:12 LD cycle and then put on a training schedule for 10½ days using skeleton photoperiod, with a 20 min air disturbance period delivered starting 15 min prior to the onset of each of the two daily 60 min light pulses. When these animals were subsequently placed in total darkness for 35 days, the two daily periods of air disturbance delivered at the same times as during training failed to maintain their entrainment to a 24 hr rest-activity cycle, showing the absence of conditioning.

Neither one of the three experiments conducted by de Groot and Rusak (2000) present any evidence for the conditioned response of the rat's circadian system to an arbitrary non-photic stimulus on behavioral and immunocytochemical measures. These data are in contradiction with the positive results of the earlier studies that also used air disturbance and light as, respectively, the CS and the US in similar conditioning paradigms (Amir & Stewart, 1996, 1998a, 1998b; Arvanitogiannis & Amir, 1999; Robinson et al., 1996). There are, however, considerable differences between experimental protocols that may account for the contradiction.

One of the differences pointed out by de Groot and Rusak (2000) involves the type of light used as the US. In their first experiment, De Groot and Rusak (2000) used the same type of light to deliver the US 3 hr after the LD transition as they used to maintain the 12:12 LD cycle. Amir and Stewart (1998b; 1996), on the other hand, in a similar protocol used an ultraviolet light as the US while maintaining the 12:12 LD cycle with regular white light. De Groot and Rusak (2000) did not consider this difference as influential, citing studies by Amir and associates in which either successful conditioning was achieved with the use of white light (Amir & Stewart, 1998a; 1998b) or no difference between the white light US and the ultraviolet light US was found (Arvanitogiannis & Amir, 1999).

However, in the study by Amir and Stewart (1998a; 1998b) that used white light as the US, each presentation thereof was preceded by the CS during training on a skeleton photoperiod schedule. Moreover, the study by Arvanitogiannis and Amir (1999) that found the same degree of conditioning with both white light and ultraviolet light used only one type of light for each given rat during training, and the light was presented only once a day and always signaled by the CS. Therefore, as de Groot and Rusak (2000) themselves pointed out, using the same type of light for both entrainment and conditioning was what prevented the rats in their first experiment from successfully building an association between the CS and the US, since exactly the same stimulus that was used as the US was also presented every day during training without being signaled by the CS. The equal number of CS-signaled and unsignaled presentations of the same US in de Groot and Rusak's (2000) first experiment effectively eliminated the ability of the CS to predict the US, which has long been shown to be a decisive factor in learning the CR (Rescorla, 1967).

Another problem appears to be responsible for the absence of conditioning in the second experiment by de Groot and Rusak (2000). This experiment tested the effect of the CS delivered at 3 hr into subjective night on free-running rest-activity cycle after conditioning under a skeleton photoperiod schedule in which the CS had been paired with each of the two light pulses used for entrainment (de Groot & Rusak, 2000). Since these light pulses were delivered at the opposite portions of the PRC, they had the opposite effects on the circadian system. As the direction of the phase-shifting effect of the CS after conditioning is dependent on the effect the US exerts on the endogenous rhythms during conditioning (Arvanitogiannis & Amir, 1999), the CS may not acquire any phase-shifting properties when paired with both phase-advancing and phase-delaying light pulses. Thus, the finding that the experimental rats' free-running rhythms were unaffected by the CS (de Groot & Rusak, 2000) is consistent with the fact that the UR brought about by light pulses during training was the opposite on every two consecutive training trials. By the same token, this finding provides confirmatory evidence to the assumption that the phase-shifting property of the CS depends on the phase-shifting property of the US during conditioning, which in turn depends on the CT of delivery.

It might be expected that the CS in de Groot and Rusak's (2000) second experiment would still increase the number of Fos-reactive cells in the SCN and the IGL on the testing night, insofar as Amir and Stewart (1998a) used the same skeleton photoperiod training procedure and found evidence of conditioning in the form of Fos expression in these areas. However, there is an important difference between these two experiments. Amir and Stewart (1998a) evaluated whether after training the CS-signaled US would produce a response different from that elicited by the US alone when tested at the same CT as during training. De Groot and Rusak (2000), on the other hand, evaluated the ability of the CS, when presented alone at a different CT than during training, to evoke a response similar to that elicited by the US alone. As already noted, during training under the skeleton photoperiod the US triggers the opposite URs. It is therefore plausible that in this experimental protocol the CS alone tested at a different CT may not trigger any response (de Groot and Rusak, 2000), whereas the response to the US alone may be different from the response to the CS-signaled US when tested at the same CT as during training (Amir & Stewart, 1998a).

Arvanitogiannis and Amir (1999) have shown that the phase-shifting effect of the CS depends on the phase-shifting effect of the US and on using the same segment of the PRC for both training and testing. However, during training under the skeleton photoperiod conditions, the phase-shifting effect of the US changes back and forth from delay to advance, insofar as the light pulses are given at the opposite segments of the PRC. Under these conditions, the cumulative effect of the two light pulses is the entrainment, not the phase-shift, of the circadian system. Therefore, to answer the question as to whether the CS that has been paired with both light pulses under the skeleton photoperiod schedule has any effect on the circadian system when presented alone, the CS should be tested for entrainment.

The third experiment by de Groot and Rusak (2000), which tested the CS alone given at the same times as during training under the skeleton photoperiod schedule, failed to demonstrate entrainment to the CS. It should be noted that on visual inspection of de Groot and Rusak's (2000) graph representing the data from their third experiment, it appears that the rats in the experimental group started to free-run in spite of the two daily presentations of the CS only about 5 days after the skeleton photoperiod had been terminated. However, the conclusion as to the absence of entrainment by the CS was made based on the analysis of the entire 35 day period of free-running (de Groot & Rusak, 2000). In a similar experiment by Robinson et al. (1996), rats, who were trained under the same skeleton photoperiod protocol and tested with the CS given twice daily in constant darkness at the same time as during training, were able to maintain entrainment with the CS alone for only 5 days and then started to free-run. The same animals failed to entrain to the CS completely after extinction was induced by presenting the CS independently of the two light pulses (US) used to maintain the skeleton photoperiod. Thus, the absence of entrainment by the CS that de Groot and Rusak (2000) report appears to be an artifact of the time scope of analysis they used, with the 35-day long testing period effectively turning into extinction after the first few days.

Overall, the protocols used by de Groot and Rusak (2000) are unsuccessful in finding evidence for conditioning for three possible reasons: due to the lack of contingency between the CS and the US in the first experiment, due to the variable nature of the response intended for conditioning in the second experiment, and due to the time scope of analysis in the third experiment. Based on this analysis, the following points appear crucial for observing the effect of conditioning on the circadian system: (a) the training and the testing stimuli should be presented during a light-sensitive phase of the circadian cycle, (b) the time of stimulus presentation should be kept constant throughout the experiment, (c) the light used for conditioning should be a distinct stimulus or the only light stimulus in the study, and (d) testing sessions should involve no more than a few presentations of the CS alone to prevent extinction of the learned response. These points will be utilized in the design of the present experiment.

The Experimental Hypothesis

The present study will attempt to test classical conditioning of the circadian system, an area that has not yet been studied in humans. A potential consequence of establishing this area of inquiry is a modification of the procedure currently used for bright light therapy of the circadian rhythm sleep disorders, which would add to the practical uses of the classical conditioning paradigm.

Based on the results of animal studies reviewed heretofore, the present experiment will utilize a procedure conceptually similar to the one described by Arvanitogiannis and Amir (2001) to test whether in humans the effect of light on melatonin secretion can be enhanced if the light is presented repeatedly at the same CT during the early night. Specifically, the acute suppression of salivary melatonin (UR and CR) will be used as a dependent measure to determine whether repeated exposure to a prolonged light stimulus (US) leads to the enhancement of the effect of a brief light stimulus given at the same CT (compound CS).

All subjects will maintain a consistent sleep schedule for the duration of the experiment. For the experimental subjects, the CT of experimental light exposure will be constant across the training sessions and the testing sessions, and will occur during the first part of the night when the endogenous melatonin levels are high and bright light has been shown to suppress salivary melatonin (Aoki et al., 1998). Control subjects will have training sessions at the same CT, but the amount of illumination provided by the control light will be below the threshold for melatonin suppression in humans. A commercially available light box will be used as a distinct light source. To ascertain whether a substantial change occurs in the CT of light exposure in the course of the experiment, the circadian phase of melatonin production will be measured at the beginning and the end of the experiment by using the time of onset of nocturnal melatonin production in saliva, or salivary dim light melatonin onset (sDLMO; Carskadon, Acebo, Richardson, Tate, & Seifer, 1996).

The present experiment will utilize procedures similar to Arvanitogiannis and Amir's (2001) study: (1) the intensity of the testing light is the same as the intensity of the experimental training light; (2) the duration of the testing stimulus is 10 times shorter than the duration of the training stimulus; and (3) the level of light intensity and the duration of exposure for the experimental training light stimulus are such that a measurable effect – here, suppression of salivary melatonin – can be expected in untrained human subjects at the appropriate CT.

Two hypotheses will be tested. First, the short duration testing light stimulus is expected to reduce salivary melatonin levels after training in experimental subjects but not in control subjects. Second, the long duration light is expected to reduce salivary melatonin levels to a greater degree after training in experimental, but not control subjects. Affirmative results would confirm the hypothesis that the association between the time of bright light exposure and the light itself leads to an enhanced response to bright light, thereby supporting the role of associative learning in the effect of bright light on circadian system in humans. In practical terms, the expected results would suggest the possibility for reducing the duration of repeated bright light exposure used for treatment of circadian rhythm disorders.

Method

Participants

Seventeen non-smoking adult paid volunteers, 10 females and 7 males, between 18 and 25 years of age (*M* = 20.7, *SD* = 1.4), on a regular sleep-wake schedule, were recruited from the ethnically diverse population of CUNY undergraduate students. Prospective subjects who during a preliminary interview reported a history of neurological, mental, or sleep disorders, shift work, substantially delayed sleep-wake schedule (habitual bedtime later than 1:30 am), or the use of sedating or stimulating medications, were excluded. Each subject was paid $400 upon completion of the experiment.

Stimuli

Three different light stimuli were employed throughout the experiment:

1. The short duration bright light stimulus was a 4.5 min pulse of bright white light of approximately 6,000 lx intensity at eye level delivered by means of a commercially available light box typically used in clinical practice for the treatment of circadian rhythm disorders. No effect of such a short light stimulus on salivary melatonin in naïve human subjects has been reported in the literature. The absence of such an effect was evaluated as described in the pretest procedures below.

2. The long duration bright light stimulus was a 45 min pulse of bright white light of approximately 6,000 lx intensity at the eye level delivered via the same light box. It has been reported that 30 min of white light exposure at 5,000 lx reduces nocturnal salivary melatonin by approximately 15% (Aoki et al., 1998). Therefore, it was expected that the long bright light stimulus, which was 50% longer and approximately 20% brighter, would

produce a reliable suppression of salivary melatonin concentration in human participants. The effect of the long bright light stimulus on salivary melatonin in naïve subjects was evaluated as described in the pretest procedures below.

3. The long duration dim light stimulus was a 45 min pulse of dim light of approximately 3 lx intensity at the eye level. This amount of illumination is well below the previously used criterion for the maximal amount of background nocturnal illumination deemed insufficient to exert any effect on human melatonin secretion (e.g., 10 lx in Aoki et al., 1998, study). The long dim light stimulus was delivered using the same light box fixture inside of which a small, low intensity light bulb with a white semi-transparent plastic cover was fitted.

The appropriate illumination levels were verified repeatedly throughout the study using a commercial digital lightmeter (Extech Instruments, Model # 403125, sensitivity: 0.01-20,000 lx, accuracy: ± 3%).

Elements of the Conditioning Protocol

The US was defined as the long duration bright light presented to participants two hours after the scheduled bedtime (BT). The UR was defined as a reduction in melatonin concentration in saliva following the US presentation. The time of light exposure was chosen because by two hours after the onset of darkness at night circulating melatonin levels in humans had been previously shown to increase by approximately 100% relative to daytime values (Arendt & Broadway, 1987). Such an increase was deemed sufficient for a measurable UR to occur. An earlier time of lights exposure was not used in order to avoid a floor effect on the melatonin suppression measure. A later time of light exposure was not used in order to minimize the possibility of considerable phase delays during the experiment, as the magnitude of the light-induced phase delay increases during the first half of the night (Czeisler, 1995).

The CS was defined as a combination of the CT of light exposure and the onset of bright light. Thus, during training the initial 4.5 min of the long duration (45 min) bright light and the CT of the light onset were considered two parts of the compound CS. During testing, the short duration (4.5 min) bright light given at the same CT as during training was considered the same compound CS.

The presence of the CR after completion of training was ascertained using two measures: a) the presence of melatonin suppression in response to the short duration bright light given at the same CT as during training, in comparison to the absence of melatonin suppression in response to the short bright light before training; and b) stronger melatonin suppression in response to the long duration bright light given at the same CT as during training, in comparison to melatonin suppression in response to the long bright light before training.

Since the CT of light exposure was an integral part of the CS in this study, a substantial shift in the melatonin cycle was undesirable because it might prevent conditioning of salivary melatonin suppression due to the change in the CT of exposure during training trials. Therefore, the time of sDLMO was ascertained at the beginning and the end of the experiment to test whether the experimental manipulations shift the circadian rhythm of melatonin secretion. The onset of nocturnal melatonin production under dim illumination as a measure of circadian phase relative to the exogenous time was developed by Lewy and associates using plasma melatonin concentration levels (Lewy & Sack; 1989; Lewy, Cutler, & Sack, 1999). Since nearly perfect correlation values were found between melatonin levels in serum and saliva samples (Laakso, Porkka-Heiskanen, Alila, Stenberg , & Johansson, 1990; Buhlmann Laboratories AG, 1998), sDLMO has been used as a measure of the CT and the amount of phase shift in a number of studies (Carskadon et al., 1996; Laakso et al., 1993; Goel, 2005).

Design and Procedure

All subjects signed a consent form. The consent form and the experimental procedures had been approved by the CCNY Institutional Review Board (Human Subjects Protocol # H-0304.1). The approval had been filed with the Office of Sponsored Research at the CUNY Graduate Center prior to data collection.

Subjects were asked to fill out a sleep log for 2 weeks to determine habitual bedtime and habitual uptime. The scheduled bedtime (BT) and uptime (UT) to be used for the duration of the study were mutually agreed upon with each subject. Subjects were asked not to vary these BT and UT by more than 30 min while at home, and their compliance was monitored with the use of sleep logs. Subjects were also instructed not to turn ceiling lights on at night in case they had to get up (e.g., to void) in the middle of the night at home; instead, the use of a small flash light or, if possible, avoidance of any artificial light source was recommended.

During the study, subjects were asked to abstain from alcohol and limit caffeine intake to an equivalent of 1 cup of coffee a day in the morning. In addition, subjects were required to abstain from eating bananas and drinking coffee, red fruit juices and artificially colored beverages on the days when the sampling of salivary melatonin was scheduled (Buhlmann Laboratories AG, 1998). Subjects were not allowed to eat or brush their teeth during saliva sampling, and 10 min prior to each sample they were asked to take a sip of water to rinse their mouths. Although mouth rinsing 15 min prior to saliva sampling is recommended (Buhlmann Laboratories AG, 1998), a 10 min period was used to prevent mouth dryness during testing sessions requiring 15 min sampling intervals, as described below.

All training and testing procedures were done at the CCNY sleep laboratory. Subjects were tested in groups of 2-3 people on the same night, but each participant had a separate soundproof bedroom where he or she was individually subjected to light exposure. Participants were instructed not to discuss their experiences with one another. Each participant was required to spend 16 nights at the CCNY sleep lab in the course of approximately 7 weeks.

The experimental manipulations performed in the lab are schematically presented in Figure 1. During the first week of the study, subjects spent three nights at the lab, 1-2 nights apart. On the first night, the time of sDLMO was measured. The subjects sat in the reclining position under the dim illumination (<10 lx) starting 4 hr before the BT and until 1 hr after the BT. They deposited a small amount of saliva into a plastic tube every 30 min. After the last sample, the subjects went to sleep, and slept until 30-60 min after the UT to compensate for 1 hr sleep loss in the evening.

