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Maternal Stress Induces Adult Reduced REM sleep and Melatonin Level

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Abstract

Objectives—We have previously reported that neonatal maternal deprivation (MD) resulted in a decrease of total sleep and an increase of orexin A in adult rats. Now, we characterized features of sleep, activity, and melatonin levels in rats neonatally treated with MD and control (MC) procedures.

Design—Adult male Sprague Dawley rats were treated with either MD or MC procedures for ten days starting at postnatal day 4. At three months of age, sleep was recorded for 48 hours in one set of MD and MC rats while another set of MD and MC rats were measured for locomotor activity (under LD=12:12). Melatonin levels in the blood, pineal gland, and hypothalamus were measured as well as clock protein level in the hypothalamus.

Results—Compared with the MC rats, REM sleep in the MD rats was significantly reduced in the light periods but not in the dark periods. Both quiet wake and total wake in the MD rats were significantly increased during the light period compared to the MC rats. The weight of the pineal gland of the MD rats was significantly smaller than in MC rats. Melatonin levels of the MD group were significantly reduced in the pineal gland and hypothalamus compared with the MC group. No significant difference was identified between groups in the expression of the clock protein in the hypothalamus.

Conclusion—Neonatal MD resulted in reduced REM sleep and melatonin levels, without changes of circadian cycle of locomotor activity and levels of clock protein.

Keywords

Insomnia; Melatonin; Clock protein; Maternal deprivation; REM sleep

Introduction

Wake and sleep cycles are regulated by both homeostatic and circadian processes (Borbely & Achermann, 1999; Espana & Scammell, 2004). The biological clock is synchronized (entrained) to the outside world primarily through light-dark cycles (Challet, 2007). The suprachiasmatic nucleus (SCN) acts as a central circadian pacemaker through autoregulatory transcriptional loops that modulate expression of specific genes, including the clock gene. This center regulates rhythmicity of a number of vital processes including sleep-wake cycles

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(Mendoza & Challet, 2009). Consistent with this role, lesions in the SCN abolish the circadian rhythms of sleep-wake states and hormone secretion (Moore & Eichler, 1972) and transplantation of fetal SCN can restore overt behavioral rhythmicity in constant environmental conditions (Ralph et al., 1990). Sleep wake cycles and locomotor activity are regulated by the SCN (Aton et al., 2006; Liu & Reppert, 2000) and may be affected by melatonin as well (Appelbaum et al., 2009; Fisher & Sugden, 2010; Krahn et al., 2002). The pineal gland is the organ that synthesizes and releases melatonin, which is then released into the cerebrospinal fluid (CSF), brain tissue, and blood. Production of melatonin is regulated by arylalkylamine-N-acetyltransferase (AANAT). A higher level of melatonin is observed in the dark phase of both diurnal species, including humans, and nocturnal species, such as rats (Challet, 2007). The data regarding the effect of melatonin on sleep are equivocal. Some studies have demonstrated sleep promoting effect of melatonin in rodents (Holmes & Sugden, 1982; Mendelson, 2002; Mendelson et al., 1980) and humans (Simonneaux & Ribelayga, 2003; Vivanco et al., 2007) while others showed that melatonin has no effect on sleep (Langebartels et al., 2001).

Chronic primary insomniacs, whose predominant complaint was difficulty in maintaining sleep, exhibited significantly lower melatonin levels during the middle of the night than healthy controls. Patients with the most severely reduced nocturnal plasma melatonin levels had a history of sleep disturbance lasting longer than five years (Hajak et al., 1995; Pandi-Perumal et al., 2007). Both exposure to bright white light at specific times during the sleep-wake cycle (Campbell, 1995) and exogenous melatonin receptor agonist administration have been shown to synchronize circadian rhythms for jet lag and sleep disturbance related to shift-work-induced sleep disorders (Folkard et al., 1993). These treatments have also been utilized for sleep disturbances caused by desynchronization of the endogenous sleep-wake cycle from altered lighting cues in blind, geriatric, and brain-damaged subjects (Buscemi *et al.*, 2005) and for early-morning awakening insomnia (Lack et al., 2005). The effectiveness of these circadian rhythm-based treatments supports further investigation of circadian regulation in the pathology of chronic insomnia.

In our previous study (Feng et al., 2007), some features of insomnia, such as reduced total sleep or increased total wake during the light period, were reported in a maternal deprivation (MD) rat model, a method originally developed as a rat model of chronic stress (Meaney *et al.*, 1991; Plotsky & Meaney, 1993). A large body of evidence has shown that neonatal MD leads to long-lasting alterations including elevated activation of the hypothalamic pituitary adrenal (HPA) axis (Plotsky & Meaney, 1993). Behaviorally, MD rats are sensitive to stressful challenge and exhibit increased anxiety-like behaviors (Boccia & Pedersen, 2001; Wigger & Neumann, 1999). At the molecular level, the MD rat has increased plasma adrenocorticotropic hormone (ACTH) and corticosterone levels at baseline as well as following stressful chanllenge (Plotsky & Meaney, 1993). Also, the MD rats exhibit elevated brain corticotrophin releasing factor (CRH) levels (Plotsky *et al.*, 2005) and hyperexpression of CRH mRNA in the hypothalamus (Ladd et al., 1996). Further analysis of this model showed that the adult rats, which were neonatally treated with MD, had features of insomnia, including decreased total sleep and increased total wake time during the light period (Feng *et al.*, 2007). In addition, hypothalamic CRH and orexin A levels are elevated in MD rats (Feng *et al.*, 2007). At least two observations support the study of circadian alterations in the MD model. First, sleep-wake cycles are controlled by both the homeostatic system and the circadian system and abnormal circadian regulation affects sleep-wake cycles. Second, the presence of sleep onset disturbance (difficulty falling asleep) and increased early-morning awakening in human insomniacs may indicate that alterations in circadian regulation could underlie chronic insomnia. Due to the presence of these hallmarks of insomnia, we tested the hypothesis that the MD rat model possesses alterations in

circadian regulation by measuring activity, sleep, and brain levels of melatonin and clock proteins.

