Spring 2012

The Effects of Prenatal Exposure to Altered Melatonin Levels on Hippocampal Gene Expression in the Male Rat

Anna Uehara
Bucknell University

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by

Anna Uehara

A Proposal Submitted to the Honors Council

For Honors in Neuroscience

April 13, 2012

Approved by:

Kathleen C Page

Adviser: Kathleen C. Page, PhD

Kathleen C Page

Department Chairperson: Kathleen C. Page, PhD

ACKNOWLEDGEMENTS

I would like to thank Carmen Acuna, PhD, for her assistance with statistical analysis of the data for this study. I am also grateful for the support of Elizabeth Marin, PhD, in helping me with my writing skills. I would also like to offer special thanks to Joshua Ripple, B.S., for inviting me into his melatonin project to which I have grown a fond interest for. Moreover, words cannot express the gratitude I have for my academic and research advisor, Kathleen C. Page, PhD, for all of her assistance in helping me understand my results and articulate my findings. Without her never-ending assistance, I would not be the student and scientist that I am now. Finally, I would like to thank my parents and friends for their undying support and assistance that has brought me to Bucknell University and allowed me to challenge myself through participating in undergraduate research. It is to these special individuals that I dedicate this study.

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ABSTRACT

The stability of the circadian rhythm for mammals depends on the levels of serotonin and melatonin, neurohormones that signal for lightness and darkness, respectively. Disruption in the stability of neurohormones has been shown to be a critical factor in psychopathological disorders in humans. For example, altering levels of melatonin *in utero* through administration of melatonin or the melatonin receptor antagonist, luzindole, has been shown to cause changes in developmental growth and adult behavior in the male rat. Analysis of relative adult hippocampal gene expression with RT-PCR revealed differences in ARNTL expression that suggested abnormality in clock gene expression of the rats that were prenatally exposed to altered levels of melatonin. Differences in the degree of plasticity as suggested by previous behavior testing did not result in differences in gene expression for GABA receptors or NMDA receptors. Morevoer, growth associated protein 43, GAP-43, a protein that is necessary for neuronal growth cones as well as long term learning has been found to be critical for axon and presynaptic terminal formation and retention in other studies, but hippocampal gene expression in our study showed no significant alteration after exposure to various maternal melatonin levels. However, ARNTL is a key regulatory component of clock genes and the circadian cycle so that alterations in the expression of thi critical gene may lead to critical changes in neuronal growth and plasticity. Our data support the conclusion that the manipulation of maternal melatonin levels alters the brain development and the circadian cycles that may lead to physiological and behavioral abnormalities in adult offspring.

INTRODUCTION

The Circadian Rhythm in Mammals

The circadian rhythm in mammals is the daily behavioral rhythm that is primarily controlled by the fluctuating levels of serotonin and melatonin. Disruption in the stability of these two neurohormones is one of the critical factors in psychopathological disorders in humans. For instance, changes in melatonin levels have been shown in schizophrenia (Rao et al., 1994), and depression (Kennedy et al., 1989) as well as other mood disorders (Srinivasan et al., 2006). It is known that melatonin is a key player in maintaining a healthy daily rhythm; however, it is not clear how abnormal fluctuations of this neurohormone influence the brain and relate to brain-related diseases.

Melatonin is synthesized from serotonin and secreted by the pineal gland during the dark period of the light-dark cycle under normal environmental conditions (Cardinali and Pevet, 1998). It is produced via a multi-step pathway beginning with the synthesis of 5-hydroxytryptophan from tryptophan via tryptophan hydroxylase. The 5-hydroxytryptophan is converted to serotonin by aromatic amino acid decarboxylase and the acetylation of serotonin by arylalkylamine N-acetyltransferase results in N-acetylserotonin. The final step involves hydroxyindole-O-methyltransferase which converts N-acetylserotonin to melatonin (Simonneuax et al., 2003). The synthetic melatonin receptor antagonist, luzindole, has a structure that is very similar to melatonin but with an additional benzyl group. This compound has been shown to competitively antagonize the melatonin receptors at a presynaptic level (Dubocovich., 1988).