On the second night, the pretest with the short, 4.5 min bright light stimulus was conducted for all subjects. Subjects went to bed at the scheduled BT, and were awakened 2 hr later. They stayed awake in their room for 1.5 h, with the background illumination of <1 lx. During this time they remained in the reclining position, facing a light box. The light box was positioned approximately 40 cm away from the subject's face, with the center of the box aligned at the subject's eye level. Subjects collected saliva samples every 15 min, starting approximately 12 min after awakening (approximately 2 min to fully awaken and assume a reclining position on an arm chair, and a 10 min waiting period to minimize the effect of gross body movements on nocturnal melatonin secretion). Immediately after the first saliva sample, they were shown the short, 4.5 min bright light. After the last saliva sample, subjects went back to sleep, and slept until 30-60 min after their habitual uptime to compensate for sleep loss during the awakening. On the third night, the pretest with the long, 45 min bright light stimulus was conducted for all subjects. The procedure was identical to that described for the second night, except that the bright light exposure lasted 45 min instead of 4.5 min.

During the following 5 weeks, subjects spent 10 nights in the lab for a total of 10 training sessions, two sessions per week, at least 1 night between any two consecutive sessions. On each of these nights, the experimental subjects went to bed at the BT, and were awakened 2 hr later for 1.5 h. During awakenings, they remained in the reclining position, with the background illumination of <1 lx, and collected saliva samples every 15 min, starting approximately 12 min after awakening. Immediately after the first saliva sample, they were shown the long, 45 min bright light. After the last saliva sample, subjects went back to sleep, and slept until 30-60 min after the UT to compensate for sleep loss during the awakening. The control subjects underwent the identical procedure, except that they were shown the long, 45 min dim light instead of bright light. The main purpose for collecting saliva samples during the training sessions was to assure that the same protocol was used across all pretest, training, and posttest sessions.

During the last week of the study, subjects spent 3 nights in the lab, 1-2 nights apart. On the first two of these last three nights, all subjects were given a posttest with a short bright light and a posttest with a long bright light. The posttest procedures were identical to the pretest procedures described above. The order of the short bright light and the long bright light on the two posttest sessions was counterbalanced between the experimental subjects, and, separately, between the control subjects. The counterbalancing of the short and the long posttest bright light stimuli was done for two reasons. First, if the long light had been given before the short light to all subjects, the control subjects could have shown some degree of melatonin suppression on the short bright light posttest due to the expected learning effect. Second, if the short light had been given before the long light to all subjects, the experimental subjects could have not shown an increase in melatonin suppression by the posttest long bright light due to extinction. On the last night of the study, the time of sDLMO was measured again using the same procedures as during the first night of the study.

Thus, during the awakening on each of the pretest, training, and posttest sessions, a total of 6 saliva samples were collected from each subject, 15 min apart. On the pretest, training, and posttest sessions involving 45 min light exposure (bright light or dim light, as required by the respective session's protocol), the first saliva sample was collected under dim illumination (<1 lx) prior to the light exposure, another 3 samples were collected during the light exposure, and 2 more samples were collected after the light exposure. On the pretest and posttest sessions involving 4.5 min bright light exposure, the first saliva sample was collected under dim illumination (<1 lx) prior to the light exposure, and the remaining 5 samples were collected after the light exposure. In other words, on all pretest and posttest sessions, sample 1 was collected immediately prior to the beginning of the light exposure, and samples 2, 3, 4, 5, and 6 were collected approximately 15 min, 30 min, 45 min, 60 min, and 75 min after the beginning of the light exposure, respectively. Table 1 provides a summary of the experimental design.

The pretest, training, and posttest sessions were scheduled to occur on the same two nights of the week for each subject. The first and the last nights of the study used to determine sDLMO were scheduled 1-2 nights prior to the first pretest night, and 1-2 nights after the second posttest night, respectively.

During the sDLMO nights, subjects were allowed to use the bathroom if at least 20 minutes remained before the next saliva sample, in general, immediately after the previous sample. During the pretest, training, and posttest nights, subjects were allowed to use the bathroom during the experimental awakening either immediately upon awakening (at least 10 minutes before the first sample) or after the last saliva sample was collected; however, subjects were asked to refrain from using the bathroom in the beginning of the awakening, if possible. Subjects were allowed to use only dim flash lights to go to the bathroom at night. Ceiling lights were turned off in the entire lab during all experimental procedures.

In the beginning of the study, subjects were told that the light presented at night may be either bright or dim, and may last either 4.5 min or 45 min. To control for the possible expectation effects, subjects were blind as to the brightness and the duration of the light to be presented on each of the awakening nights. The brightness became known to the subject as soon as the light switch was turned on, and the duration was determined by the experimenter immediately thereafter by opening a sealed envelope that contained the “duration code” for the night. The experimenter always knew what type of light was scheduled to occur on a given night.

Data Analysis

Salivary melatonin assays and individual data patterns.

Saliva melatonin samples from the sDLMO sessions, the pretest sessions, and the posttest sessions were analyzed in the Endocrine-Hypertension Laboratory at the Brigham and Women's Hospital in Boston. Buhlmann Direct Saliva Melatonin radioimmunoassay was used for analysis. Intra-assay and inter-assay precision measures for evening and nighttime melatonin samples do not exceed 5% and 9%, respectively, and the minimum detectable melatonin concentration value is 0.2 pg/ml (Buhlmann Laboratories AG, 1998).

Prior to employing inferential statistics, melatonin patterns of individual subjects were analyzed to determine whether nocturnal melatonin production was within the expected range, whether a substantial phase shift in sDLMO had occurred in the course of the experiment, and whether the long bright light suppressed melatonin on the pretest.

Nocturnal melatonin production was determined for each subject based on whether the subject's salivary melatonin level prior to the light exposure on each of the two nocturnal pretest sessions was above the threshold of 3 pg/ml. This threshold was used because daytime salivary melatonin values are expected to be below 3 pg/ml, and nocturnal values, although vary substantially between individuals, typically range between 3 and 50 pg/ml (Buhlmann Laboratories AG, 1998).

To determine the amount of phase shift, the time of sDLMO on each sDLMO session was defined as the interpolated time when saliva melatonin content reached the threshold of 3 pg/ml and continued to increase on subsequent samples (Buhlmann Laboratories AG, 1998). The difference between the time of sDLMO at the beginning of the experiment and the time of sDLMO at the end of the experiment should not have exceeded an arbitrary threshold of 60 min for each subject. This threshold allowed for some variability in sDLMO during the period of several weeks needed to complete the experiment, and defined the amount of phase shift that was considered detrimental to the process of conditioning.

Melatonin suppression during the long bright light pretest was confirmed if melatonin levels on at least one of the samples taken after the light onset fell below the pretest nocturnal baseline (pre-NBL) by more than a threshold amount. The pre-NBL was defined individually by taking an average of two samples, the first sample from the short light pretest and the first sample from the long light pretest. The threshold was also determined individually for each subject by multiplying by 2 the standard deviation for the six melatonin values collected on the short light pretest night, when no effect of light was expected and any within-subject variability in melatonin values was considered random. The arbitrary threshold of 2 standard deviations was used to minimize the possibility of interpreting random between-sample variability within one individual as evidence of melatonin suppression, and to establish consistency in the determination of melatonin suppression. Thus, the following formula was used to calculate the value below which melatonin levels during the long light pretest had to drop for melatonin suppression deemed to be present: [(Sample 1 from the short light pretest + Sample 1 from the long light pretest) / 2] – (*SD* of the six samples from the short light pretest x 2).

In addition to the pre-NBL, the posttest nocturnal baseline (post-NBL) was calculated for each subject by taking an average of the first sample from the short light posttest and the first sample from the long light posttest. The post-NBL values were used in statistical analyses of between-group differences.

Statistical analyses.

The presence of a phase shift in sDLMO was analyzed with the use of *t* tests. The amount of phase shift in each group was compared to the null hypothesis value (the absence of phase shift). Also, the amount of phase shift was compared between the groups. As in the present experiment the absence of phase shift was required, the increased probability of Type I error due to the use of multiple *t* tests was not of a concern for this analysis.

The effect of the long light on melatonin secretion during the pretest was analyzed using a 6 x 2 (Sample x Group) ANOVA, where the first level of the sample variable was the pre-NBL and 5 subsequent levels were, respectively, sample numbers 2, 3, 4, 5, and 6 that were taken after the onset of light. Similarly, the effect of the short light on melatonin secretion during the pretest was analyzed using a 6 x 2 (Sample x Group) ANOVA. The pre-NBL and 5 samples taken after the onset of the short light were the levels of the sample variable. Greenhouse-Geisser epsilon was used to correct the *df* values whenever the homogeneity of covariance assumption was violated. In addition, to see whether the nocturnal melatonin baseline was different between the groups or changed differentially between the pretest and the posttest, the comparison between the pre-NBL and the post-NBL was made using a 2 x 2 (Pretest-posttest x Group) ANOVA.

To evaluate the first hypothesis that the short light would result in melatonin suppression in the experimental group on the posttest, a 2 x 6 x 2 (Pretest-posttest x Sample x Group) ANOVA was used. For the short light pretest and the short light posttest sessions, the first level of the sample variable was the pre-NBL or the post-NBL, respectively. The remaining 5 levels of the sample variable were the 5 samples taken after the onset of the short light.

Similarly, the second hypothesis that the long light would produce a stronger melatonin suppression on the posttest in the experimental group was evaluated with the use of a 2 x 6 x 2 (Pretest-posttest x Sample x Group) ANOVA. For the long light pretest and the long light posttest sessions, the first level of the sample variable was the pre-NBL or the post-NBL, respectively. The remaining 5 levels of the sample variable were the 5 samples taken after the onset of the long light. Greenhouse-Geisser epsilon was used to correct the *df* values whenever the homogeneity of covariance assumption was violated.

Results

A total of 17 subjects were studied, 9 experimental and 8 control. Two of these subjects, one experimental, and one control, were excluded from further analysis because they were very low melatonin producers: they did not reach sDLMO criterion of 3 pg/ml on either one of the two sDLMO sessions, and they had the pre-light nocturnal salivary melatonin value of <3 pg/ml on at least one of the pretest sessions. Therefore, only data for 8 experimental and 7 control subjects were analyzed. These subjects' nocturnal salivary melatonin profiles were within normal limits, with the nighttime values ranging between 3.2 pg/ml and 36.2 pg/ml, and the interpolated times of sDLMO ranging between 242 min before BT to 34 min after BT.

Analysis of Individual Subjects' Data Patterns

Prior to employing inferential statistics for group comparisons, each subject's pattern was analyzed individually. First, each subject's data were analyzed to determine if the subject met two criteria, as described in *Data Analysis*: (a) the absence of a large phase-shift in sDLMO during the experiment, which indicated the constancy of the CS-US relationship throughout the training and testing sessions; and (b) the presence of melatonin suppression in response to the long bright light on the pretest, which indicated the presence of the UR. Successful conditioning was not expected in subjects who did not meet both of these criteria.

Second, individual pretest-posttest data patterns were reviewed. The review relied primarily on visual inspection of the graphical representations of each subject's melatonin levels from the two pretest nights and the two posttest nights. The aim of this review was to determine whether a pattern of pretest-posttest differences would emerge among experimental subjects that would suggest an increase in melatonin suppression on the posttest nights.

Magnitude of sDLMO shift and the presence of UR.

Three experimental subjects shifted their sDLMO by more than 60 min in the course of the experiment, showing 111 min delay, 85 min advance, and 80 min advance. The same three experimental subjects did not suppress melatonin in response to the long light on the pretest. Except these three, no other subjects evidenced the absence of the UR on the long light pretest. Among control subjects, everyone showed melatonin suppression on the long light pretest; however, two control subjects had a phase advance of 69 min and 109 min in the course of the experiment. Overall, out of 8 experimental and 7 control subjects, 3 experimental subjects had a large shift in sDLMO and did not have the UR, and 2 control subjects had a large shift in sDLMO.

Individual pretest-posttest patterns.

Individual subjects' results are graphically presented in Figures 2 through 16. The overall pattern can be summarized as follows. In the experimental group, the three subjects who did not meet the sDLMO and the UR criteria presented no evidence of melatonin suppression on either of the pretest or posttest sessions (see Figures 4, 8, and 9, experimental subjects 3, 7, and 8). Among the other five experimental subjects, none evidenced melatonin suppression on the short light pretest. On the short light posttest, only one experimental subject's data suggested a decrease in melatonin relative to post-NBL; however, this appears to be an artifact of generally lower values this subject had during the entire short light posttest session (see Figure 3, experimental subject 2). On the long light posttest, only one out of 5 experimental subjects showed stronger melatonin suppression (see Figure 6, experimental subject 5). One experimental subject who responded to the long light on the pretest did not evidence melatonin suppression on the long light posttest, with the first two samples after the light onset unexpectedly showing a nearly 50% increase in melatonin level (see Figure 5, experimental subject 4). In the control group, 3 subjects appeared to have decreased melatonin levels on the short light pretest, while none of the control subjects showed such a decrease on the short light posttest. All 7 control subjects suppressed melatonin in response to the long light both on the pretest and on the posttest. Two control subjects suppressed melatonin more on the long light posttest in comparison to the pretest (see Figures 12 and 13, control subjects 3 and 4), while the other five suppressed melatonin to a comparable extent on the posttest. On visual inspection, the possible changes in melatonin suppression between the pretest and the posttest did not appear to relate to the magnitude or direction of sDLMO phase-shifts. These analyses of individual subjects' data did not support the hypothesized effects of conditioning on the experimental subjects.

Statistical Analyses of Group Data

Exclusion from statistical analyses of 3 experimental and 2 control subjects who did not meet the sDLMO and the UR criteria was considered, since these criteria were believed to be essential for successful conditioning in the present experiment. However, based on the data collected in this experiment, no explanation could be provided to the questions as to why these 5 subjects demonstrated considerable phase shifts and why 3 of them showed no evidence of the UR on the pretest. Therefore, instead of excluding these subjects from the analyses of group data, each statistical analysis of the between-group differences was conducted twice, with and without the 5 subjects who did not meet the sDLMO and the UR criteria. The tables referenced below report group mean and standard deviation values for all 15 subjects. However, as the overall pattern of results does not change with the exclusion of these 5 subjects who did not meet the sDLMO and the UR criteria, graphical representations of group data for the sake of visual clarity do not include these 5 subjects.

Phase shift in sDLMO.

The possibility of a phase-shift in the course of the experiment was analyzed by comparing the time of sDLMO on the first night and on the last night of the experiment. When all 15 subjects were included in the analysis, it appeared that the experimental procedures did not induce a significant phase shift in the experimental subjects (Mean delay = 6 min, SD = 66 min, $t(7) = 0.2$, n.s.), or in the control subjects (Mean advance = 21 min, SD = 53 min, $t(6) = 1.0$, n.s.), nor did the phase shift differ between the groups ($t(13) = 0.8$, n.s.). When 5 subjects (3 experimental and 2 control) whose sDLMO shifted by more than 60 min in the course of the experiment were excluded, it reduced the variance but did not change the pattern of results. The analysis of the remaining 10 subjects showed no significant phase shift in the experimental group (Mean delay = 20 min, SD = 27 min, $t(4) = 1.6$, n.s.) or in the control group (Mean delay = 6 min, SD = 29 min, $t(4) = 0.5$, n.s.), nor were the groups different from each other ($t(8) = 0.8$, n.s.).

Long light pretest.

The effect of the long light on melatonin secretion during the pretest was analyzed using a 6 x 2 (Sample x Group) ANOVA. When all 15 subjects were included, the main effect of the sample variable was significant, $F(2, 26) = 17.3$, $p<.0005$, showing melatonin suppression by the long light. There was no overall difference between the groups, and no Sample x Group interaction (*F* ratios <1, n.s.), which indicated that the response to the long light was essentially the same in both groups (*M* and *SD* values for the group and sample variables are presented in Table 2). The comparison between pre-NBL and each of the samples revealed that sample 2 was similar to pre-NBL ($F(1,13) = 1.2$, n.s.), while all subsequent samples had significantly lower melatonin concentration values relative to pre-NBL (all $F(1,13)$ values > 13.0, and respective p values < .005). Melatonin levels began to increase between sample 5 and sample 6 ($F(1,13) = 13.6$, $p = .003$); however, sample 6 remained significantly lower than the pre-NBL ($F(1,13) = 16.4$, $p = .001$).

The same analysis was performed for the 5 experimental and 5 control subjects whose shift in sDLMO did not exceed the 60 min criterion. The main effect of the sample variable was significant, $F(2, 16) = 23.1$, $p<.0005$. There was no main effect of the group variable or Sample x Group interaction (*F* ratios <1, n.s, *M* and *SD* values are presented in Table 3). The comparison between pre-NBL and each of the samples revealed that sample 2 was similar to pre-NBL ($F(1,8) = 0.3$, n.s.), while all subsequent samples had significantly lower melatonin concentration values relative to pre-NBL (all $F(1,8)$ values > 13.0, and respective p values < .007).