Materials and Methods

Animals and maternal deprivation treatment

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University and the Louis Stokes Cleveland Veterans Affairs Medical Center. Male Sprague Dawley rats were acquired with their mother at postnatal day 2 (PN2) from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Animals were housed in the animal facility of Cleveland VA Medical Center under a 12:12 hour light:dark schedule. Since the neonatal period is a stress hyporesponsive period (SHRP) and the feature of SHRP is critically maintained by maternal interaction and less by other factors (Levine, 2001; Levine, 2002), environmental change induced stress response would be neglectible. Thus, a short (two days) period of recovery were given before the start of MD. At PN4, rat pups from each litter were equally divided into groups of MD and control (MC) group. The MD procedure separates rats from their mother for six hours per day for ten days as detailed in our previous publication (Feng *et al.*, 2007). Six hours of separation were divided into two 3-hour sessions, a morning session and an afternoon session. Each session was started by removing both MD and MC pups from the home cage and putting them to the special separation cage. Then, the control pups were immediately placed back into the home cage. The MD pups were returned after the end of the separation session. After the neonatal treatment, rats were not disturbed until the start of adult experiments, usually at three months of age or older [see: (Feng *et al.*, 2007)]. The MD and MC group were divided into two sets of animals. One set of MD and MC rats were used for the sleep study, while the other set of MD and MC rats were used for measurement of activity, melatonin, and hypothalamic clock protein.

Activity measurements and tissue collection

Activity measurements were collected for 48 hours for 3 –5 month old MD (n = 10) and MC (n = 10) rats using the MotorMonitor (Kinder Scientific, Inc., Poway, CA). This system consists of a rectangular frame with beam detecting sensors installed in the surrounding arms. Motion signals inside the frame are detected by the sensors and the frame is interfaced with computer for storage and analysis. To initiate the test, a standard rat housing cage was placed inside the frame, allowing detection of the animal's activities. As previously mentioned, all rats were subjected to 12: 12 hour light:dark periods, with the lights turning on at 9:00 A.M. and off at 9:00 P.M. Movement metrics for data analysis include basic movements, X and Y ambulations, immobility, event time, total beam breaks, event count for rearing, hole pokes, etc. For this study, basic movement was analyzed and reported as activity only. Rats were sacrificed within a small time window from 10:00 A.M. – 12:00 P.M. during the light phase. This window corresponds to human evening and is a period with the greatest alteration of stress related hormones, including ACTH and cortisol, in chronic insomniacs (Vgontzas *et al.*, 2001). Brain and blood samples were collected for quantification of melatonin and clock protein.

Polysomnography (PSG) recording and sleep scoring

At three months of age, rats (MD rats: n =15; MC rats: n =16) were anesthetized with pentobarbital (50 mg/kg, i.p.), shaved with an electric clipper, and restrained with a stereotaxic instrument. Betadine and alcohol were applied to the shaved portion of the head to disinfect the incision site. Marcaine, a local anesthetic, was then injected around the skin margins of the skull. A 3.5–4 cm incision was made along the midline of the head, roughly 1–1.5 cm from the brow ridge, to expose the skull. Three holes were drilled into the skull in

the frontal and parietal regions to accommodate three stainless steel jewel screws that served as EEG electrodes, as described in our previous publication (Feng *et al.*, 2007). These electrodes were bonded to the dorsal surface of the skull with C&B Metabond bonding agent, which adheres extremely well to bone. Similar to previous studies, the EMG recorded from stainless steel wires soldered onto stainless steel springs. Electrodes were sutured onto the neck muscles. The skin overlaying the skull and posterior muscles was reapposed, allowing for a cable to extend through the skin and connect to the polysomnograph. Rats were monitored continuously until recovery from anesthesia, every 2 hours for the first day and at least twice a day thereafter for a week. After ten days of recovery and adaptation, 48 hours of sleep-wake was recorded and analyzed. Data was scored as REM sleep, NREM sleep, quiet wake (wake period without activity) and active wake (wake with more activity, according to our previous definitions (Feng *et al.*, 2007) and was separated into light (lights on) and dark (lights off) periods. Total sleep was calculated by adding REM sleep and NREM sleep time of each time period of each rat together and displayed as mean ± se.

Brain and blood sample collection

Rats were deeply anesthetized with CO2 gas and then decapitated. From each rat, 2.5 ml of trunk blood was collected in tubes containing EDTA (to prohibit coagulation) and centrifuged for 15 min at 1800×*g* at 4°C for plasma separation. The plasma was stored at −80°C for future analysis. Immediately after opening the skull, the dura was carefully cut for exposure of the pineal gland. The intact pineal gland was carefully dissected out using small forceps and extra fluid was absorbed with surgical tissue. Then, it was transferred to a microtube and carefully weighed using a balance that has a sensitivity of 0.01 mg. The hypothalamus was dissected according to our previous description (Feng *et al.*, 2007), and separated at the midline into two sets: one set was used for melatonin measurement while the other set was used for quantification of the clock protein using Western blot. All samples were immediately stored individually at −80 °C for future processing.

Melatonin extraction and radioimmunoassay (RIA) quantification of melatonin levels

Melatonin extraction from blood was conducted according to a commercial protocol from ALPCO Diagnostics (Windham, NH). Melatonin from brain tissue homogenate was extracted using a modified protocol based on a previous publication (Prada et al., 2005). Briefly, after washing the C18 column with methanol and water, pre-processed stock solution from brain samples (220 μl) or blood samples (1 ml plasma per rat) was added to the column. The column was centrifuged for 1 min at 500×*g*. Then, the columns were washed repeatedly with 10% methanol and with 100% hexane, followed by centrifugation for 1 min at 500×*g* after each wash. Next, samples were eluted with 100% methanol by centrifugation for 1 min at 200×*g*. The solution containing melatonin was evaporated using the Eppendorf Vacufuge Concentrator (Model 5301, Eppendorf North America, Inc., Westbury, NY) at 4°C. The dried samples were stored at −80°C for subsequent quantification.