Figure 1: Simplified diagram of the circadian rhythm monitored by suprachiasmatic nucleus (adapted from Drake, 2010). Light induces retinal ganglion cells to signal to the suprachiasmatic nucleus along the retinohypothalamic tract. The suprachaiasmatic nucleus, which contains the primary circadian molecular clock processes inputs from the retinal ganglion cells as well as other brain regions and elicits the release of melatonin by the pineal gland on top of outputs to other brain regions. Melatonin is then released into the cerebrospinal fluid and blood, spiking in levels at night.

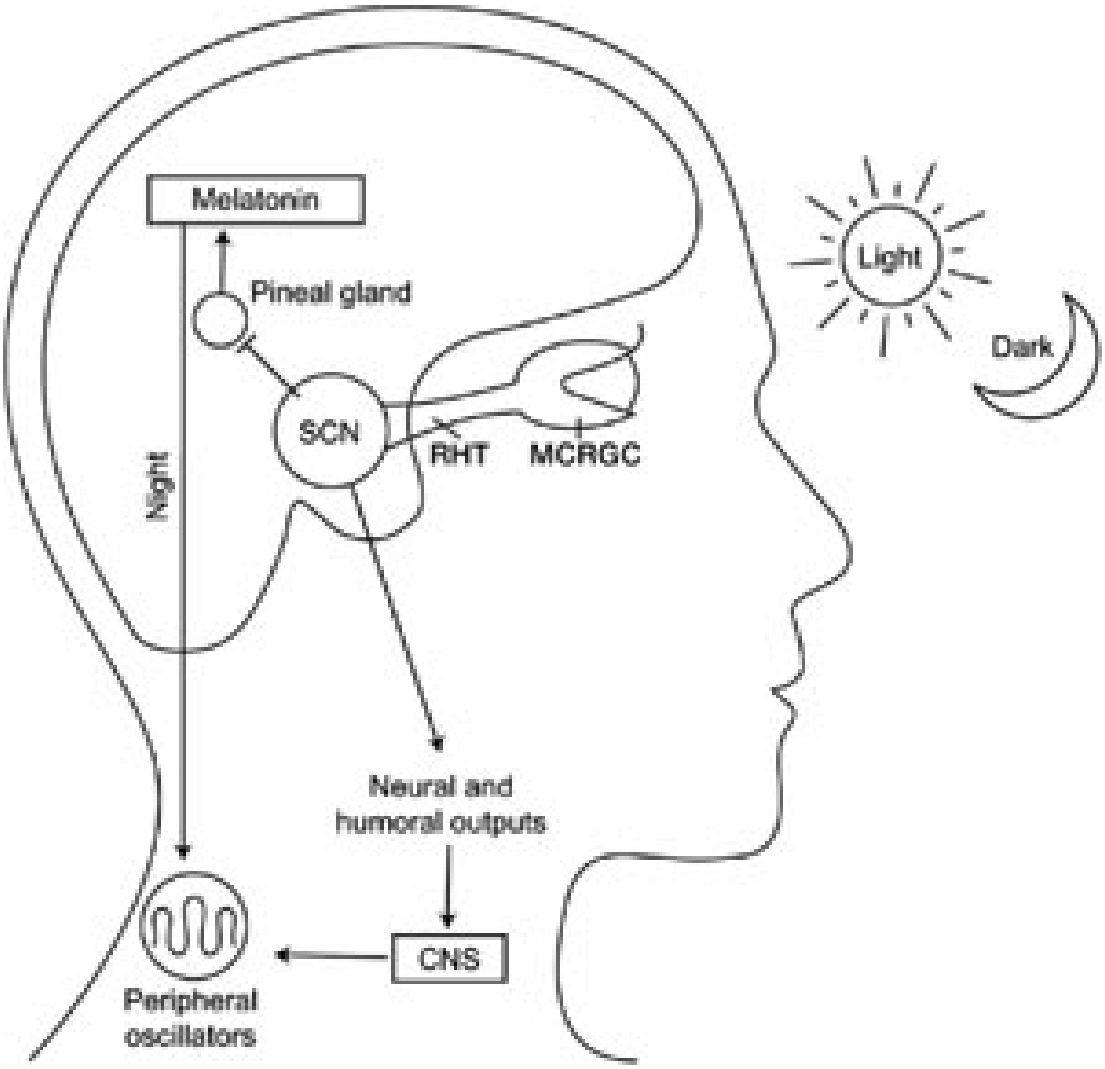
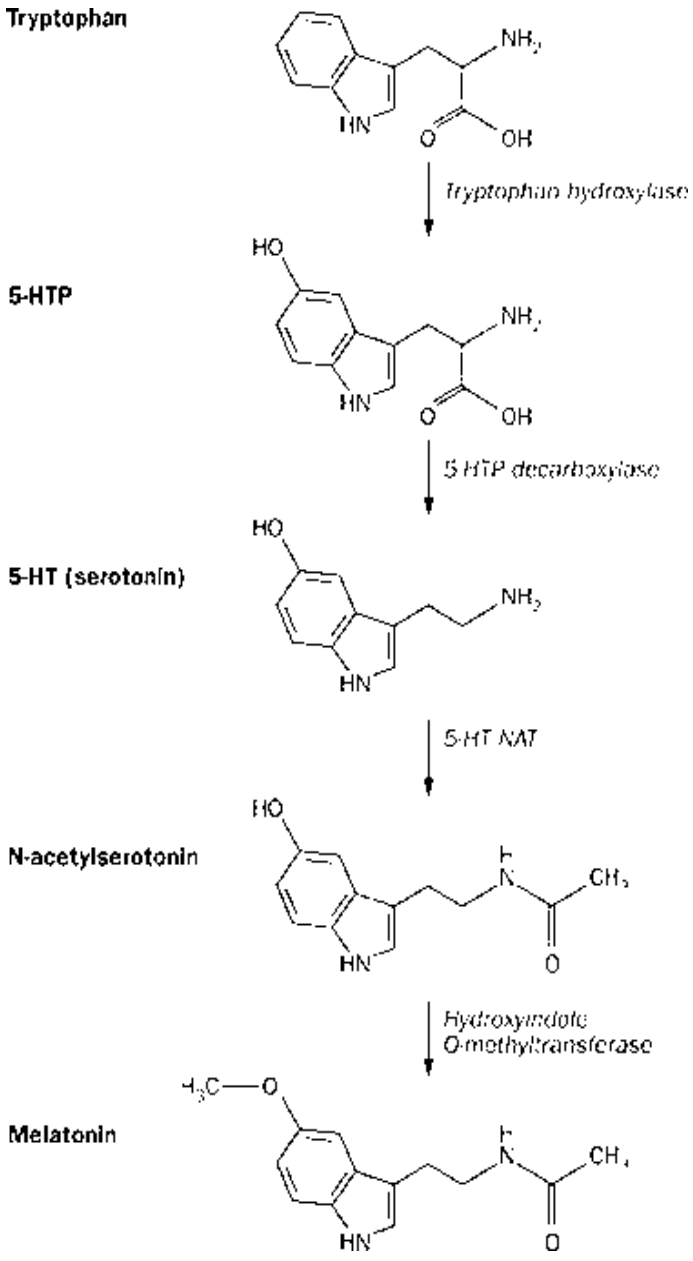
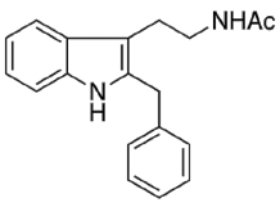