Overall, during the pretest with the long light, both groups had similar baseline melatonin levels, which were reduced to a similar extent by exposure to the long light. It took approximately 30 min of light exposure to achieve a significant decline in salivary melatonin. Melatonin levels remained significantly below the baseline up to 30 min after the light was turned off, although melatonin began to rise between 15 and 30 min after the light was off. The effect of the pretest long light exposure is graphically presented in Figures 17 (5 experimental subjects) and 18 (5 control subjects).

Short light pretest.

The effect of the short light on melatonin secretion during the pretest was analyzed using a 6 x 2 (Sample x Group) ANOVA. No statistically significant main effects or interactions were detected regardless of whether the subjects who did not meet the sDLMO shift criterion were included or excluded (all respective F ratios < 1.2, n.s.). These results showed that there was no consistent effect of the short light prior to conditioning in either group. The mean and standard deviation values are shown in Table 3 (all 15 subjects), and the patterns of melatonin levels during the short light pretest are graphically presented in Figures 19 (5 experimental subjects) and 20 (5 control subjects).

Nocturnal melatonin baseline on the pretest and the posttest.

The experimental hypotheses concerned the effects of light exposure on the posttest. As the pretest samples were referenced to the pre-NBL and the posttest samples were referenced to the post-NBL, a comparison was made between the pre-NBL and the post-NBL with the use of a 2 x 2 (Pretest-posttest x Group) ANOVA. No main effects and no interaction were detected, regardless of whether or not the subjects who did not meet the sDLMO criterion were included (all F ratios < 1, n.s.). Thus, the experimental and the control group were not different from one another in terms of nocturnal melatonin baseline values. In addition, no change in nocturnal baseline melatonin values occurred between the pretest and the posttest in either of the groups. The mean and standard deviation values are shown in Tables 2 and 3 (pre-NBL), and in Tables 4 and 5 (post-NBL).

Short light: pretest-posttest comparison.

According to the first experimental hypothesis, the effect of the short light on melatonin was expected to be different between experimental and control subjects on the posttest, while no difference between the groups was expected on the pretest. To evaluate this hypothesis, a 2 x 6 x 2 (Pretest-posttest x Sample x Group) ANOVA was used. The interaction of interest was the interaction between all three variables, which, if significant, would show that the groups responded differently to the short light on one of the testing sessions but not on the other. The homogeneity of covariance assumption was not violated for these data.

The short light posttest mean and standard deviation values are presented in Table 4 (all 15 subjects), and the patterns of melatonin levels during the short light posttest are graphically presented in Figures 21 (5 experimental subjects) and 22 (5 control subjects). The ANOVA results indicated no main effects and no interactions, regardless of whether or not the subjects who did not meet the sDLMO criterion were included (all respective F ratios < 1.6, n.s.). Thus, the first hypothesis was not supported.

Long light: pretest-posttest comparison.

According to the second experimental hypothesis, the effect of the long bright light was expected to be different between the groups on the posttest, but not on the pretest. To evaluate this hypothesis, a 2 x 6 x 2 (Pretest-posttest x Sample x Group) ANOVA was used. The first level of the sample variable was the pre-NBL or the post-NBL, respectively. The remaining 5 levels of the sample variable were the 5 samples taken after the onset of light. The interaction of interest was the interaction between all three variables, which, if significant, would show that the groups responded differently to the long light on one testing session but not the other.

The long light posttest mean and standard deviation values are presented in Table 5 (all 15 subjects), and the patterns of melatonin levels during the long light posttest are graphically presented in Figures 23 (5 experimental subjects) and 24 (5 control subjects). When all 15 subjects were included in the analysis, it was found that only the main effect of the sample variable was statistically significant, $F(2, 32) = 18.3$, $p < .0005$. No other main effects or interactions were found (all F ratios < 2.0, n.s.). The same pattern of results was found when only 10 subjects were included in the analysis (main effect of the Sample factor: $F(2, 16) = 24.8$, $p < .0005$; other main effects and interactions: all F ratios < 2, n.s.).

Similar to the results of the long light pretest, the pretest and the posttest data combined revealed that a significant decrease in melatonin level first occurred on the sample 3 taken approximately 30 minutes after the onset of light (all 15 subjects: $F(1,13) = 11.6$, $p = .005$; only 10 subjects: $F(1,8) = 7.7$, $p = .02$). The subsequent samples remained significantly below the baseline values (for 15 subjects or 10 subjects, all F ratios > 15.0, all p values < .003). The absence of main effects of the pretest-posttest variable and the group variable, and the absence of any interactions indicate that melatonin was suppressed by the long light to a similar degree in both groups on the pretest as well as on the posttest. Thus, the second hypothesis was not supported.

Exploratory analysis.

The analysis of the short light pretest and posttest sessions revealed no significant change in melatonin levels relative to the respective pre- or post-NBL in either experimental or control subjects, as described above. During the long light exposure, however, both groups showed a significant and similar decrease in melatonin levels on both the pretest and the posttest. As there were individual differences in the degree and direction of the sDLMO shift during the experiment, and in the change between the pre-NBL and the post-NBL values, a possibility existed that differences between the groups on the long light posttest might have been obscured by sDLMO shifts or changes in melatonin baseline. Therefore, an exploratory analysis was performed to examine whether the change in response to the long light between the pretest and the posttest was related to the change in the nocturnal melatonin baseline or to the shift in sDLMO.

First, for each subject the lowest melatonin value on long light posttest was subtracted from the lowest value on the long light pretest to obtain a measure of the change in melatonin suppression. Similarly, for each subject the post-NBL was subtracted from the pre-NBL to obtain a measure of the change in nocturnal baseline. These two measures were correlated with each other and with the shift in sDLMO using Pearson product-moment correlation. None of these three correlations were significant, regardless of whether or not the subjects who did not meet the sDLMO and the UR criteria were excluded (all r values $< .45$, all p values $> .09$).

Thus, any changes that may have occurred between the pretest and the posttest in melatonin suppression by the long light were not related to changes in nocturnal melatonin baseline or to sDLMO shifts. Therefore, the absence of statistically significant difference between the groups on the long light posttest cannot be due to phase shifts in sDLMO or to changes in baseline melatonin levels.

Discussion

The aim of the present experiment was to evaluate whether repeated exposure of human subjects to 45 min of bright light at the same time in the early subjective night would enhance the response to bright light given at this time of night. It was expected that after training with repeated exposure to the 45 min bright light pulse, 4.5 min of bright light given at the same time of night would measurably suppress melatonin secretion, and that 45 min of bright light given at the same time of night would suppress melatonin to a greater extent than in the beginning of training. These effects were expected to occur due to the learned association between the time of light exposure and the light itself. To control for procedure effects associated with repeated measures, control subjects were exposed to 45 min of dim light at the same time of night.

The data provide no evidence that repeated exposure to the 45 min long pulse of bright light 2 hr after the bedtime alters melatonin response to bright light. On the pretest, both groups showed an equal amount of melatonin suppression during the long light exposure. Neither group evidenced melatonin suppression on the pretest with the short light. Similarly to the pretest sessions, the posttests with the short bright light and the long bright light yielded no significant differences between the groups. Melatonin suppression was absent in both groups during the short light posttest, and both groups showed equal amounts of suppression during the long light posttest. In addition, the baseline melatonin values were not changed between the pretest and the posttest and did not differ between the groups. These results show no effect of conditioning and lend no support to the hypothesis that the time of light delivery may serve as an associative cue during light exposure in humans.

In addition to the statistical analysis of group data, the review of the individual subjects' patterns does not suggest meaningful differences between the pretest and the posttest in the experimental subjects. For example, one experimental subject, whose sDLMO remained almost exactly the same and whose melatonin suppression by the long light on the pretest was perhaps the clearest, did not show any evidence of conditioning on the short light or the long light posttest sessions (Figure 2). A pattern of another experimental subject seems to suggest the possibility of a stronger response on the long light posttest (Figure 6); however, a tendency towards a stronger response on the long light posttest can also be found in at least one control subject (Figure 12).

The negative results of the present study are in contradiction with animal data on conditioning of the circadian system (Amir & Stewart, 1996; 1998a; 1998b; Arvanitogiannis & Amir, 1999; 2001; Golombek at al, 1994; Robinson et al., 1996). Although De Groot and Rusak (2000) failed to replicate conditioned responses in rats, methodological reasons may account for their negative findings, as discussed in the introduction. Therefore, assuming that animal data have revealed a true phenomenon, the absence of evidence for conditioned melatonin suppression in humans in the present study may be explained in several different ways.

Several methodological issues may be considered in attempt to account for the negative findings. First, as animal data suggest, the CT of light exposure is an important associative cue. The present experimental hypothesis is built on this notion. However, unlike animal studies, subjects in the present experiment, albeit required to keep a rigid bedtime and uptime schedule while at home, were frequently unable to do so due to the constraints imposed on them by everyday life (school, work, and family obligations).

Numerous times different subjects reported that they had to stay up late or wake up early, and on occasion a given subject may not even have reported the schedule change to avoid being excluded from the study. Although subjects went to bed in the lab at the same time on all training and testing sessions, and were always awakened exactly 2 hr after the bedtime, changing their sleep schedule at home may have resulted in substantial changes in their melatonin rhythms throughout the experiment. These changes may have led to variations in the CT when the bright light was presented at night in the lab. Varying the CT of light presentation would prevent building associations between the CT and light exposure by reducing the predictive value of the CT. In short, if participants experienced self-induced circadian shifts, it would undermine the role of time as a CS and thus would prevent learning. This explanation seems to be supported by the observation that the magnitude and the direction of the phase shift in the time of sDLMO between the first and the last nights of the study varied substantially between subjects. A phase-shift in sDLMO by more than 60 min in either direction was considered detrimental to the process of conditioning, as it would indicate that the CT of light exposure, one of the two parts of the compound CS, changed substantially in the course of the experiment. Five subjects, three experimental and two control, demonstrated sDLMO shifts of more than one hour. The fact that these five subjects either did not have the UR or were in the control group (Figures 4, 8, 9, 13, 14) supports the notion that phase shifts were largely self-induced and not due to the repeated bright light exposure at night. However, the overall pattern of results did not change when subjects who had shown a phase shift of more than one hour were excluded from analyses. Phase shifts in sDLMO were also not related to the amount of melatonin suppression on the long light posttest or to the posttest melatonin baseline levels. In addition, the one experimental subject who had virtually no phase shift in sDLMO did not show any evidence of melatonin suppression on the short light posttest, or any response enhancement on the long light posttest (Figure 2). It therefore appears that the shift in the rhythm of melatonin secretion, as assessed by sDLMO, does not contribute to the posttest response to bright light. While this conclusion does not eliminate the possibility that the expected learning process in the experimental group was hindered by the variability in the CT of exposure, it makes this possibility less likely.

Second, the present negative results may have to do with individual variability in the response to the long bright light. The absence of melatonin suppression in response to the long light on the pretest was considered indicative of the absence of the UR, which would make conditioning impossible. Accordingly, the finding that three out of 15 subjects did not show any evidence of melatonin suppression by 45 min of bright light on either the pretest or the posttest suggests that the training stimulus may not have been potent enough to induce a consistent UR. Thus, the absence of the UR during learning trials would preclude learning of a CR. This explanation does not seem likely, however, because the other 12 subjects showed a measurable suppression of salivary melatonin, and the overall effect of the long light was highly significant even when all 15 subjects were analyzed. Moreover, when statistical analyses included only the subjects who had the UR, there was still no evidence of the enhanced response to the long light or any response to the short light during the posttest sessions among experimental subjects in comparison to control subjects. Therefore, individual differences in the strength of the

UR cannot fully account for the lack of evidence for experimental subjects' greater melatonin suppression on the posttest sessions.

Third, it is possible that the expected learning effect did not take place because the number of training sessions was not sufficient, or the sessions were scheduled too far apart. In animal experiments, the number of training sessions varied between 7 (Golombeck et al., 1994) and 40 (Arvanitogiannis & Amir, 1999), while 10 training sessions were used most often (Amir & Stewart, 1996; 1998a; 1998b; Arvanitogiannis & Amir, 2001). In the present experiment, which was conceptually similar to Arvanitogiannis and Amir (2001), 10 training sessions were used. However, unlike the cited animal experiments where training sessions were conducted every day, in the present experiment training sessions occurred only twice a week to minimize sleep deprivation and to avoid a perpetual phase delay among experimental subjects due to repeated long bright light exposure at night. Allowing 2-3 melatonin cycles to occur between every two consecutive training sessions may have undermined the learning process. Indeed, if a CR is to be learned, the CS must be consistently paired with the US, whereas in the present experiment the time of light exposure was actually paired with light only twice out of every 7 occurrences. An argument against this possibility lies in the dual nature of the present CS. Similar to Arvanitogiannis and Amir's (2001) study, the CS in the present experiment was a compound stimulus, a combination of the CT of light exposure and the onset of light. While the CT used for training was paired with the long bright light stimulus only intermittently, subjects experienced bright light in the middle of the night only in the laboratory. (Even if a subject turned on ceiling lights at home in the middle of the night by mistake, the brightness of a typical ceiling light – 150 to 300 lx – is approximately 20 to 40 times lower than the brightness of the experimental light; in addition, it would occur only rarely and in a different context.) Thus, the onset of the experimental bright light as part of the compound CS was a distinct cue, and the compound CS in the present experiment was still distinctive and present only in the context of experimental manipulations, which should have facilitated the conditioning process. Therefore, while it is possible that scheduling training sessions only twice a week could play a role in the lack of evidence for conditioning, the extent of this possibility remains unclear.

And forth, the type of light used as the experimental stimulus may not have been optimal for successful conditioning. As discussed in the introduction, the use of the same type of light for both conditioning and entrainment may have prevented De Groot and Rusak (2000) from finding a CR in their first experiment due to the variable effect of light presented at two opposite segments of the PRC. In a similar experiment by Amir and Stewart (1996; 1998b), an ultraviolet light was used for conditioning as a distinct light type, and white light was used for entrainment. In the present experiment, ambient white light was the main source of entrainment for subjects, and an artificial white light was used for conditioning sessions. The similarity between the entrainment light and the experimental light may have resulted in the experimental light stimulus not being a distinct enough US. It should be noted that in De Groot and Rusak's (2000) study the same light fixture was used to deliver light of the same spectral characteristics and the same brightness to rats that remained in the same cages throughout the entire experiment, including entrainment and conditioning. In the present study, the light fixture used for conditioning was a novel stimulus, and the experimental light, although similar in appearance, was still different in spectral characteristics and in brightness from ambient light. Moreover, the laboratory context in which conditioning took place was different from the context in which entrainment took place. These physical and contextual differences between ambient light and the experimental light were deemed sufficient to create a distinct US. However, the possibility remains that the experimental light was not distinct enough for conditioning to take place.

Conceptual differences between the present study and animal experiments may also help explain the negative results. It is possible that the lack of conditioning in human subjects may be due to a distinct role of social and cognitive factors. For example, some subjects seemed to have understood the pretest-posttest structure. A single-blind procedure employed in the present study was meant to control for this demand characteristic. However, due to the necessity of repeated measures and multiple training sessions associated with conditioning, a number of subjects developed expectations as to the type of light to be delivered, which was reflected in their comments in the evening or immediately after awakening. Although a distractor task could possibly be employed, it is unlikely that any task would be sufficient to divert subjects' attention away from such an obvious stimulus as bright light in the middle of the night without compromising dim background illumination and relative immobility required for salivary melatonin sampling. In addition, subjects were tested in groups of 2-3 people and may have discussed the light exposure experiences with each other when leaving the lab despite the explicit instruction not to do so. It is unclear how expectations regarding experimental procedures might have affected subjects' responses.

Another conceptual issue that may play a role in the process of conditioning has to do with the nature of the CR. For example, a pattern presented by one experimental subject suggests a possibility of conditioned tolerance. This subject showed evidence of melatonin suppression in response to the long light during the pretest but not during the posttest (Figure 5). A theory of conditioned tolerance is based on opponent processes model and predicts that in some forms of classical conditioning the CR appears to be the opposite of the UR (Brandon & Wagner, 1991). The CR in such cases represents a later part of the UR when the organism compensates for the initial disruption of homeostatic balance (Brandon & Wagner, 1991). In agreement with this theory, the absence of melatonin suppression by the long light on the posttest in the subject who suppressed melatonin on the pretest may be explained by learning to produce more melatonin to counteract the effect of light exposure at night. It is unclear, however, why similar effect was not present in other experimental subjects, as seen from the absence of a statistically significant difference between the pretest and the posttest. In addition, when this individual subject was removed from the statistical analysis, the results of comparisons between the groups and between the testing sessions did not change. Therefore, the suggestion of conditioned tolerance is not confirmed by the data collected in the present experiment, and the pattern presented by this individual subject has to be considered a random variation.