Melatonin levels in the plasma, pineal gland, and hypothalamus were determined using the radioimmunoassay (^{125}I) method with a standard RK-MEL2 RIA kit (ALPCO Diagnostics; Windham, NH). Detecting sensitivity was 0.3 pg/ml using 400 ul of extracted solution. A pilot assay using two untreated rats was conducted to determine the optimal dilution ratio. Then, dry samples from both brain tissue and plasma were reconstituted using RIA buffer provided with the kit. The protocol was carefully followed according to the manufacturer's instruction. The level of melatonin in the tissue was converted into pg/mg (wet tissue) while the amount of melatonin in the blood was converted to pg/mL blood based on the original samples.

Western Blot for semi-quantitative analysis of clock expression

Western blotting was performed to semi-quantitatively analyze clock protein expression using the method described in our previous publications (Feng et al., 2003; Feng et al., 2007) with minor modifications. Briefly, stored tissue samples from the hypothalamus were sonicated in cold RIPA Buffer (Cat# 89901, Fisher Scientific, Inc) equal to 10 times the tissue weight containing protease inhibitor cocktail for 5 seconds, followed by incubation on ice for 10–15 seconds. The sonication was repeated three times. The resulting homogenate was centrifuged for 30 minutes at 25000×*g* at 4°C. The supernatant was carefully transferred to a new tube and stored at −80°C for subsequent use. The protein concentration of the samples was determined using the Bradford assay (BioRad). A volume containing 22.5 μg of total protein was loaded in each lane of a 10% SDS-PAGE gel. The SDS-PAGE gel was run at 200V for 1 hour, followed by transfer to a nitrocellulose membrane at 100V for 1 hour. The membrane was blocked in 5% non-fat milk in TBS-Tween at room temperature for 30 minutes, followed by incubation with goat anti-clock pAb (1:1000 dilution in 5% Non-fat milk TBS-Tween) at 4°C overnight. The membrane was washed with TBS-T for 3 × 10 minutes, followed by incubation with a horseradish peroxidase-conjugated secondary antibody at room temperature for 2 hours. After three 10 minute washes with TBS-Tween, a chemiluminescent substrate (Pierce Biotechnology) was added to the membranes to visualize bound antibodies. The membrane was then exposed to X-ray film, and the bands were quantified and normalized to actin. The expression ratio (ER) of clock protein was calculated by dividing the optical density of the clock protein band (OD_{clock}) by the optical density of the band for the house-keeping protein β-actin (OD_{Actin}). For example, the ER of the MC group, ER_{MC}, was calculated as OD_{clock}/OD_{Actin} of the MC group

Statistical analyses

Sleep and activity data were analyzed by two-way (treatment X circadian phase) ANOVA and was further analyzed using multiple comparison procedures (Bonferroni t-test). with a software of Sigmastat (Version 3.1.1, Systat Software, Inc. Point Richmond, CA). Differences in the tissue weights of the pineal glands were analyzed using t-test for two samples assuming equal variances (because F-test showed that $p>0.05$). Melatonin levels of the pineal gland and hypothalamus were analyzed using a t-test for two samples assuming unequal variances because $p<0.05$ in F-test. Significance was considered at $p < or = 0.05$. Data was displayed as mean ± standard error.

Results

Maternal deprivation results in changes in REM sleep and in wake

NREM sleep was significantly more in the light period than in the dark period in both MC and MD groups (group x circadian phase: $F=91.238$, $P = <0.001$). The NREM sleep in the dark period was approximately 60% of that in the light period for both groups (Figure 1). Overall, no significant difference in NREM sleep was observed between MC and MD rats in either the light or dark periods. REM sleep in the MD rats was significantly reduced in both light periods in comparison to the control MC rats (Figure 2). The MD rats exhibited a 20.45% reduction ($t=2.234$, $p=0.027$) and a 26.61% reduction ($t=2.889$, $p=0.005$) in time spent in REM sleep in the first and second light periods, respectively. However, differences in REM sleep between the groups during both dark periods were not significant ($p=0.905$ and $p=0.303$ for comparison between MC and MD groups in first and second dark phase, respectively).

Two way ANOVA showed that the difference of total sleep between MC and MD groups were not large but statistically significant in the second light period ($t=2.478$, $p=0.015$; data

was not shown). These differences were not statistically significant in the first light period (t=1.769, p=0.080) and all dark period.

Consistent with our previous publication, we scored the wake state as quiet (QW) and active wake (AW) according to the differences of EMG activity (Feng *et al.*, 2007). In the dark period, the mean time spent in active wake and total wake in the MD rats was slightly less than that of the MC rats. However, these differences were not statistically significant (not shown). In contrast, the MD rats spent 40.99% more time in the quiet wake and 21.21% more time in the total wake state in the light period compared to MC rats over the same time periods (Figure 3). Two way ANOVA showed that these differences were significant for comparisons between MC and MD rats in both, QW (t=3.032, p=0.004) and total wake (t=2.443, p=0.018) during the light period.

MD rats exhibit no significant changes in ligh-dark patterns of locomotor activity

Locomotor activity was recorded for 48 hours under regular light:dark cycle. Basic activity exhibited typical rhythmic patterns corresponding to the alterations in light:dark cycles. The mean of activity was lower during the light phase (9:00 A.M.–9:00 P.M.) and higher during the dark phase (9:00 P.M.– 9:00 A.M.) in all rats. No differences in activity or phase disturbances were observed between the MC and MD rats during any particular time period (Figure 4). To quantify activity, data was calculated in 6-hour sections for a total of 48 hours (Table 1). Two-way (time X treatment) ANOVA showed that differences in the mean values between the two groups were not large enough (p=0.079) to exclude the possibility that the difference was simply due to random sampling variability after allowing for the effects of differences in time. Thus, despite the observation that the mean activity of the MD rats was higher than that of the MC rats in each of the four 6-hour sessions, the differences were not statistically significant (P = 0.255). However, the differences in the mean values over the different time periods were statistically significant (P = <0.001). Pairwise multiple comparison procedures using the Bonferroni t-test showed that there was no significant difference between treatment groups (MD vs. MC, t=1.142, p=0.255), but that the activity in the light period was significantly different from that of the dark period in both MC and MD groups.