Melatonin
Pineal gland
SCN
RHT
MCRGC
Night
Light
Dark
Neural and
humoral outputs
CNS
Peripheral
oscillators

Figure 2: Diagram of melatonin synthesis (a) (adapted from Koch et al., 2009) and molecular diagram of melatonin receptor antagonist, luzindole (b). Within the pineal gland, 5-hydroxytryptophan is synthesized from tryptophan by tryptophan hydroxylase. 5-HTP decarboxylase transforms 5-HTP into 5-HT, or serotonin. Acetylation of serotonin by arylalkylamine N-acetyltransferase produces N-acetylserotonin. Melatonin is produced from N-acetylserotonin by hydroxyindole-O-methyltransferase.

(a)


Tryptophan
NH2
HN
O
OH
Tryptophan hydroxylase
5-HTP
HO
NH2
HN
O
OH
5-HTP decarboxylase
5-HT (serotonin)
HO
NH2
HN
5-HT NAT
N-acetylserotonin
HO
H
N
CH3
O
HN
Hydroxyindole
O-methyltransferase
Melatonin
H3C
O
H
N
CH3
O
HN

(b)


NHAc
N
H

The secretion of melatonin is controlled by the circadian clock within the suprachiasmatic nuclei (SCN) and is entrained to a light-dark cycle that is roughly 24 hours long (Claustrat et al., 2005). SCN activity is responsive to the amount of light from the environment via signals from the retinal ganglion cells that project through the optic chiasm and onto the SCN. The SCN then projects indirectly to the pineal gland, where it is able to control the nightly secretion of melatonin. Cells in the SCN are also capable of responding to the pineal gland's melatonin secretion via melatonin receptors. Hence, SCN control of melatonin secretion occurs through a feedback loop that consists primarily of inputs from the retinal ganglion cells, but also from endogenous melatonin secreted from the pineal and acting back on the SCN.

Clock Genes

Within the SCN, a circadian rhythm is generated by the oscillations in various regulatory protein levels. Brain and muscle aryl hydrocarbon receptor translocator (ARNT)-like (BMAL1) and Circadian Locomotor Output Cycle Kaput (CLOCK) form a heterodimer that binds to E-boxes which are regulatory sites within the sequence of DNA that allows the expression of Period (PER) and Cryptochrome (CRY) mRNA as well as other clock controlled genes. PER and CRY protein form a dimer (PER/CRY), which then feedback to inhibit the BMAL1/CLOCK dimer function (Okamura et al., 2002). Furthermore, inhibition of the BMAL1/CLOCK cycle is also conducted by a protein known as nuclear receptor subfamily 1, group D, member 1 (NR1D1). NR1D1 works by indirectly inhibiting the expression of the BMAL1 gene by blocking its transcription, thereby decreasing the amount of BMAL1 protein (Layeghifard et al., 2008).

Figure 3: Simplified model of clock gene network in the suprachiasmatic nucles of mammals (adapted from Richter et al., 2004). BMAL1/CLOCK heterodimer binds to E-boxes on the DNA transcribing clock genes and clock controlled genes. Clock genes, PER1 and CRY form a dimer that then blocks BMAL1/CLOCK from binding to the E-box, thereby inhibiting transcription of the clock genes and clock controlled genes.