Lastly, the dependent variable used in the present study was qualitatively different from outcome measures used in animal studies. For example, Amir and associates (Amir & Stewart, 1996, 1998a, 1998b; Arvanitogiannis & Amir, 1999, 2001) used rest-activity and body temperature cycles as well as immunocytochemical measures. These animal experiments employed true circadian variables to successfully show the effect of conditioning on the circadian system. Presently, however, the effect of conditioning was measured by using acute suppression of salivary melatonin, which is not a direct measure of the circadian system's response to light.

The involvement of acute melatonin suppression by light in circadian regulation has been hypothesized based on the presence of melatonin receptors in the SCN (Arendt, 1995). These receptors that have been found in rodents and humans (Reppert at al., 1988; Vanecek & Watanabe, 1999) provide the feedback information about circulating melatonin levels. This feedback mechanism is thought to be involved in the phase-shifting effect of exogenous melatonin described in animals and humans, because the effect of exogenous melatonin depends on the intact SCN (Arendt, 1995; Cassone et al., 1986; Lewy et al., 1998). Moreover, since light presented at a shift-sensitive time produces an acute reduction in melatonin, the feedback of the information about circulating melatonin levels to the SCN may also be involved in the process by which environmental light entrains and shifts circadian rhythms (Arendt, 1995). Suppression of melatonin by light has been referred to as "a proxy for the effects of light on the human circadian pacemaker" (Zeitzer et al., 2000, p. 699).

Following this logic, the present work was based on the supposition that melatonin suppression reflects the activation of a process by which environmental light entrains and phase-shifts the circadian system. However, the possibility that acute melatonin response to light is involved in circadian regulation has not been empirically clarified, mainly due to the confounding nature of light as a stimulus that exerts both acute and phase-shifting effects simultaneously. Therefore, it is conceivable that acute melatonin suppression and circadian regulation may be physiologically independent. In that case, experiments by Amir and associates (Amir & Stewart, 1996, 1998a, 1998b; Arvanitogiannis & Amir, 1999, 2001) and the present experiment tap into different physiological processes, which accounts for differences in findings.

Golombeck et al. (1994) used melatonin as a dependent measure, which was similar to the present study. However, they conditioned production of melatonin using the offset of light as a US, while in the present experiment an attempt was made to condition suppression of melatonin. It is possible that melatonin production may be conditioned during early night when melatonin naturally increases (Golombeck et al., 1994), whereas melatonin suppression during early night is not amenable to conditioning (present findings). This reasoning is consistent with the role of the CT in the process of conditioning of the circadian system (Arvanitogiannis & Amir, 1999, 2001) discussed in the introduction. Indeed, since the state of the circadian system changes as a function of the CT, the relationship between the CT and the circadian system's response to environmental zeitgebers may be determined by the state of readiness of the system to produce a given response. Therefore, the limits of conditioning, that is, the type of CR that can occur, may also depend on the internal state of the circadian system. Thus, while the acute UR, melatonin suppression, was clearly evident in the present study, the attempted CR could not take place, as the state of the circadian system was consistent with melatonin production at the time when conditioning was taking place.

To address procedural and conceptual issued discussed herein, several steps can be taken in future studies. First, participants have to be screened for nocturnal melatonin production, for the circadian phase position such as the time of salivary or plasma

DLMO, and for the UR to the bright light stimulus intended to be used as a US. Second, participants' compliance with the experimentally imposed schedule has to be ensured by requiring participants to live in the lab for the duration of the experiment. Third, the training sessions have to take place once per 24 h cycle. Forth, a spectrally distinct type of light, such as blue light (Wright, Lack, & Kennaway, 2004), should be used as a US. And finally, in addition to the direct melatonin suppression, other measures of the circadian system's response to light have to be employed, such as the circadian phase of the melatonin cycle and the body temperature cycle, for which constant routine protocols may be needed before and after the training sessions.

In conclusion, it should be noted that a number of general conditions in the present study, including individual differences in response to light, schedule disruptions due to everyday life events, and cognitive expectations, were roughly similar to what a person with a circadian rhythm disorder might experience during treatment. Therefore, even if the possibility of conditioning of the human circadian system is confirmed in the future under more controlled experimental conditions, the results of the present study suggest that the clinical usefulness of the conditioning procedure in the bright light treatment may be limited at best.

Table 1. A Summary of the Experimental Design

Night #	Procedure	Description of procedure	Subjects used
1	sDLMO	Eleven saliva samples in 30 min intervals starting 4 hr before BT and ending 1 hr after BT	All subjects
2	Pretest #1, short bright light	Six saliva samples in 15 min intervals starting approximately 2.2 hr after BT; 4.5 minutes of bright light after the first sample	All subjects
3	Pretest #2, long bright light	Six saliva samples in 15 minute intervals starting approximately 2.2 hr after BT; 45 minutes of bright light after the first sample	All subjects
4-13	Training with long bright light	Six saliva samples in 15 minute intervals starting approximately 2.2 hr after BT; 45 minutes of bright light after the first sample	Experimental subjects
	Training with long dim light	Six saliva samples in 15 minute intervals starting approximately 2.2 hr after BT; 45 minutes of dim light after the first sample	Control subjects
14	Posttest #1	Six saliva samples in 15 minute intervals starting approximately 2.2 hr after BT; either 4.5 or 45 minutes of bright light after the first sample	All subjects
15	Posttest #2	Six saliva samples in 15 minute intervals starting approximately 2.2 hr after BT; either 4.5 or 45 minutes of bright light after the first sample	All subjects
16	sDLMO	Eleven saliva samples in 30 min intervals starting 4 hr before BT and ending 1 hr after BT	All subjects

Note. Abbreviations: sDLMO = salivary dim light melatonin onset; BT = bedtime.

Table 2.

Mean Salivary Melatonin Values on the Long Light Pretest

	Experimental Group (n=8)	Control Group (n=7)
Pre-NBL	17.0 (7.3)	18.7 (6.9)
Sample 2	16.8 (8.6)	17.0 (5.2)
Sample 3	13.2 (4.3)	13.1 (2.4)
Sample 4	10.8 (3.8)	10.9 (3.1)
Sample 5	10.4 (3.3)	8.8 (1.7)
Sample 6	12.7 (3.9)	11.1 (2.0)

Note. The table presents group means (standard deviations in parentheses) for the pretest nocturnal melatonin baseline (pre-NBL) and for the melatonin contents of saliva samples collected after the onset of the long bright light on the pretest night (samples 2 through 6). Salivary melatonin contents are measured in picograms per milliliter. Pre-NBL was calculated for each subject by averaging sample 1 from the short light pretest and sample 1 from the long light pretest. The light was turned on immediately after sample 1 and approximately 15 min before sample 2. The light exposure lasted 45 min. Samples were collected in 15 min intervals.

Table 3.

Mean Salivary Melatonin Values on the Short Light Pretest

	Experimental Group (n=8)	Control Group (n=7)
Pre-NBL	17.0 (7.3)	18.7 (6.9)
Sample 2	16.9 (7.8)	18.4 (7.4)
Sample 3	18.1 (7.2)	18.5 (8.8)
Sample 4	16.7 (7.9)	19.5 (9.4)
Sample 5	19.5 (7.3)	19.1 (9.8)
Sample 6	19.9 (7.9)	19.1 (9.5)

Note. The table presents group means (standard deviations in parentheses) for the pretest nocturnal melatonin baseline (pre-NBL) and for the melatonin contents of saliva samples collected after the onset of the short bright light on the pretest night (samples 2 through 6). Salivary melatonin contents are measured in picograms per milliliter. Pre-NBL was calculated for each subject by averaging sample 1 from the short light pretest and sample 1 from the long light pretest. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 4.5 min. Samples were collected in 15 min intervals.

Table 4.

Mean Salivary Melatonin Values on the Short Light Posttest

	Experimental Group (n=8)	Control Group (n=7)
Post-NBL	16.5 (7.3)	20.5 (6.7)
Sample 2	16.8 (8.8)	22.6 (7.8)
Sample 3	16.3 (8.2)	22.2 (8.3)
Sample 4	18.0 (7.1)	21.9 (8.4)
Sample 5	19.0 (11.3)	23.3 (8.7)
Sample 6	17.5 (7.5)	22.6 (7.5)

Note. The table presents group means (standard deviations in parentheses) for the posttest nocturnal melatonin baseline (post-NBL) and for the melatonin contents of saliva samples collected after the onset of the short bright light on the posttest night (samples 2 through 6). Salivary melatonin contents are measured in picograms per milliliter. Post-NBL was calculated for each subject by averaging sample 1 from the short light posttest and sample 1 from the long light posttest. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 4.5 min. Samples were collected in 15 min intervals.

Table 5.

Mean Salivary Melatonin Values on the Long Light Posttest

	Experimental Group (n=8)	Control Group (n=7)
Post-NBL	16.5 (7.3)	20.5 (6.7)
Sample 2	18.4 (9.4)	16.2 (6.9)
Sample 3	15.2 (9.2)	14.3 (7.1)
Sample 4	11.5 (5.8)	11.0 (6.1)
Sample 5	13.0 (7.7)	9.6 (5.5)
Sample 6	15.9 (8.4)	12.7 (6.4)

Note. The table presents group means (standard deviations in parentheses) for the posttest nocturnal melatonin baseline (post-NBL) and for the melatonin contents of saliva samples collected after the onset of the long bright light on the posttest night (samples 2 through 6). Salivary melatonin contents are measured in picograms per milliliter. Post-NBL was calculated for each subject by averaging sample 1 from the short light posttest and sample 1 from the long light posttest. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 45 min. Samples were collected in 15 min intervals.

Figure 1. Graphical representation of experimental procedures. The down and up arrows on the top of the figure represent the scheduled bedtime and the uptime, respectively. Each horizontal bar represents a procedure performed in the lab. White areas on each bar represent lights on and out of bed, black areas represent lights off and in bed, and gray areas represent dim illumination and out of bed in a reclining position. The vertical lines protruding down from each bar indicate the times of saliva sampling. The white or gray rectangles embedded in the bars represent the times of bright light or dim light exposure, respectively. The text to the left of each bar indicates the night number, the procedure performed, and the subjects tested. Abbreviations: Nt = night; sDLMO = salivary dim light melatonin onset; Ss = subjects; Exp = experimental; Cont = control.

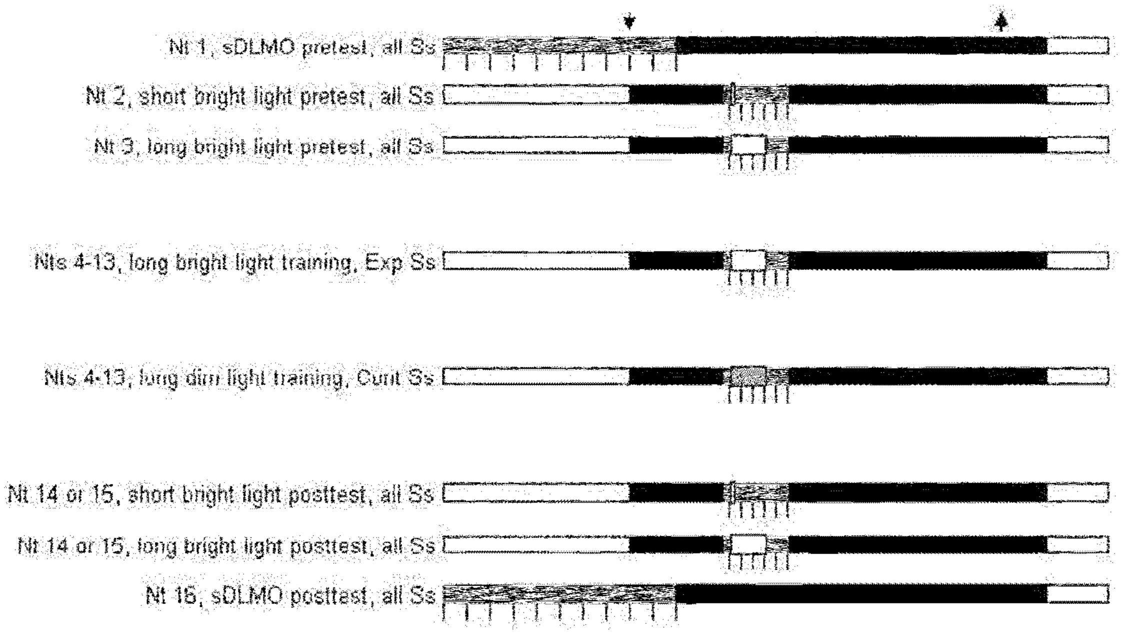


Figure 2. Melatonin values on four test nights for experimental subject 1. The subject showed similar melatonin suppression on the long light pretest and the posttest. No evidence of melatonin suppression was present on the short light pretest or the posttest.

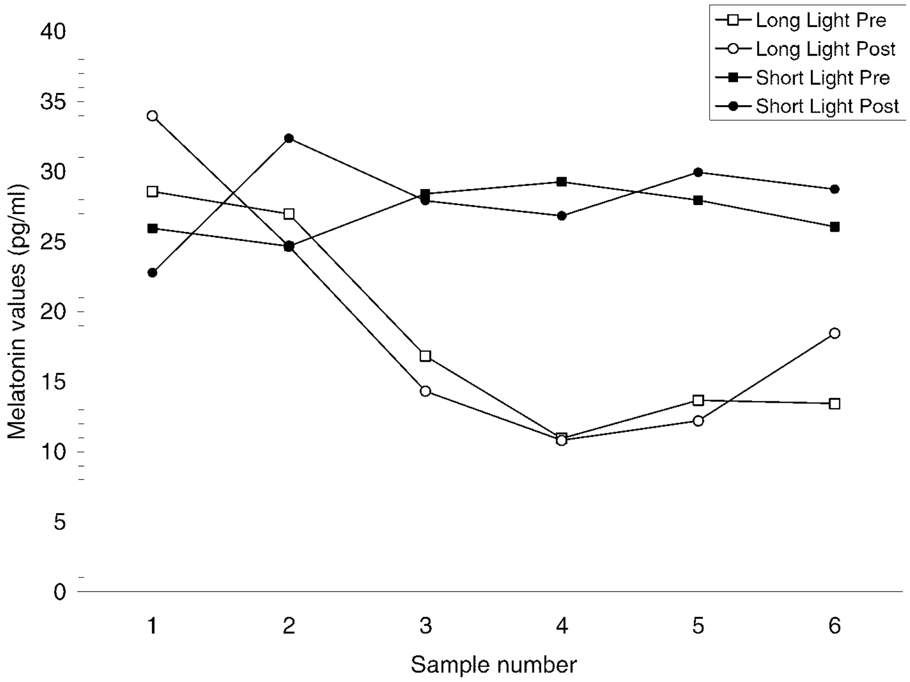


Figure 3. Melatonin values on four test nights for experimental subject 2. The subject showed approximately equal melatonin suppression on each of the long light nights, and no melatonin suppression on the short light pretest. Melatonin values were overall lower during the short light posttest in comparison to the short light pretest.

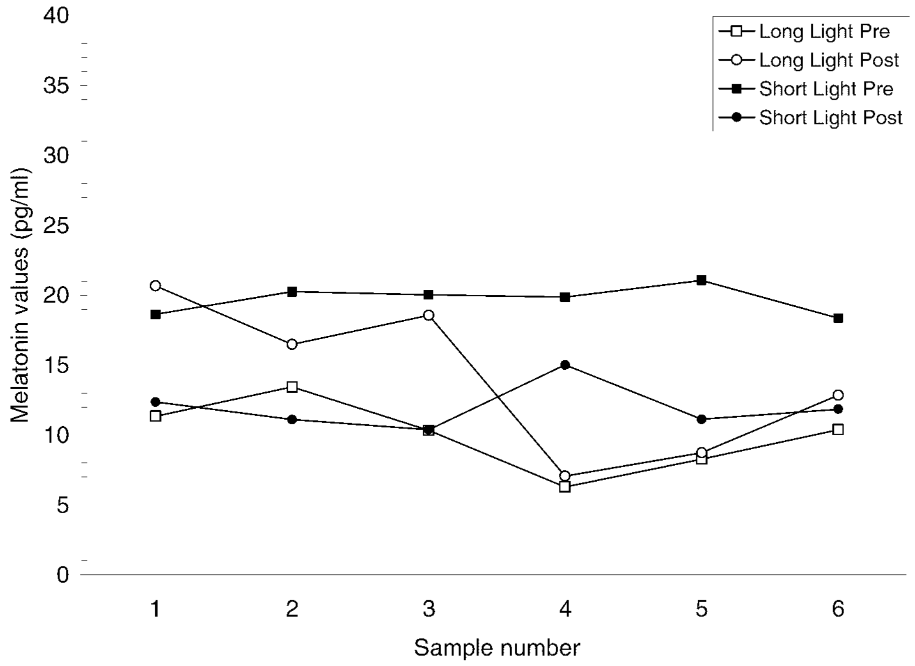


Figure 4. Melatonin values on four test nights for experimental subject 3. The subject showed no melatonin suppression on either of the long light nights, and no melatonin suppression on either of the short light nights.