MD rats exhibited decreases in the size of the pineal gland and in melatonin levels in the hypothalamus

Only six samples from plasma of MC group and six samples from the plasma of the MD groups were successfully processed due to accidental sample loss during the samples processing. The mean plasma melatonin was 9.43 ± 0.78 pg/ml in the MC group and 9.15 ± 1.25 pg/ml in the MD group. Statistical analysis showed that the difference of the mean between these two groups was not significant (t=2.228 and p= 0.87). Although the number of animals was relatively small (n=6), the negligible difference between the means of the MC and MD indicated that the use of additional animals was not likely to produce a significant difference. Therefore, no additional animals were added.

Prior to analysis of melatonin content in the pineal gland, intact pineal glands isolated from each group of animals were weighed. The mean organ weights of the MD and MC groups were plotted and statistically analyzed (Figure 5A). Interestingly, the mean organ weight in the MD group (1.95 ± 0.10 g) was 14.7% smaller than that of the MC group (2.28 ±0.12 g), (t = 2.08, P=0.04, n=11 for both groups). To characterize the amount of melatonin present in the pineal gland, melatonin in each dried sample of the entire organ was analyzed. Majority of the samples had the value in the linier part of the stabdart cuve. One out layer in the MD group was removed due to extremely high value more than two fold of the standart deviation. The mean of the total melatonin content per pineal gland was 21.19 ± 2.91 pg for

the MC group (n=10) and 13.78 ± 1.38 pg for the MD group (n=11) (Figure 5B). MD group exhibited a significant (t=1.73, p=0.032) reduction of melation than the control group.

Left side of the hypothalamus from each rat was used to quantify hypothalamic melatonin. The amount of hypothalamic melatonin was 8.64 ± 1.19 pg/g in the MC group (n=9) and 5.06 ± 0.81 pg/g in the MD group (n=10; Figure 6). In comparison to the MC group, the hypothalamic melatonin level was reduced by 41.44% in the MD group, which was statistically significant (t=2.11, p=0.026).

Clock protein levels in the hypothalamus are similar in MD and control rats

Right side of the hypothalamus was used for clock protein quantification. The specificity of the polyclonal antibody against clock (sc-6972, Santa Cruz Biotechnology, Inc., Santa Cruz, CA.) was verified using control blocking peptides (Figure 7A). A band in the expected molecular weight (100kda) of clock protein was observed in addition to the expected band at 43 kda for the housekeeping protein actin, which was used as a loading control (Figure 7B). The mean ER_{MC} value was 0.3105 ± 0.0320 and the mean ER_{MD} value was 0.3265 ± 0.0328. The difference between ER_{MC} and ER_{MD} was not found to be statistically significant (t=2.074, p=0.7; Figure 7C).

Discussion

The major findings of this study include the following: (1) the MD rats had significantly less REM sleep and greater quiet wake than MC rats during the light period; (2) the MD rats possessed smaller pineal glands and expressed less melatonin than MC rats in both pineal gland and hypothalamus. However, (3) the circulating levels of melatonin in the bloodstream and the levels of clock protein in the hypothalamus were not different between the groups.

Alteration in sleep-wake behaviors and the model of insomnia

Previously, we reported that neonatal MD resulted in a decrease in total sleep, an increase in total wake during the light period, and a large increase in hypothalamic orexin A (but not orexin B) and CRH levels in the adult MD rats (Feng *et al.*, 2007). The differences of REM and NREM sleep between MD and MC rats were not significant. In the current study, we found that the MD rats exhibited a significant decrease in REM (but not NREM) sleep and a significant increase in quiet wake and total wake during the light period. On one hand, these findings were consistent with our previous findings and confirmed the observation that the MD rats had less total sleep and more wake during the animals' night (light period), but not during their "subjective" day, i.e., dark period and supports the idea that the MD rat is a model of chronic insomnia. One consideration for the reason of a small reduction of total sleep in the MD rats might be that our control group was the home cage control instead of the previously reported maternal handling group (Plotsky & Meaney, 1993). On the other hand, the observation that significant decreases in REM sleep occurred in the MD group differed from the previous study which showed that the mean REM sleep in MD rats was 19.1% less than that of the MC group during the light period but the differences was not statistically significant (Feng *et al.*, 2007), One explanation is that the previous study had a smaller group size (11 MD and 12 MC rats) compared with that of current study (MC=15 and MD= 16). We believe that the increase in the group size in the present study has made some contribution to the differences in current study. Data from the previous study and the current study support the conclusion that sleep changes are present in MD rats. Specifically, neonatal MD results in a reduction of total sleep in adult rats, in particular REM sleep, during the light period, i.e. subjective night. Based on findings in this and our previous paper (Feng *et al.*, 2007), where MD rats had consistent reduction of total sleep and more

pronounced reduction of REM sleep observed during 3–6 month of age, the MD rat can be considered as a model of chronic insomnia. The word “chronic” applied in this model is in contrast to any likely model that had insomnia-like changes right after a treatment with either a drug or behavioral manipulation, such as acute stress, but it is not intended to claim that the insomnia-like changes found in the MD rat are life-long. The limitation of the MD rat as a model of insomnia includes (1) the size of the group needs to be relative large (n>=15) in order to show the difference of total sleep between MD and control rats, (2) this model of insomnia may only reflect pathology of stress induced insomnia and (3) the reduction of sleep is more from a reduction of REM sleep rather than NREM sleep. Despite the reduction of total sleep is mild, we still consider this is the best rodent model of chronic insomnia beause (1) human insomniacs may not nessecarily have a reduction of total sleep or sleep efficiency measured by PSG (Lichstein et al., 1994), (2) this model links to changes of HPA axis and orexins and (3) the pathology of this model has a choronic feature.