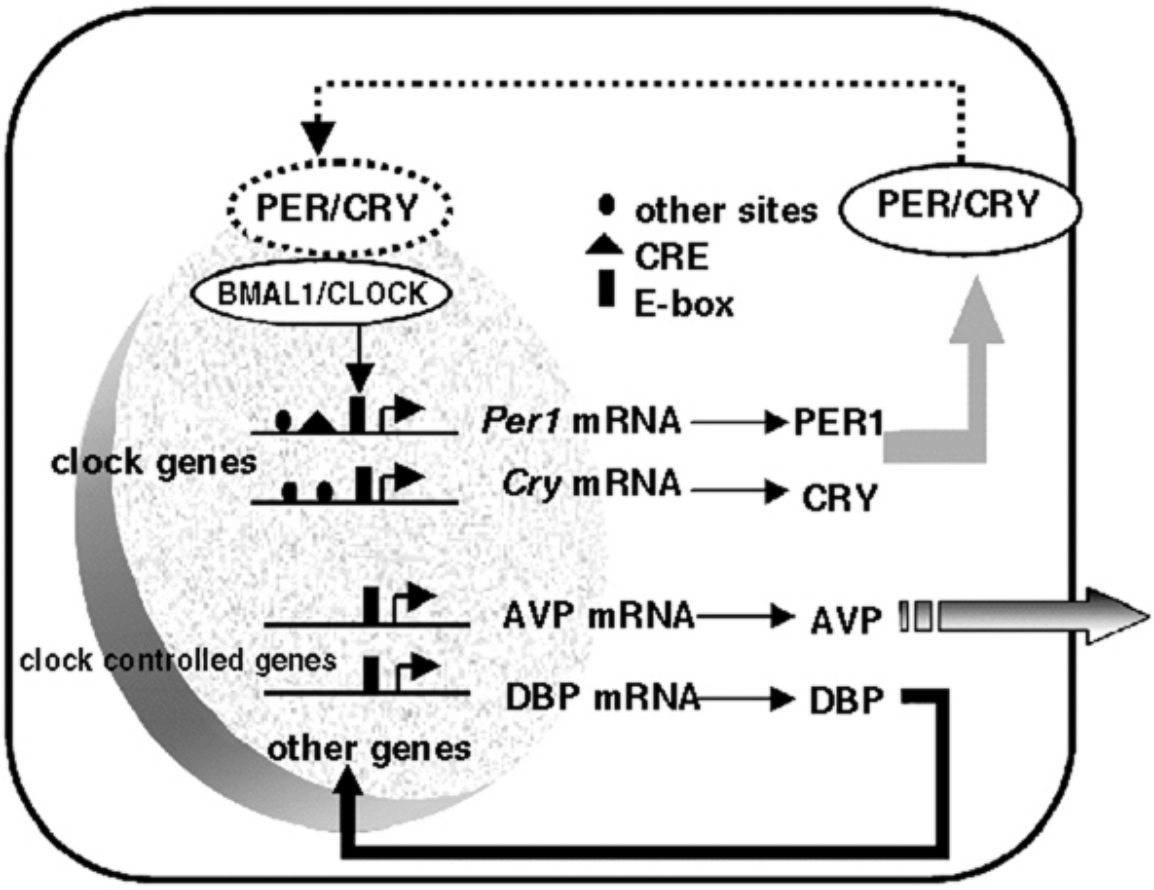
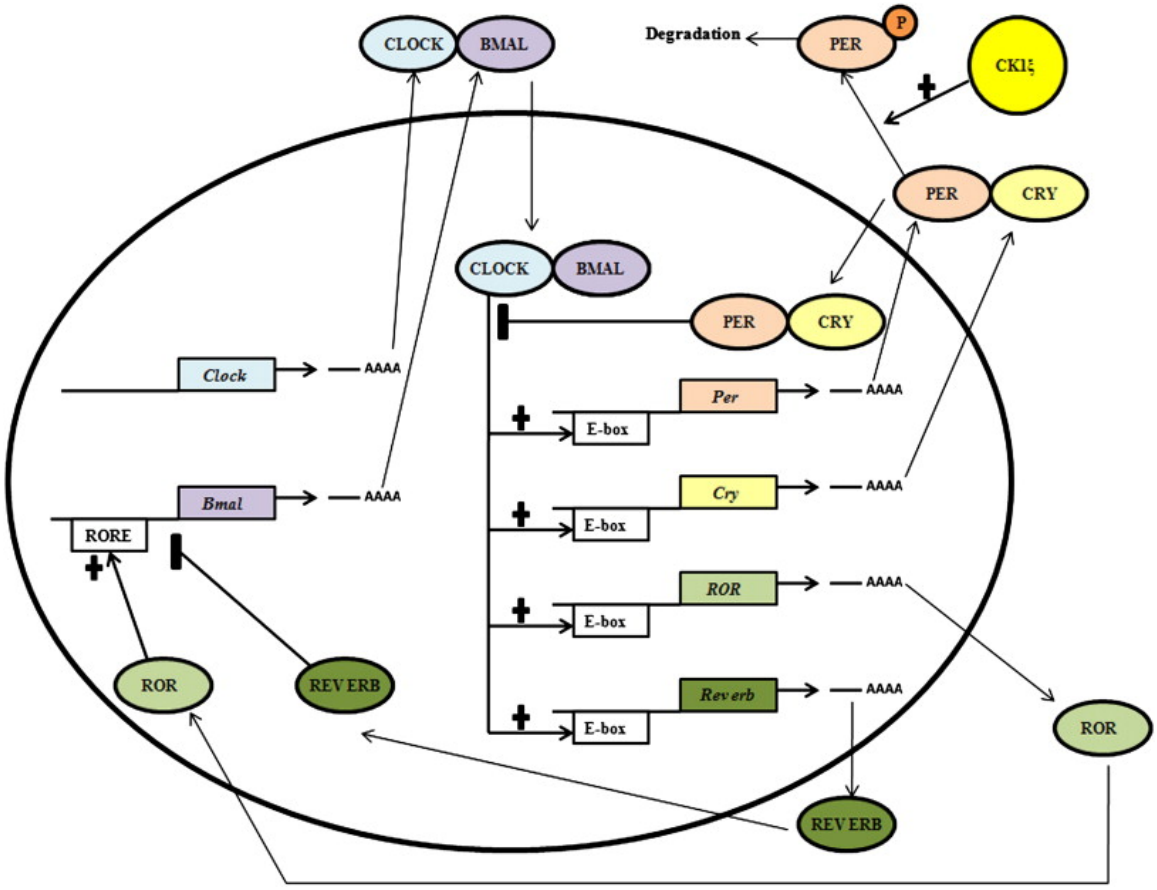