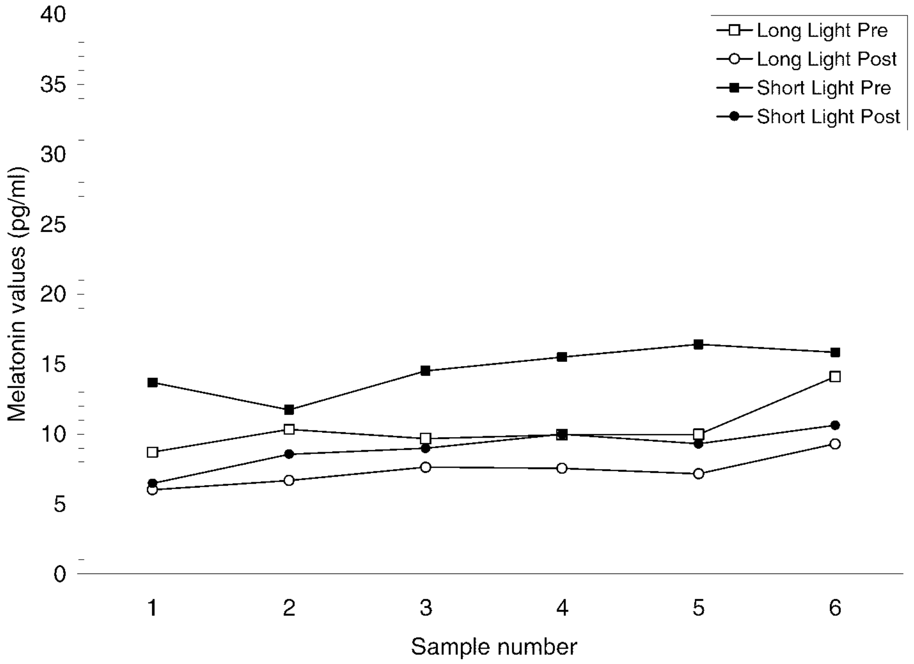


Figure 5. Melatonin values on four test nights for experimental subject 4. The subject showed melatonin suppression on the long light pretest but not on the long light posttest; no melatonin suppression was evident on either of the short light nights.

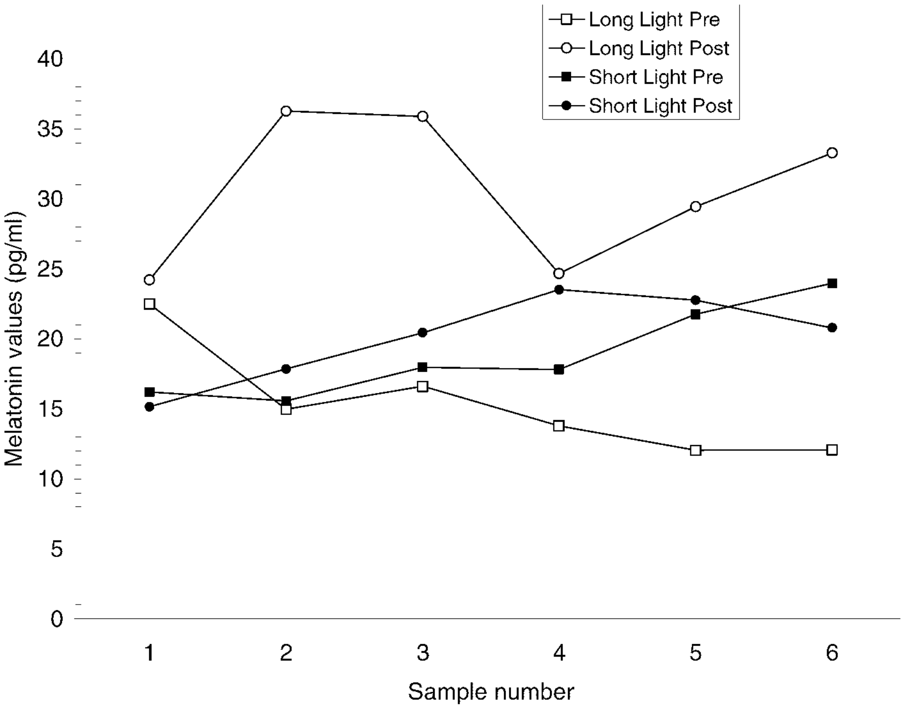


Figure 6. Melatonin values on four test nights for experimental subject 5. Melatonin suppression during the long light appeared to be stronger on the posttest in comparison to the pretest. No melatonin suppression was evident on either of the short light nights.

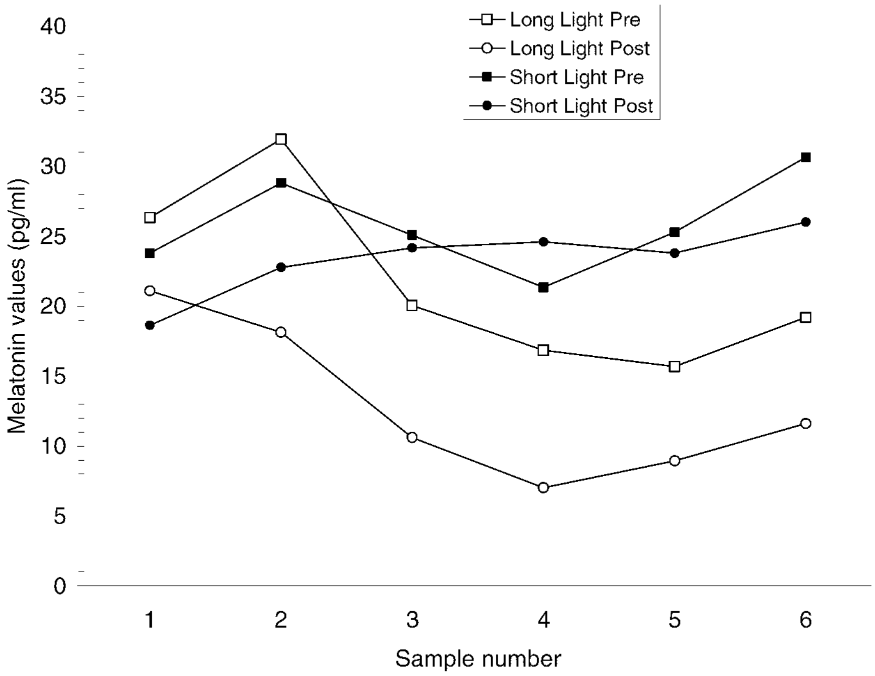


Figure 7. Melatonin values on four test nights for experimental subject 6. Melatonin suppression was comparable during the long light exposure on the pretest and on the posttest. No clear evidence of melatonin suppression was present on either of the short light nights.

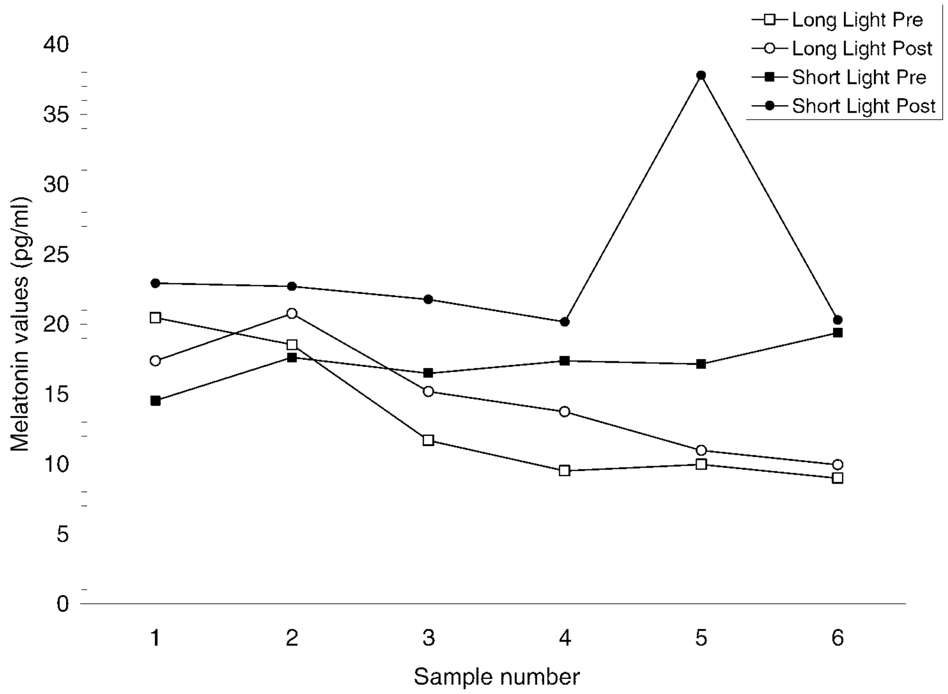


Figure 8. Melatonin values on four test nights for experimental subject 7. This subject's melatonin values were quite variable and no consistent suppression could be found on all 4 nights.

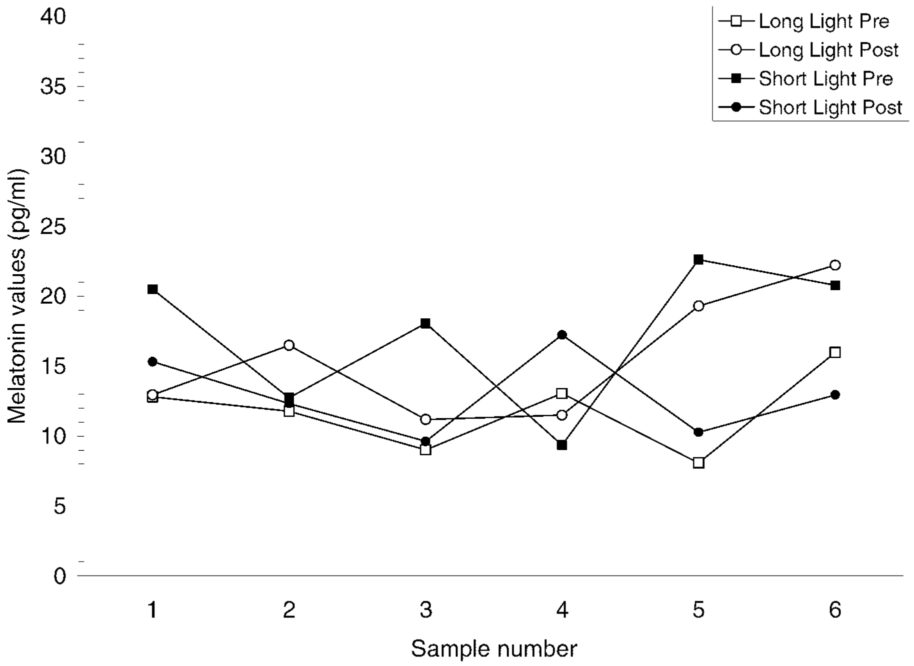


Figure 9. Melatonin values on four test nights for experimental subject 8. The subject showed no melatonin suppression on either of the long light nights, and no melatonin suppression on either of the short light nights.

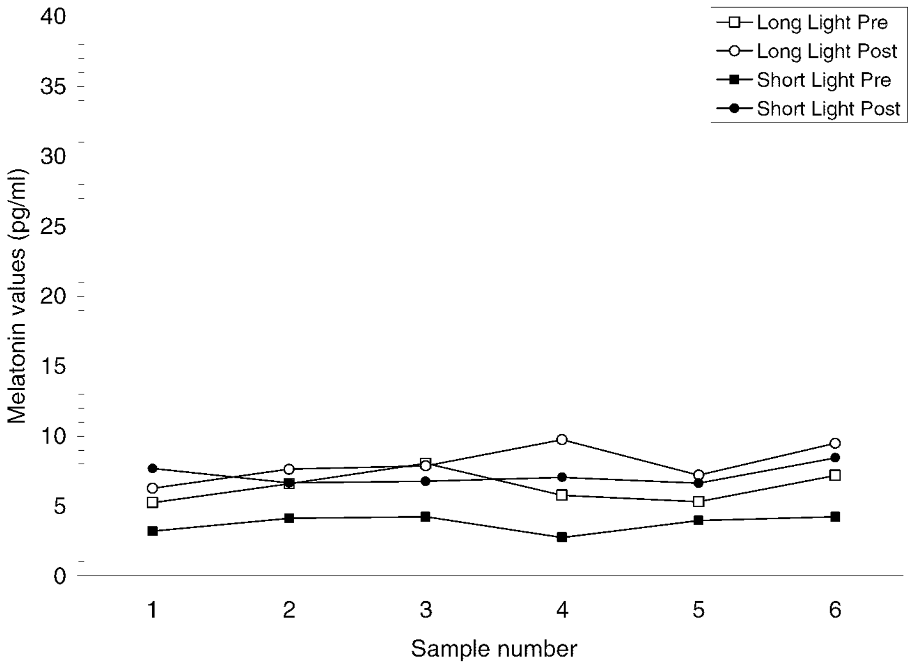


Figure 10. Melatonin values on four test nights for control subject 1. The subject showed similar melatonin suppression on both the long light pretest and the posttest nights, and no melatonin suppression on either of the short light nights.

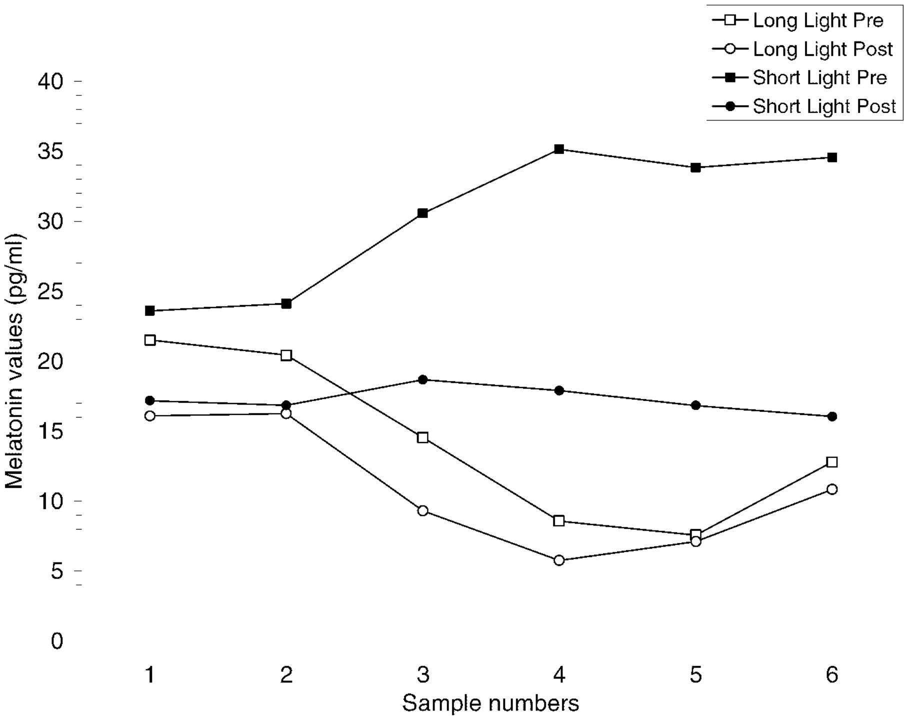


Figure 11. Melatonin values on four test nights for control subject 2. The subject showed similar melatonin suppression on both the long light pretest and the posttest nights, and no melatonin suppression on either of the short light nights.