Alterations in pineal gland weight and melatonin levels

The weight of the pineal gland was significantly smaller in the MD group. This result indicates that neonatal MD may induce maldevelopment of the pineal gland and reduced productive capacity. No similar studies have previously observed this change in the pineal gland. However, one previous study that focused on the pineal gland showed that ten days of continuous lighting increased the gland weight (Guzman et al., 1983). Another previous study found that eight consecutive days of chronic stress induced by physical immobilization significantly increased pineal melatonin levels on experimental days 3 and 6 and significantly suppressed serotonin N-acetyltransferase (NAT) activity on experimental days 1, 3, and 5, with a slight reduction on day 7, but this study did not report the gland weight (Vollrath & Welker, 1988). These observations imply that the effects of neonatal MD on the pineal gland are likely different from that of other types of stress and/or stress treatments in mature animals.

The effect of stress on melatonin levels has been analyzed previously. Acute stress and permanent lighting have been reported to increase melatonin levels. In human, stress, CRH administration, and swimming increase melatonin levels, but exercise at night significantly blunts the nocturnal increase in plasma melatonin levels (Monteleone et al., 1990). Daytime swimming elicited no major changes in enzyme activity or pineal melatonin, but swimming at night prevented the normal rises in NAT activity and pineal melatonin (Tannenbaum *et al.*, 1989). One study showed that swimming caused a rapid and highly significant drop in the melatonin content in the pineal gland (Yaga et al., 1993). Stress induced by a saline injection depressed both NAT activity and the melatonin content of the pineal gland (Troiani et al., 1987). Physical immobilization for eight consecutive days suppressed the NAT activity, but slightly increased pineal melatonin levels (Vollrath & Welker, 1988). Direct intravenous injection of CRH in humans induced an inhibitory effect on pineal secretion of melatonin (Kellner *et al.*, 1997). Immobilization for 30 minutes significantly increased pineal melatonin content, as well as plasma melatonin concentrations, in rats bearing intact pineal glands (Chiba *et al.*, 1998). Together, this evidence implies that neonatal chronic stress by MD has a negative impact on the maturation of pineal gland and the levels of melatonin in the hypothalamus. Whether the impact leads to disturbance of REM sleep during the light period remains to be explored.

Melatonin levels were determined for samples from the plasma, pineal gland, and hypothalamus at the early light phase, within a window of 1 to 2 hours after the light was turned on. Despite the fact that melatonin levels are significantly higher during the dark phase, the underlying reason for selection of this time point was the fact that this model has been proposed to be a model of insomnia and human insomniacs have symptoms of difficulty in falling asleep and highest blood hormones of ACTH and cortisol during evening

or early night (Drake *et al.*, 2004; Saletu-Zyhlarz *et al.*, 1997; Stepanski *et al.*, 1988; Vgontzas *et al.*, 2001), a period corresponding to rat's early light period. Thus, a reduction of sleep in the light period could indicate a reduction in melatonin levels due to melatonin having a promoting effect on sleep (Bendz & Scates, 2010; Holmes & Sugden, 1982; Mendelson, 2002; Mendelson *et al.*, 1980) or an increase of brain CRH or orexin A because CRH and orexin A are increased in this model (Feng et al., 2007). Levels of plasma melatonin in our study were comparable to previous reports by other labs, which have shown that melatonin in normal rats exhibits a large variation from 11–180 pg/mL in serum (50–800 pmol/L, mw 232) (Peschke *et al.*, 2006) to 200 pg/mL in plasma (Jaworek *et al.*, 2004). The variance is possibly due to using different strains and/or methods for melatonin analysis. Our results indicate that neonatal MD does not produce an alteration in blood levels of melatonin. This is different from alterations of melatonin level in the hypothalamus. The mean melatonin levels in the pineal gland of MD animals were also decreased to about 32% of controls. One expaination to the negative finding of blood melatonin in contrast to a significant reduction of melationin in the hypothalamus and pineal gland is that melatonin needs to cross brain blood bariar and distributed in the entire circulating system. The limitation of the result of blood level of melatonin was that the number of total animals was only 6 for each group. However, we still believe that the result of blood level of melatonin is reliable due to the very close means and small standard error. Overall, differences in the weight of the pineal gland and in the melatonin levels in both hypothalamus and pineal gland highly support the hypothesis that production of melatonin in the MD group is decreased. These observations are consistent with the findings that melatonin (Brzezinski et al., 2005; Cardinali et al., 2005) or melatonin receptor agonist (Zammit *et al.*, 2009) advances sleep onset, and that MD rats exhibited decreased total sleep during the light (sleep) period (Feng *et al.*, 2007).

Clock protein levels and circadian rhythms in the MD rat

In the rodent, the rhythmic synthesis of melatonin by the pineal gland is tightly controlled by the master "clock" located in the SCN via nocturnal release of norepinephrine that triggers AANAT transcription (Feillet et al., 2008). *Clock* gene is one of the multiple genes that regulate circadian activities including period 1 and period 2 (Dardente et al., 2007; DeBruyne et al., 2007). One of the major interests in studying the change of clock protein level is that it can affect behaviors. *Clock-d19* mutant mice display an increase in cocaine reward behavior, increased excitability of dopamine neurons in the midbrain ventral tegmental area, a key brain reward region, and an increased expression and phosphorylation of tyrosine hydroxylase (the rate-limiting enzyme in dopamine synthesis), as well as changes in several genes known to regulate dopamine activity in the ventral tegmental area (McClung *et al.*, 2005). *Clock-d19* mutant mice also showed decreased sleep, lowered depression-like behavior, lower anxiety, and an increase in the reward value for cocaine, sucrose, and medial forebrain bundle stimulation (Roybal *et al.*, 2007). Knockdown of Clock using siRNA specifically in the VTA, results in hyperactivity and a reduction in anxiety-related behavior (Mukherjee et al.). The levels of clock protein in the hypothalamus of the MD group were not significantly different from that of the control group indicating that maturation of clock protein production is not as sensitive as the maturation of melatonin production to maternal stress. This result is consistent with our other data of the MD rats having no phase misalignment measured by 24 hours of locomotor activity. This result is also consistent with the observation that acute physical stress elevated Period1 (Per1) mRNA expression in mouse peripheral organs, but not behavioral rhythms and peripheral molecular clocks (Yamamoto *et al.*, 2005).

In summary, our study indicates that neonatal MD significantly suppresses the maturation of the pineal gland and reduces brain melatonin levels without affecting the clock protein,

activity profiles, or plasma melatonin levels. These alterations likely contribute to the phenotype in which MD rats exhibit reduced total sleep and REM sleep during the light period without a change in circadian phase.

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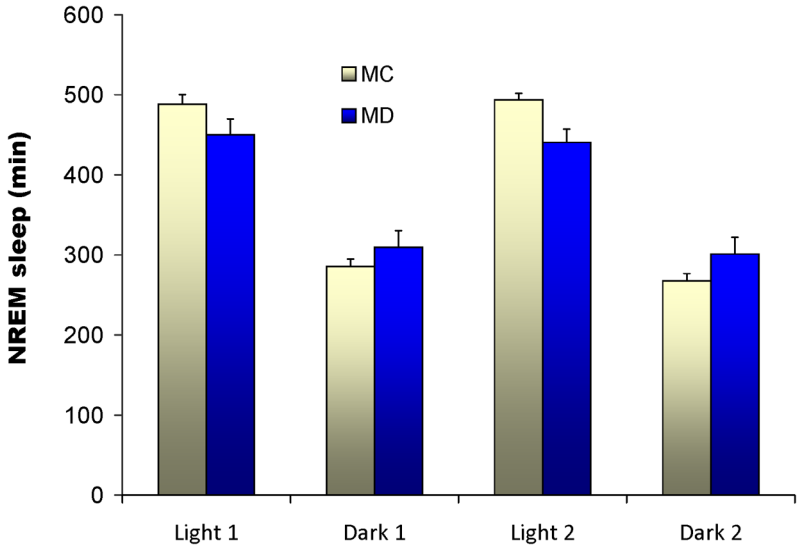

Figure 1.

48 hours of NREM Sleep plotted in 12-hour light and dark phases. Data presented as mean ± se. ***: $p<0.001$. The percentage of NREM sleep was significantly higher in the light phase than the dark phase in both MD (n=15) and MC rats (n=16), but differences between the groups in the same phase were not significant.

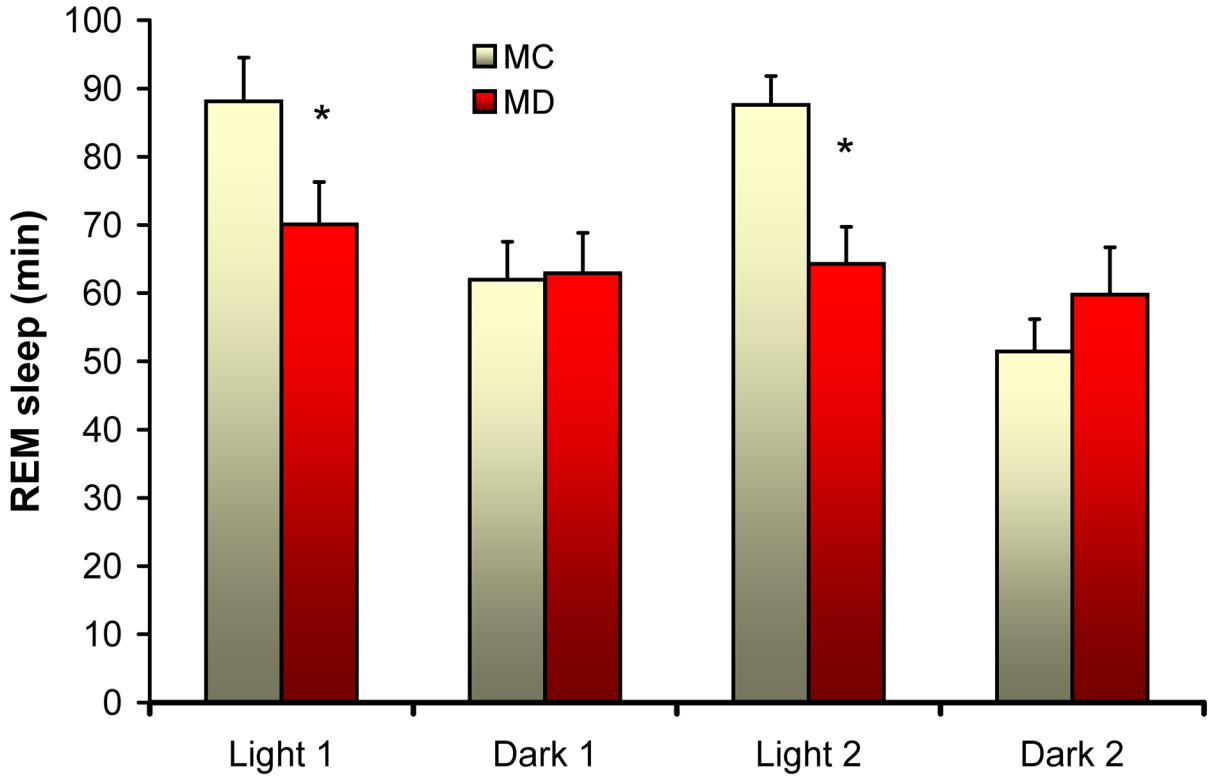

Figure 2.
REM sleep during 12-hour light and dark phases in MC (n=16) and MD rats (n=15). Data showed that the MC rats spent a significantly higher amount of time in REM sleep during the light phase compared to the dark phase. However, the time spent in REM sleep in MD rats was similar in the light phase and in the dark phase. In comparison to MC rats, the amount of time spent in REM sleep of the MD rats was significantly higher in both light phases, but similar in the dark phases. *: $p<0.05$.