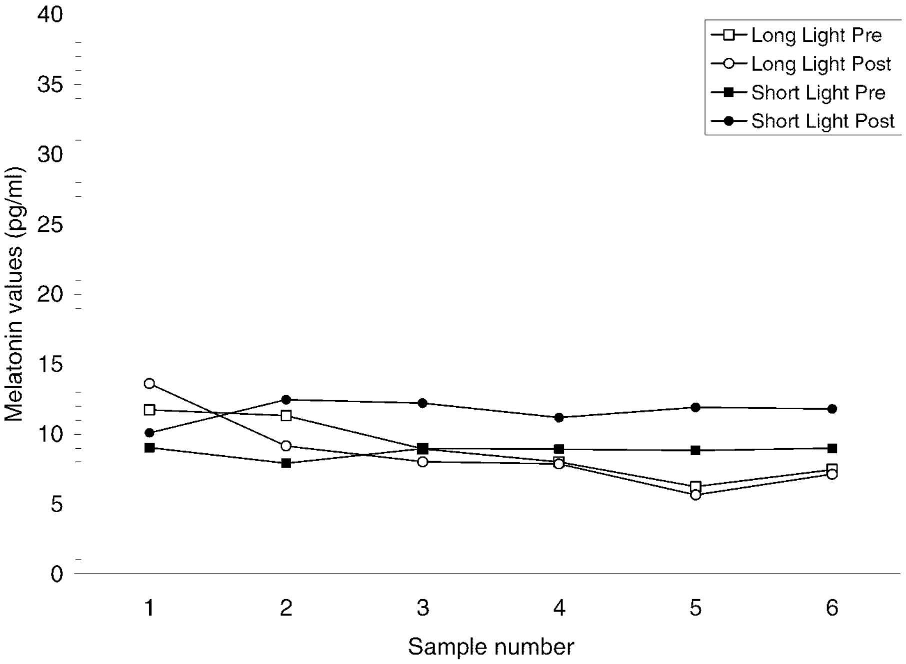


Figure 12. Melatonin values on four test nights for control subject 3. The subject showed stronger melatonin suppression on the long light posttest than on the long light pretest. No consistent evidence of melatonin suppression was found on either of the short light nights.

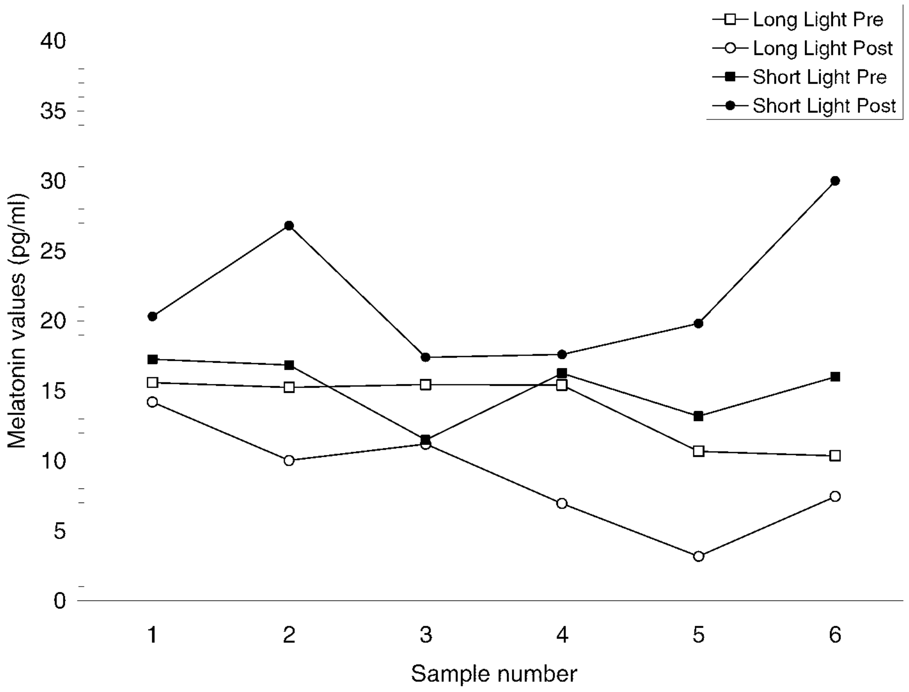


Figure 13. Melatonin values on four test nights for control subject 4. The subject showed stronger melatonin suppression on the long light posttest than on the long light pretest. No consistent evidence of melatonin suppression was found on either of the short light nights.

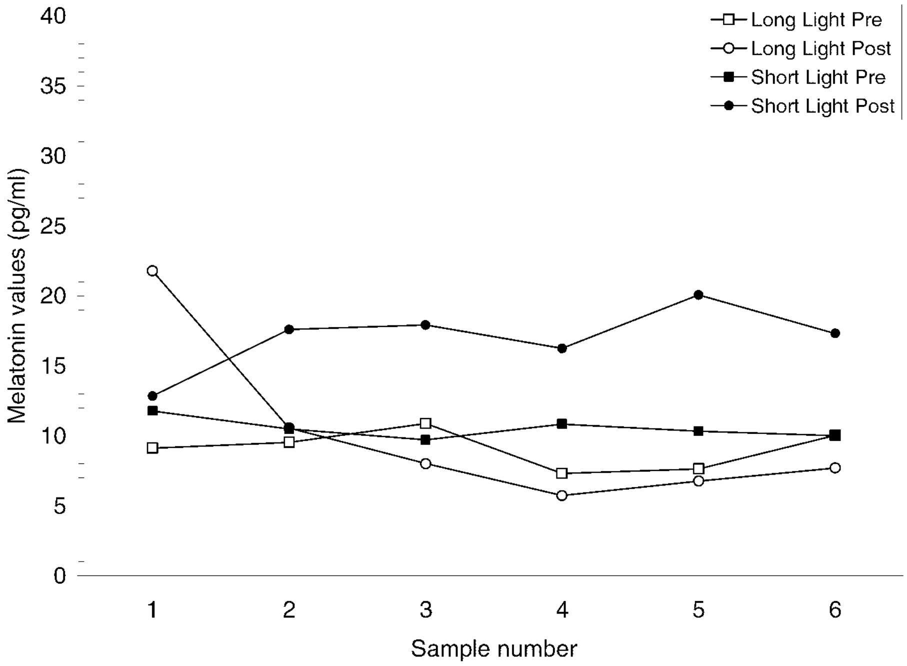


Figure 14. Melatonin values on four test nights for control subject 5. The subject showed similar melatonin suppression during the long light on both the pretest and the posttest. Melatonin suppression also appeared on the short light pretest, but not on the short light posttest.

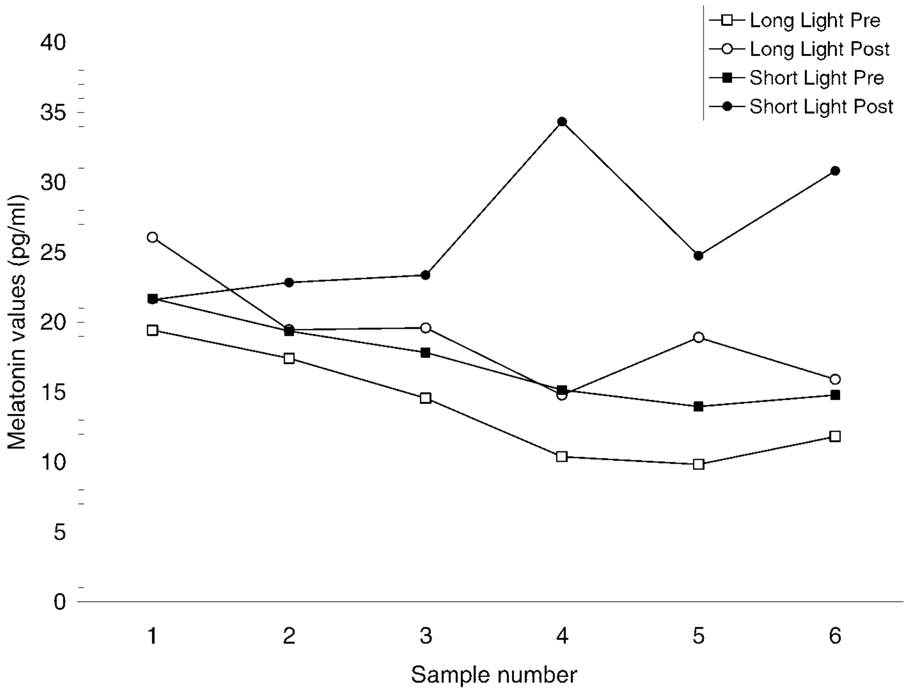


Figure 15. Melatonin values on four test nights for control subject 6. The subject showed similar melatonin suppression during the long light on both the pretest and the posttest. Melatonin suppression also appeared on the short light pretest, but not on the short light posttest.

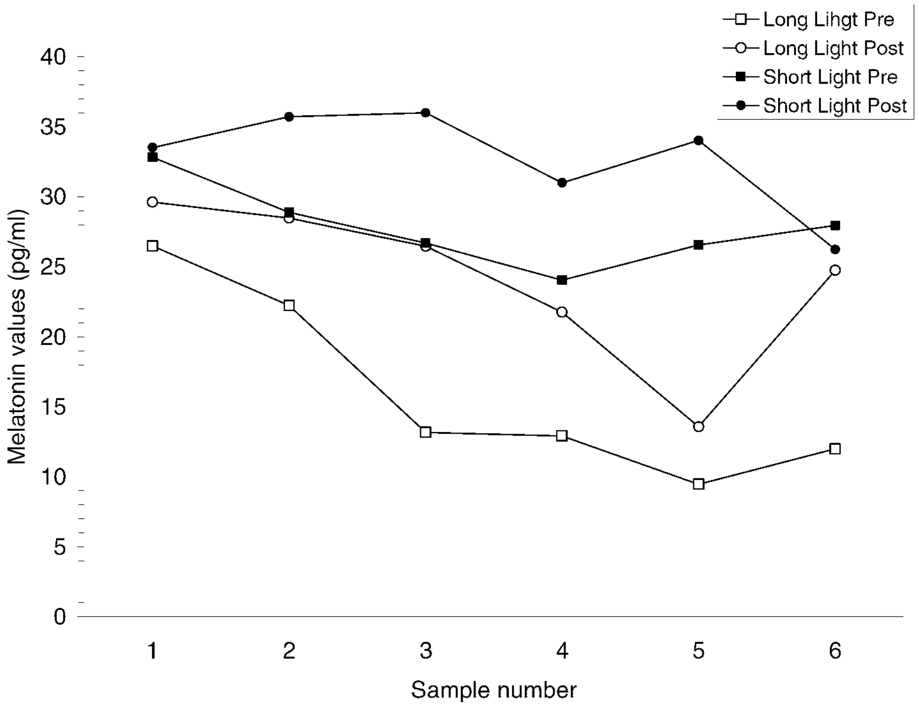


Figure 16. Melatonin values on four test nights for control subject 7. The subject showed similar melatonin suppression during the long light on both the pretest and the posttest. No melatonin suppression was evident on the short light pretest or the posttest.

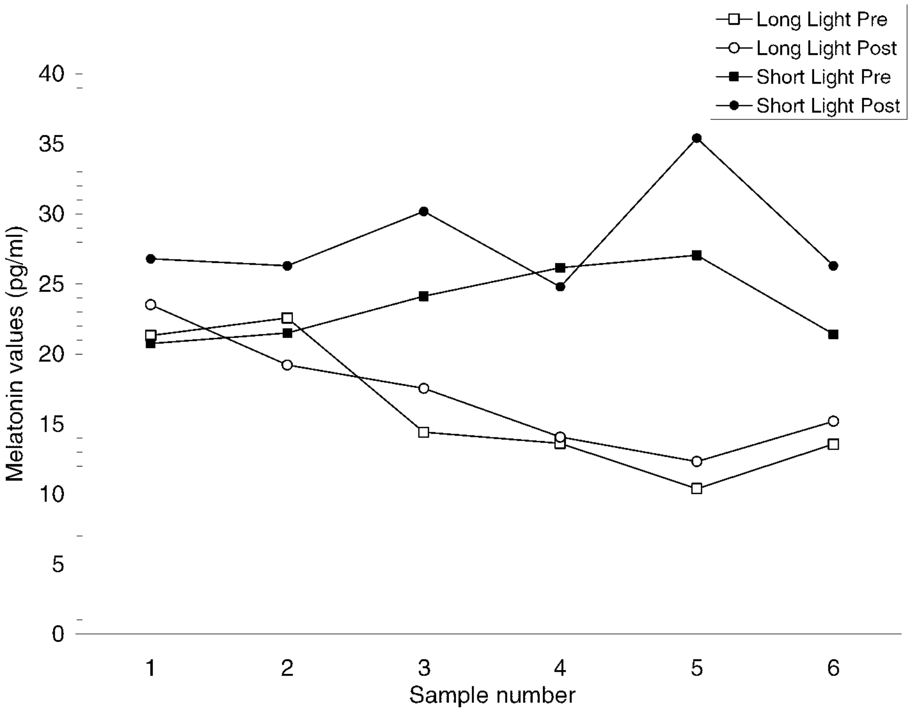


Figure 17. Experimental subjects' melatonin values on the long light pretest. Only 5 experimental subjects who had no substantial phase shift during the experiment are included. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 45 min. Samples were collected in 15 min intervals.

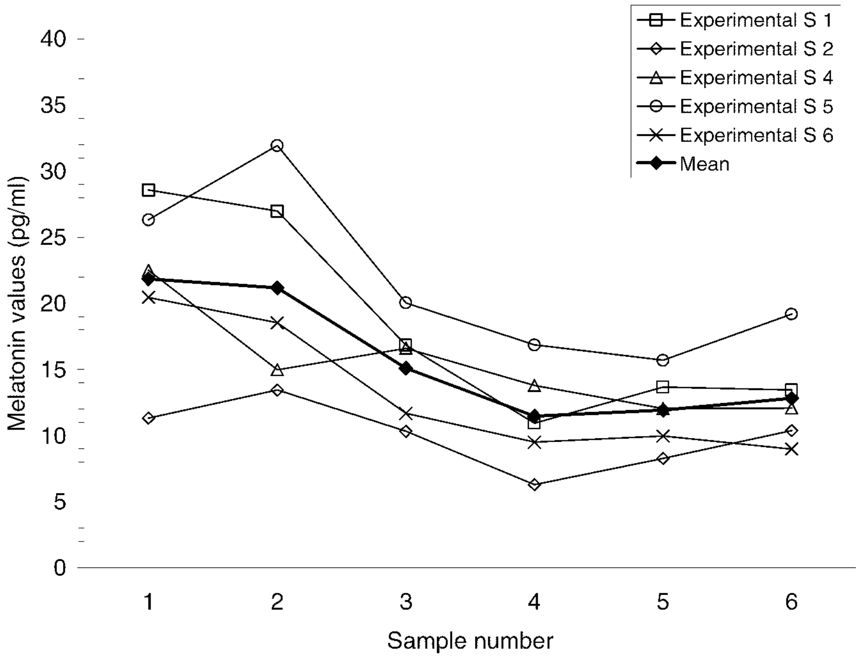


Figure 18. Control subjects' melatonin values on the long light pretest. Only 5 control subjects who had no substantial phase shift during the experiment are included. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 45 min. Samples were collected in 15 min intervals.

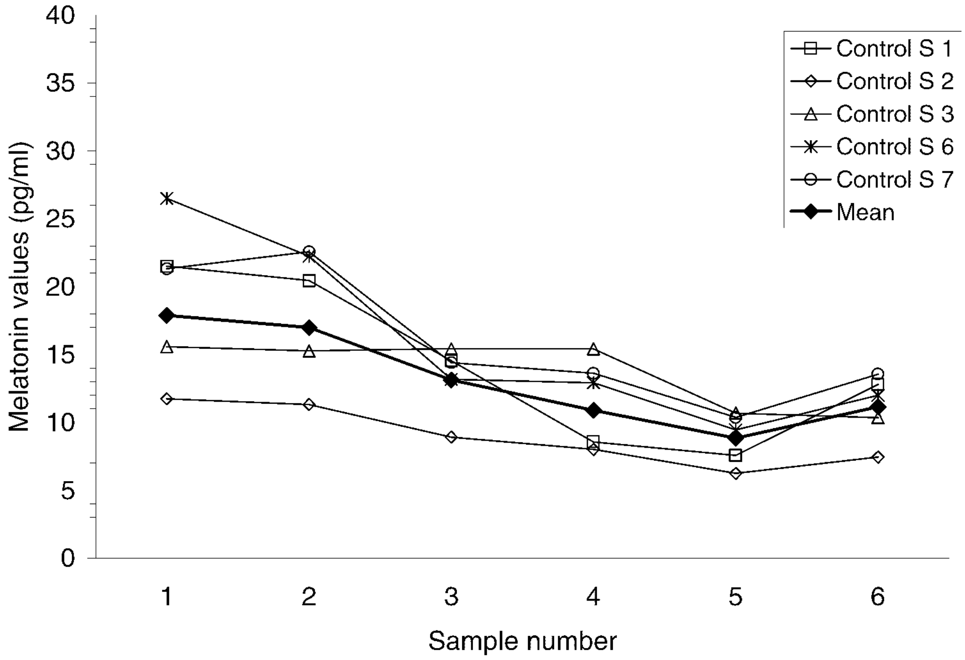


Figure 19. Experimental subjects' melatonin values on the short light pretest. Only 5 experimental subjects who had no substantial phase shift during the experiment are included. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 4.5 min. Samples were collected in 15 min intervals.