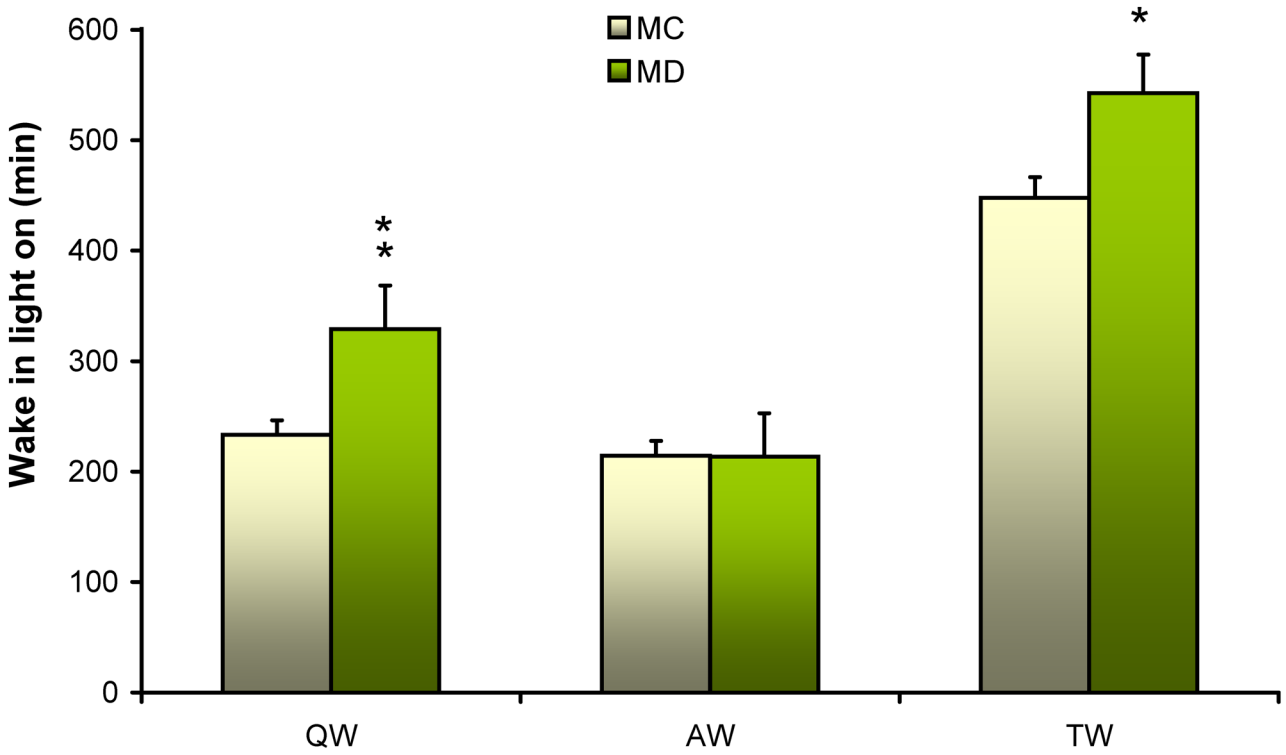

Figure 3.
Quiet wake (QW) and total wake time (TW) during the light period in the MD groups (n=15) were significantly higher than that of the MC group (n=16). AW: active wake. *: $p<0.05$.

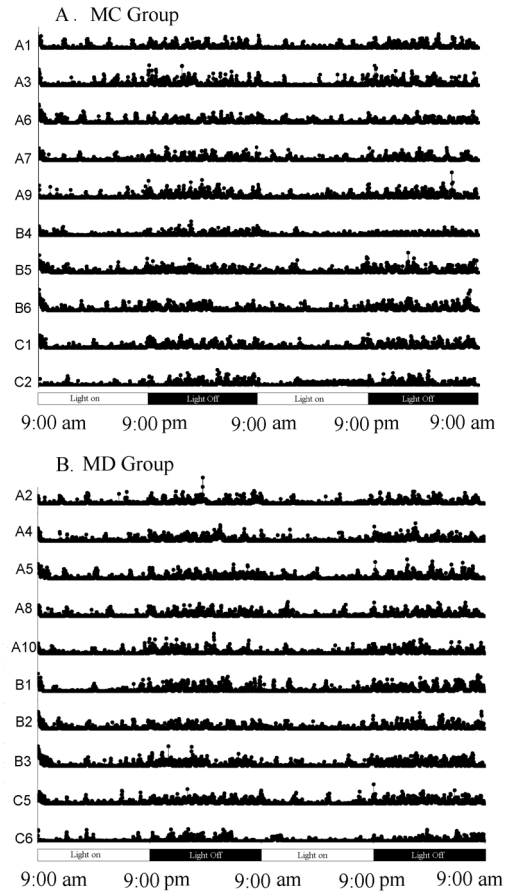

Figure 4.

Locomotor activity was measured continuously for 48 hours in the home cage. A. Activity of ten MC rats. B. Activity of ten MD rats. The lable on the left side of each line was the ID of the rat. More activity occurred in the dark phase than in the light phase in both MC rats (A) and MD rats (B). Overall, circadian rhythmic pattern was normal. No significant differences were observed between MC and MD rats.

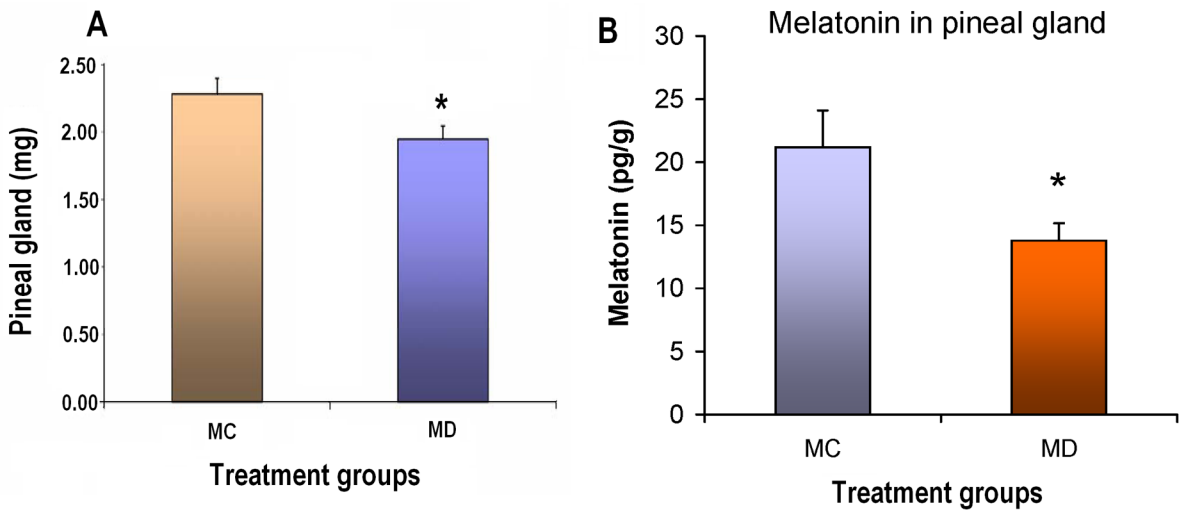

Figure 5.
The mean weight of the pineal gland in the MD group (n=11) was 15% smaller than that of the MC group (n=11). Although the difference was small, it was statistically significant ($p<0.05$). The difference in melatonin levels in the pineal gland between the MC (n=11) and MD rats (n=10) was also statistically significant ($p=0.032$).

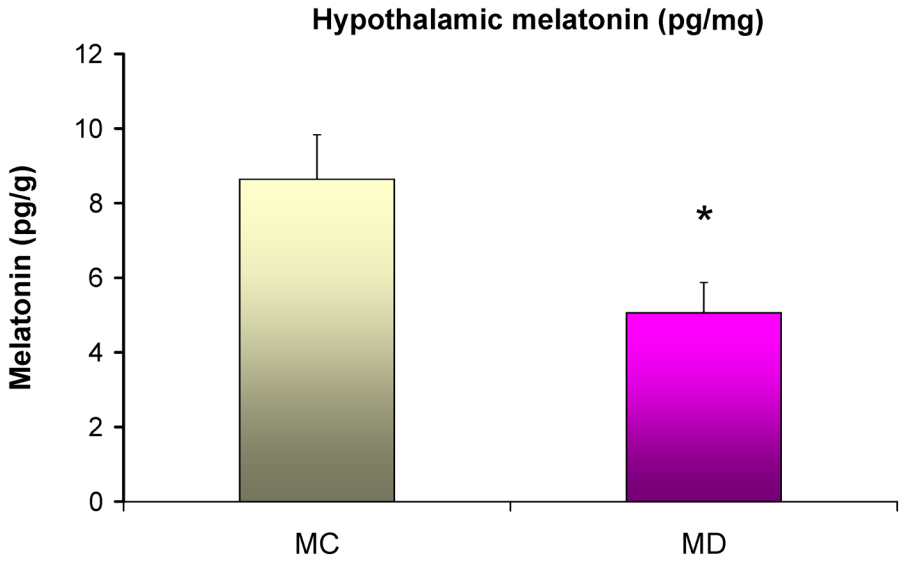

Figure 6.
Melatonin levels in the hypothalamus. The mean melatonin level in the hypothalamus of MD (n=9) rats was significantly decreased by more than 40% than that of MC rats (n=10). The difference was statistically significant ($p<0.05$).

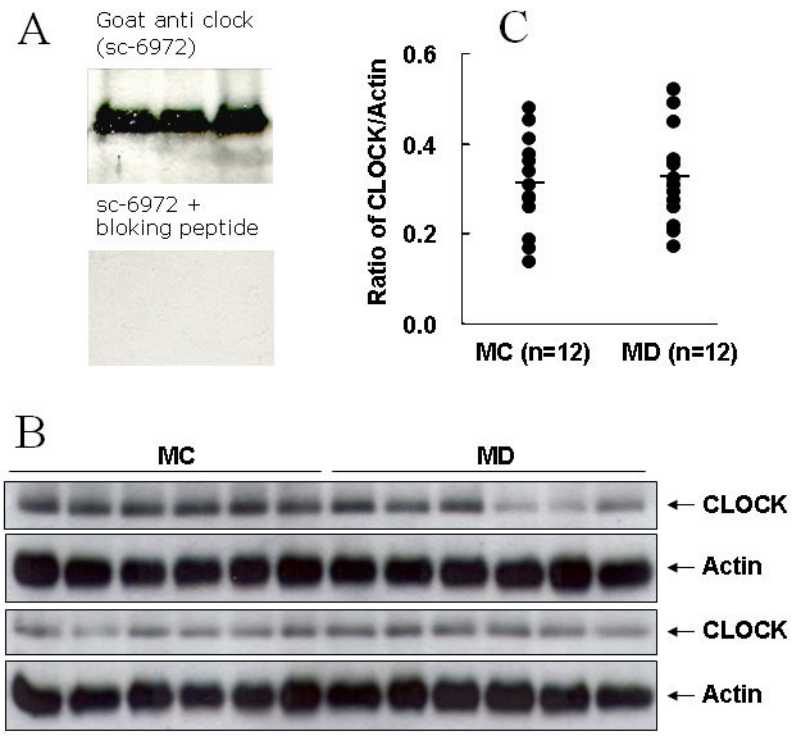

Figure 7.

Clock protein levels in the hypothalamus. A. A band of the appropriate molecular weight for the clock protein was evident in Western blots incubated with the goat anti-clock antibody (sc-6972), but not when blots were pretreated with the sc-6972 blocking peptide. B. Western blots of clock for MC and MD rats. Actin blots are shown as a loading control for normalization purposes. C. Densities of the clock and actin bands were quantified by densitometry. Clock levels were normalized to actin levels. The mean normalized levels of clock expression were similar in the MC and MD groups. No significant difference was observed ($p=0.73$).

Table 1

48 hours of locomotor activity

Basic movement of activity was quanbtified by 6 hours. The value in the table represents the mean ± se of each group. None of the differences between groups in the same time period was statistically significant.

	Time period	MC	MD
Day 1	9 am– 3 pm	3505 ±194	3780±161
	3 pm– 9 pm	2413±104	2398±68
	9 pm– 3 am	7868±204	8722±167
	3 am– 9 am	6497±134	6787±162
Day 2	9 am– 3 pm	2374±76	2485±62
	3 pm– 9 pm	2689±132	2243±88
	9 pm– 3 am	7992±223	8477±254
	3 am– 9 pm	6913±174	7577±195