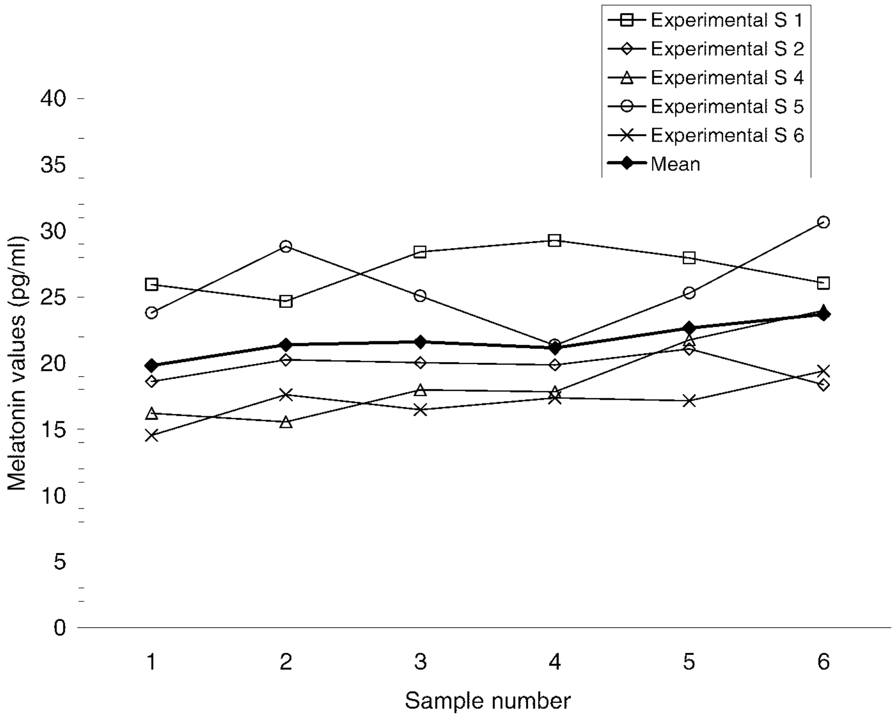


Figure 20. Control subjects' melatonin values on the short light pretest. Only 5 control subjects who had no substantial phase shift during the experiment are included. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 4.5 min. Samples were collected in 15 min intervals.

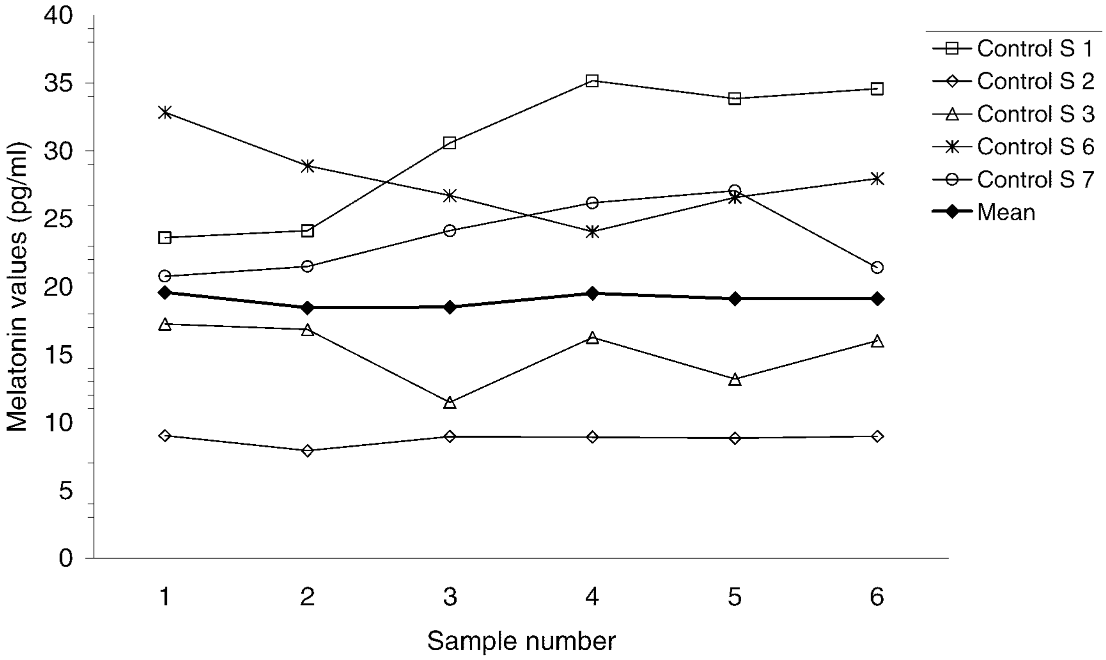


Figure 21. Experimental subjects' melatonin values on the short light posttest. Only 5 experimental subjects who had no substantial phase shift during the experiment are included. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 4.5 min. Samples were collected in 15 min intervals.

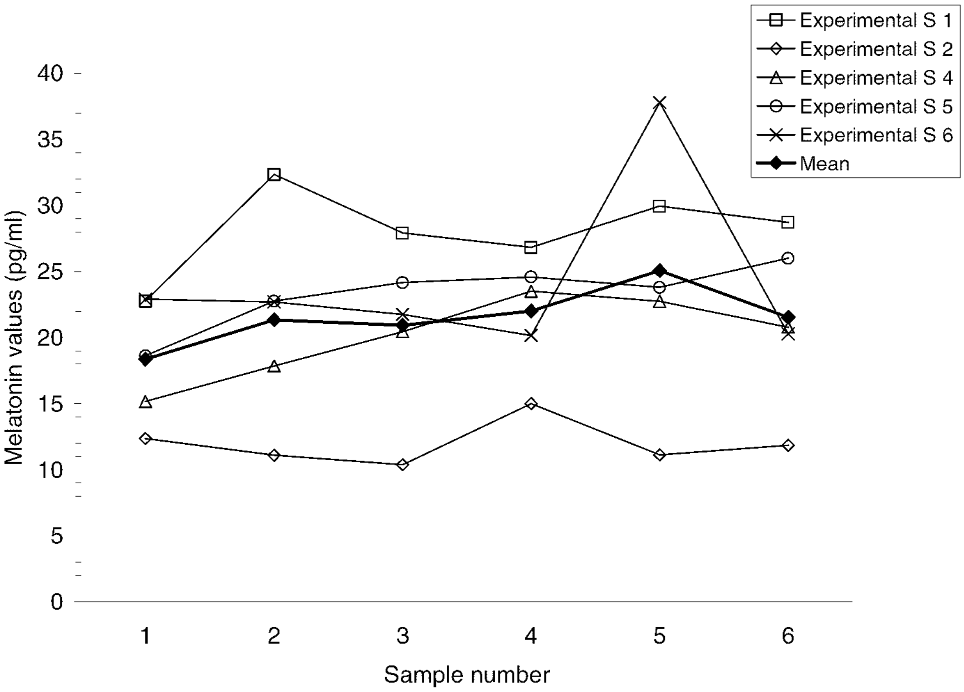


Figure 22. Control subjects' melatonin values on the short light posttest. Only 5 control subjects who had no substantial phase shift during the experiment are included. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 4.5 min. Samples were collected in 15 min intervals.

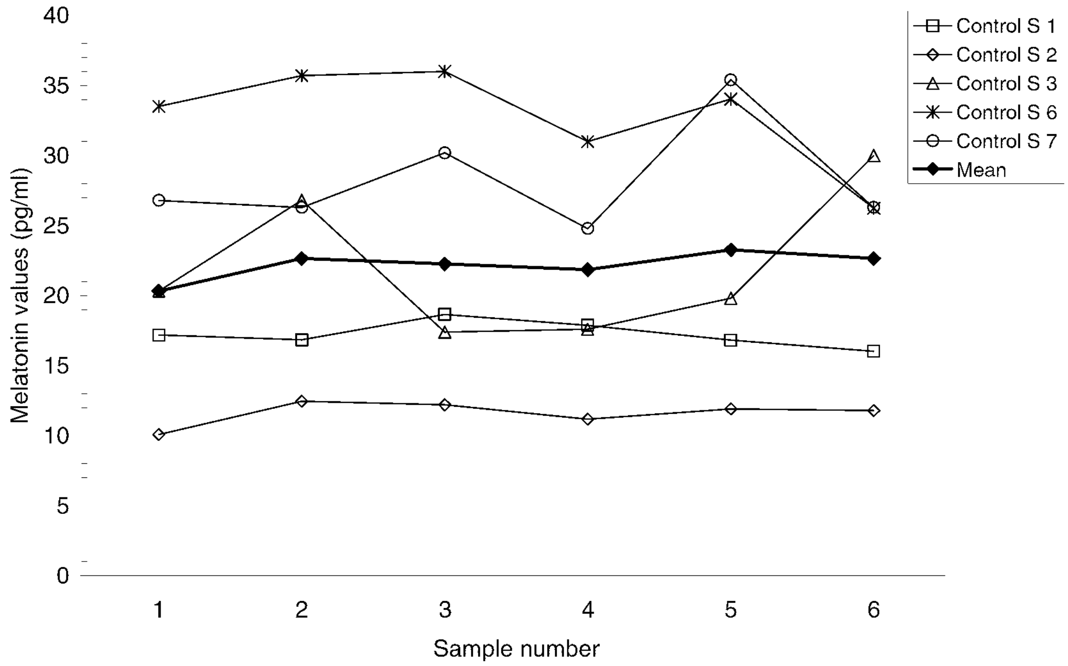


Figure 23. Experimental subjects' melatonin values on the long light posttest. Only 5 experimental subjects who had no substantial phase shift during the experiment are included. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 45 min. Samples were collected in 15 min intervals.

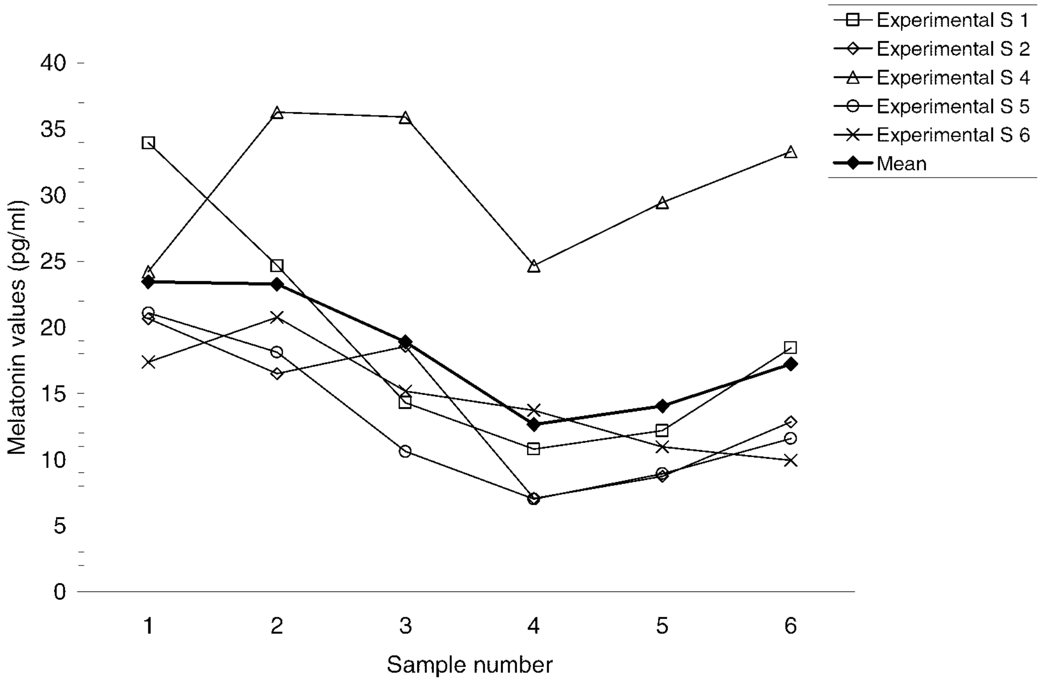
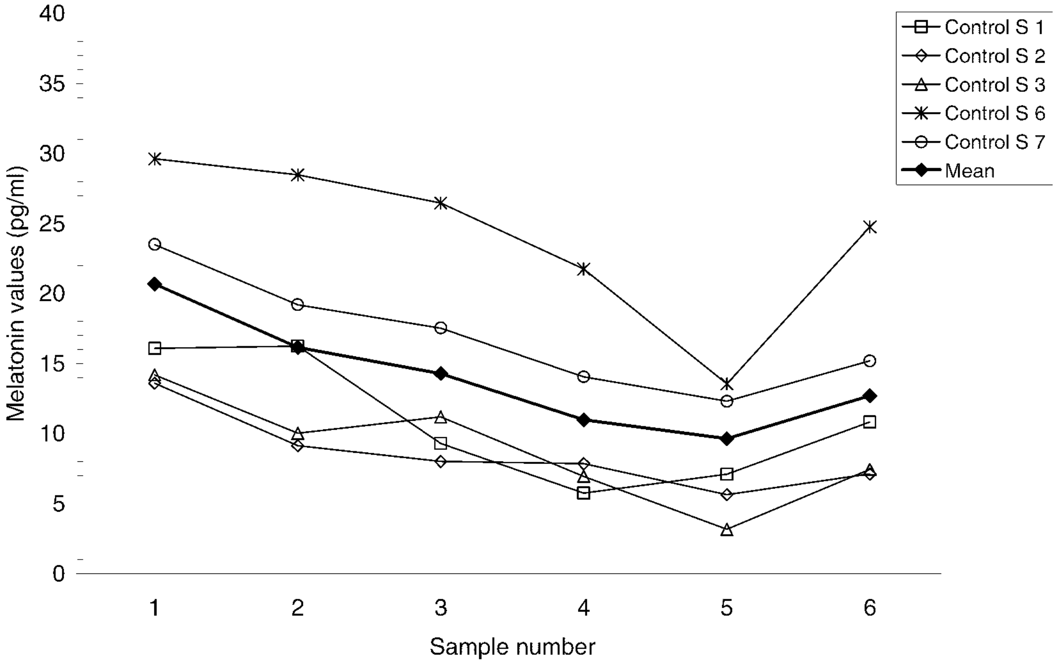


Figure 24. Control subjects' melatonin values on the long light posttest. Only 5 control subjects who had no substantial phase shift during the experiment are included. The light was turned on immediately after the first sample and approximately 15 min before sample 2. The light exposure lasted 45 min. Samples were collected in 15 min intervals.



References

Amir, S. & Stewart, J. (1996). Resetting of the circadian clock by a conditioned stimulus. *Nature, 379*, 542-545.

Amir, S. & Stewart, J. (1998a). Induction of Fos expression in the circadian system by unsignaled light is attenuated as a result of previous experience with signaled light: A role for Pavlovian conditioning. *Neuroscience, 83*, 657-661.

Amir, S. & Stewart, J. (1998b). Conditioning in the circadian system. *Chronobiology International, 15*, 447-456.

Aoki, H, Yamada, N., Ozeki, Y., Yamane, H., & Kato, N. (1998). Minimum light intensity required to suppress nocturnal melatonin concentration in human saliva. *Neuroscience Letters, 252*(2), 91-94.

Arendt, J. (1995). *Melatonin and the mammalian pineal gland.* London: Chapman Hall.

Arendt, J. & Broadway, J. (1987). Light and melatonin as zeitgebers in man. *Chronobiology International, 4*, 273-282.

Arvanitogiannis, A. & Amir, S. (1999). Conditioned stimulus control in the rat circadian system depends on clock resetting during conditioning. *Behavioral Neuroscience, 113*, 1297-1300.

Arvanitogiannis, A. & Amir, S. (2001). A novel, associative process modulating photic resetting of the circadian clock. *Neuroscience, 104*, 615-618.

Boivin, D. B., Duffy, J. F., Kronauer, R. E., & Czeisler, C. A. (1996). Dose-response relationships for resetting of human circadian clock by light. *Nature, 379*, 540-542.

Bojkowski, C. J., Aldhous, M. E., English, J. Franey, C., Poulton, A. L., Skene, D. J., et al. (1987). Suppression of nocturnal plasma melatonin and 6-sulphatoxymelatonin by bright and dim light in man. *Hormone and Metabolic Research, 19*(9), 437-440.

Brainard, G. C., Richardson, B. A., Petterborg, L. J., & Reiter, R. J. (1982). The effect of different light intensities on pineal melatonin content. *Brain Research, 233*(1), 75-81.

Brandon, S. E., & Wagner, A. R. (1991). Modulation of a discrete Pavlovian conditioned reflex by a putative emotive Pavlovian conditioned stimulus. *Journal of Experimental Psychology: Animal Behavior Process, 17*, 299-311.

Buhlmann Laboratories AG (1998). *The Buhlmann direct saliva melatonin radioimmunoassay test kit package insert*. Switzerland: Author.

Burish, T. G., & Redd, W. H (1983). Behavioral approaches to reducing conditioned responses to chemotherapy in adult cancer patients. *Behavioral Medicine Update, 5*(2-3), 12-16.

Buske-Kirschbaum, A., Kirschbaum, C., Stierle, H., Jabaij, L., & Hellhammer, D. (1994). Conditioned manipulation of natural killer (NK) cells in humans using a discriminative learning protocol. *Biological Psychology, 38*(2-3), 143-155.

Buske-Kirschbaum, A., Kirschbaum, C., Stierle, H., Lehnert, H., & Hellhammer, D. (1992) Conditioned increase of natural killer cell activity (NKCA) in humans. *Psychosomatic Medicine, 54*(2), 123-132.

Campbell, S., Dawson, D., & Anderson, M. (1993). Alleviation of sleep maintenance insomnia with timed exposure to bright light. *Journal of the American Geriatric Society, 41*, 829-836.

Carskadon, M. A., Acebo, C., Richardson, G. S., Tate, B. A., & Seifer, R. (1996). An approach to studying circadian rhythms of adolescent humans. *Journal of Biological Rhythms, 12*(3), 278-289.

Cassone, Vincent M., Chesworth, Michael J. Armstrong, & Stuart M. (1986). Entrainment of rat circadian rhythms by daily injection of melatonin depends upon the hypothalamic suprachiasmatic nuclei. *Physiology and Behavior, 36*(6), 1111-1121.

Childress, A. R., Ehrman, R., McLellan, T. A., & O'Brien, C. P. (1986). Extinguishing conditioned responses during opiate dependence treatment. Turning laboratory findings into clinical procedures. *Journal of Substance Abuse Treatment, 3*, 33-40.

Czeisler, C. A. (1995). The effect of light on the human circadian pacemaker. *Ciba Foundation Symposium, 183*, 254-302.

Czeisler, C. A., Allan, J. S., Strogatz, S. H., Ronda, J. M., Sanchez, R., Rios, C. D., et al. (1986). Bright light resets the human circadian pacemaker independent of the timing of the sleep-wake cycle. *Science, 233*, 667-671.

Czeisler, C. A., Kronauer, R. E., Allan, J. S., Duffy, J. F., Jewett, M. E., Brown, E. N., et al. (1989). Bright light induction of strong (type 0) resetting of the human circadian pacemaker. *Science, 244*, 1328-1333.

Czeisler, C. A., Richardson, G. S., Coleman, R. M., Zimmerman, J. C., Moore-Ede, M. C., Dement, W. C., et al. (1981). Chronotherapy: Resetting the circadian clock of patients with delayed sleep phase insomnia. *Sleep, 4*, 1-21.

De Groot, M. H. M. & Rusak, B. (2000). Responses of the circadian system of rats to conditioned and unconditioned stimuli. *Journal of Biological Rhythms, 15*, 277-291.

Dijk, D. J., Cajochen, C., & Borbely, A. A. (1991). Effect of a single 3-hour exposure to bright light on core body temperature and sleep in humans. *Neuroscience Letters, 121*(1-2), 59-62.

Duffy, J. F., Kronauer, R. E, & Czeisler, C. A. (1996). Phase-shifting human circadian rhythms: influence of sleep timing, social contact and light exposure. *Journal of Physiology, 495*, 289-97.

Edelstein, K. & Amir, S. (1999). The role of the intergeniculate leaflet in entrainment of circadian rhythms to a skeleton photoperiod. *The Journal of Neuroscience, 19*, 372-380.

Fukuhara, C., Aguzzi, J., Bullock, N., & Tosini, G. (2005). Effect of long-term exposure to constant dim light on the circadian system of rats. *Neurosignals, 14*(3), 117-125.

Gauci, M., Husband, A. J., Saxarra, H., & King, M. G. (1994) Pavlovian conditioning of nasal tryptase release in human subjects with allergic rhinitis. *Physiology and Behavior, 55*(5), 823-825.

Gillette, M. U. & Tischkau, S. A. (1999), Suprachiasmatic nucleus: the brain's circadian clock. *Recent Progress in Hormonal Research, 54*, 33-59.

Goel, N. (2005). Late-night presentation of an auditory stimulus phase delays human circadian rhythms. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology, 289*(1), R209-R216.

Golombeck, D. A., Chuluyan, H, E., Canterewicz, B. I., & Cardinali, D. P. (1994). Increased pineal melatonin content coupled to restricted water availability in a Pavlovian conditioning paradigm in rats. *Journal of Neural Transmission, General Section, 98*, 237-246.

Harley, J. P. (1973). Temporal conditioning as a function of instructions and intertribal interval. *Journal of Experimental Psychology, 100*(1), 178-184.

Hatonen, T., Alila-Johansson, A., Mustanoja, S., & Laakso, M. L. (1999). Suppression of melatonin by 2000-lux light in humans with closed eyelids. *Biological Psychiatry, 46*(6), 827-831.

Honma, K., Honma, S., & Wada, T. (1987). Phase-dependent shift of free-running human circadian rhythms in response to a single bright light pulse. *Experientia, 43*, 1205-1207.

Howard, M. O. (2001). Pharmacological aversion treatment of alcohol dependence. I. Production and prediction of conditioned alcohol aversion. *The American Journal of Drug and Alcohol Abuse, 27*(3), 561-585.

Ikemi, Y. & Nakagawa, S. (1962). A psychosomatic study of contagious dermatitis. *Kyushu Journal of Medical Science, 13*, 335-350.

Kirkpatrick, K. & Church, R. M. (2000). Stimulus and temporal cues in classical conditioning. *Journal of Experimental Psychology: Animal Behavior Processes, 269*(2), 206-219.

Kornhauser, J. M., Mayo, K. E., & Takahashi, J. S. (1996). Light, immediat-early genes, and circadian rhythms. *Behavioral Genetics, 26*, 221-240.

Krauchi, K. & Wirz-Justice, A. (1994). Circadian rhythm of heat production, heart rate, and skin and core temperature under unmasking conditions in men. *American Journal of Physiology, 267*, (3 Pt 2), R819-829.

Laakso, M. L., Alila, A., Hatonen, T., & Mustanoja, S. M. (1996). Ontogeny of pineal melatonin rhythm in rats under 12:12 and 14:14-hr light:dark conditions. *Journal of Pineal Research, 21*, 155-164.

Laakso, M., L., Hatonen, T., Stenberg, D., Alila, A., & Smith, S. (1993). One-hour exposure to moderate illuminance (500 lux) shifts the human melatonin rhythm. *Journal of Pineal Research, 15*(1), 21-26.

Laakso, M. L., Leinonen, L., Joutsiniemi, S. L., Porkka-Heiskanen, T., & Stenberg, D. (1992). Pineal melatonin and locomotor activity of rats under gradual illuminance transitions. *Physiology and Behavior, 52*, 621-628.

Laakso, M. L., Porkka-Heiskanen, T., Alila, A., Stenberg, D., & Johansson, G. (1990). Correlation between salivary and serum melatonin: dependence on serum melatonin levels. *Journal of Pineal Research, 9*, 39-50.

Lack, L., & Wright, H. (1993). The effect of evening bright light in delaying the circadian rhythms and lengthening the sleep of early morning awakening insomniacs. *Sleep, 16*(5), 436-443.

Lewy A. J., Bauer, V. K., Ahmed, S., Thomas, K. D., Cutler, N. L., Singer, C. M. et al. (1998). The human phase response curve (PRC) to melatonin is about 12 hours out of phase with the PRC to light. *Chronobiology International, 15*(1), 71-83.

Lewy, A. J., Cutler, N. L., & Sack, R. L. (1999). The endogenous melatonin profile as a marker for circadian phase position. *Journal of Biological Rhythms, 14*(3), 227-236.

Lewy, A. J., & Sack, R. L. (1989). The dim light melatonin onset as a marker for circadian phase position. *Chronobiology International, 6*(1), 93-102.

Lewy, A. J., Sack, R. L., Blood, M. L., Bauer, V. K., Cutler, N. L., & Thomas, K. H. (1995). Melatonin marks circadian phase position and resets the endogenous circadian pacemaker in humans. *Ciba Foundation Symposium, 183*, 303-321.

Lewy, A. J., Wehr, T. A., Goodwin, F. K., Newsome, D. A., & Markey, S. P. (1980). Light suppresses melatonin secretion in humans. *Science, 210*, 1267-1269.

Lerchl, A. (1995). Sustained reponse of pineal melatonin synthesis to one-minute light pulse during night in Djungarian hamsters (phodopus sungorus). *Neuroscience Letters, 198*, 65-67.

Liu, T. & Borjigin, J. (2005) Free-running rhythms of pineal circadian output. *Journal of Biological Rhythms, 20*(5), 430-440.

Lockhart, R. A. (1966). Temporal conditioning of GSR. *Journal of Experimental Psychology, 71*(3), 438-446.

Maeda, K. I. & Lincoln, G. A. (1990). Phase shifts in the circadian rhythm in plasma concentrations of melatonin in rams induced by a 1-hour light pulse. *Journal of Biological Rhythms, 5*, 97-106.

Meijer, J. H., Watanabe, K., Detari, L., de Vries, M. J., Albus, H., Treep, J. A., et al. (1996). Light entrainment of the mammalian biological clock. *Progress in Brain Research, 111*, 175-190.

Mellor, C. S. & White, H. P. (1978). Taste aversions to alcoholic beverages conditioned by motion sickness. *American Journal of Psychiatry, 135*(1), 125-126.

Middleton, B., Arendt, J., & Stone, B. M. (1996). Human circadian rhythms in constant dim light (8 lux) with knowledge of clock time. *Journal of Sleep Research, 5*(2), 69-76.

Middleton, B., Arendt, J., & Stone, B. M. (1997). Complex effects of melatonin on human circadian rhythms in constant dim light. *Journal of Biological Rhythms, 12*(5), 467-77.

Moga, M. M., Weis, R. P., & Moore, R. Y. (1995). Efferent projections of the paraventricular thalamic nucleus in the rat. *The Journal of Comparative Neurology, 359*, 221-238.

Moore, R. Y., Weis, R., & Moga, M. M. (2000). Efferent projections of the intergeniculate leaflet and the ventral lateral geniculate nucleus in the rat. *The Journal of Comparative Neurology, 420*, 398-418.

Morin, L, P., Goodless-Sanchez, N., Smale, L., & Moore, R. Y. (1994). Projections of the suprachiasmatic nuclei, subparaventricular zone and retrochiasmatic area in the golden hamster. *Neuroscience, 61*, 391-410.

Murphy, P. J., & Campbell, S. S. (1996). Physiology of the circadian system in animals and humans. *Journal of Clinical Neurophysiology, 13*(1), 2-16.

Olness, K. & Ader, R. (1992). Conditioning as an adjunct in the pharmacotherapy of lupus erythematosus. *Journal of Developmental and Behavioral Pediatrics, 13*, 124-125.

Paakkonen, T., Makinen, T. M., Leppaluoto, J., Vakkuri, O., Rintamaki, H., Palinkas, L. A. et al. (2006). Urinary melatonin: a noninvasive method to follow human pineal function as studied in three experimental conditions. *Journal of Pineal Research, 40*(2), 110-115.

Pavlov, I. P. (1927). *Conditioned reflexes*. London: Oxford University Press.

Reppert, S. M., Weaver, D. R., Rivkees, S. A., & Stopa, E. G. (1988). Putative melatonin receptors in a human biological clock. *Science, 242*, 78-81.

Rescorla, R. A. (1967). Pavlovian conditioning and its proper control procedures. *Psychological Review, 74*, 71-80.

Rescorla, R. A. & Wagner, A. R. (1972). A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonresinforcement. In A. H. Black & W. F. Prokasy (Eds.), *Classical conditioning II* (pp. 64-99). New York: Appleton-Centrury-Crofts.

Reuss S. (1996). Components and connections of the circadian timing system in mammals. *Cell Tissue Research, 285*, 353-78.

Robinson, B., Stewart, J. & Amir, S. (1996). Entrainment of circadian rhythms by a conditioned stimulus [Abstract]. *Society for Neuroscience Abstracts, 22*, 2056.

Rusak, B., Robertson, H. A., Wisden, W., & Hunt, S. P. (1990). Light pulses that shift rhythms induce gene expression in the suprachiasmatic nucleus. *Science, 248*, 1237-1240.

Sadun, A. A., Schaechter, J. D., & Smith, L. E. (1984). A retinohypothalamic pathway in man: light mediation of circadian rhythms. *Brain Research, 302*, 371-377.

Schaechter, J. D., & Sadun, A. A. (1985). A second hypothalamic nucleus receiving retinal input in man: the paraventricual nucleus. *Brain Research, 340*, 243-250.

Scheer, F.A., Pirovano, C., Van Someren, E. J., & Buijs, R. M. (2005) Environmental light and suprachiasmatic nucleus interact in the regulation of body temperature. *Neuroscience, 132*(2), 465-477.

Schwartz, W. J., Takeuchi, J., Shannon, W., Davis, E. M., & Aronin, N. (1994). Temporal regulation of light-induced Fos and Fos-like protein expression in the ventrolateral subdivision of the rat suprachiasmatic nucleus. *Neuroscience, 58*(3), 573-583.

Shanahan, T. L., Kronauer, R. E., Duffy, J. F., Williams, G. H., & Czeisler, C. A. (1999). Melatonin rhythm observed throughout a three-cycle bright-light stimulus designed to reset the human circadian pacemaker. *Journal of Biological Rhythms, 14*, 237-253.

Shanahan, T. L., Zeitzer, J. M. & Czeisler, C. A. (1997). Resetting the melatoning rhythm with light in humans. *Journal of Biological Rhythms, 12*, 556-567.

Steinlechner, S. (1996). Melatonin as a chronobiotic: Pros and cons. *Acta neurobiologiae experimentalis, 56*(1), 363-372.

Stockhorst, U., Mahl, N., Krueger, M., Huenig, A., Schottenfeld-Naor, Y., Huebinger, A., et al. (2004) Classical conditioning and conditionability of insulin and glucose effects in healthy humans. *Physiology and Behavior, 81*(3), 375-388.

Stockhorst, U., Steingrüber, H.-J., Scherbaum, W. A. (2000). Classically conditioned responses following repeated insulin and glucose administration in humans. *Behavioural Brain Research, 110*(1-2), 143-159.

Takahashi, J. S., & Zatz, M. (1982). Regulation of circadian rhythmicity. *Science, 2178*, 1104-1111.

Vanecek, J. & Watanabe, K. (1999). Mechanisms of melatonin action in the pituitary and SCN. *Advances in experimental medicine and biology, 460*, 191-198.

Wehr, T. A. (1997). Melatonin and seasonal rhythms. *Journal of Biological Rhythms, 12*(6), 518-527.

Wever, R. A., Polasek, J., Wildgruber, C. M. (1983). Bright light affects human circadian rhythms. *Pflügers Archive: European Journal of Physiology, 396*(1), 85-87.

Williams, D. A., Frame, K. A., & LoLordo, V. M. (1992). Discrete signals for the unconditioned stimulus fail to overshadow contextual or temporal conditioning. *Journal of Experimental Psychology: Animal Behavior Processes, 18*(1), 41-55.

Williams, D. A. & LoLordo, V. M. (1995). Time cues block the CS, but the CS does not block time cues. *The Quarterly Journal of Experimental Psychology, 48B*(2), 97-116.

Wolpe, J. (1982). *The practice of behavior therapy* (3rd ed.). Oxford: Pergamon.

Wright, H. R., Lack., L. C., & Kennaway, D. J. (2004). Differential effects of light wavelength in phase advancing the melatonin rhythm. *Journal of Pineal Research, 36*(2), 140-144.

Yang, C.-M. & Spielman, A. J. (1999). The role of behavioral treatment in the management of insomnia. *Disease Management and Health Outcomes, 5*(4), 209-224.

Zeitzer, J. M., Dijk, D. J., Kronauer, R., Brown, E., & Czeisler, C. (2000). Sensitivity of the human circadian pacemaker to nocturnal light: Melatonin phase resetting and suppression. *The Journal of Physiology, 526*(3), 695-702.