PER/CRY
BMAL1/CLOCK
other sites
CRE
E-box
PER/CRY
clock genes
Per1 mRNA
PER1
Cry mRNA
CRY
AVP mRNA
AVP
clock controlled genes
DBP mRNA
DBP
other genes

Figure 4: Simplified model of clock gene network in the suprachiasmatic nucles of mammals in relation to NR1D1's inhibitory effects on BMAL1 (adapted from Sukumaran et al., 2010). NR1D1, noted as REVERB in diagram, has inhibitory effects on the transcription of BMAL. High levels of REVERB will inhibit the transcription of BMAL, thereby decreasing the possibility of forming a BMAL/CLOCK heterodimer, affecting the level or protein production.


CLOCK
BMAL
Degradation
PER
P
CK1ε
PER
CRY
CLOCK
BMAL
PER
CRY
Clock
AAAA
Per
AAAA
E-box
Bmal
AAAA
Cry
AAAA
RORE
E-box
ROR
AAAA
E-box
ROR
REV ERB
Rev erb
AAAA
E-box
ROR
REV ERB

The fluctuating concentration differences of the BMAL1/CLOCK dimer in relation to the PER1/CRY dimer created by the activation and repression of downstream gene targets are what create circadian rhythm within the SCN.

Melatonin and the Circadian Rhythm

There are two main hypotheses of how melatonin peaks are positively correlated with the length of the dark phase during the light-dark cycle. In what is known as the "duration hypothesis", the duration of the nocturnal melatonin peak is considered to be the critical parameter in understanding the light phase of the light-dark cycle (Cardinali and Pevet, 1993). In contrast, the "coincidence hypothesis", states that circadian variation of sensitivity to melatonin is involved with the light response. When intact Syrian hamsters were kept at long photoperiods and administered exogenous melatonin, their circadian response modeled those that were kept at normal photoperiods, indicating that the exogenous melatonin advanced the circadian sensitive phase (Stetson and Watson-Whitmyre, 1985). It is unclear whether or not either of these hypotheses is an accurate explanation for SCN interaction with the photoperiods and regulation of melatonin levels; however, both hypotheses underline the ties between the SCN, photoperiods cued by the retinal ganglion cells, and the melatonin that is released from the pineal gland.

In addition to its association with the daily behavioral rhythm, melatonin is also known to be a regulator of various bodily functions that oscillate with the daily cycle of light and dark. For instance, core body temperature in humans, which exhibits a maximum during the day and a minimum at night, has been shown to be coupled with the sleep-wake cycle in normal conditions (Cagnacci et al., 1992). Experiments have also been conducted for those suffering from insomnia

or circadian rhythm disorders and the data show that low-dose melatonin treatments promoted regulation in sleep onset and sleep maintenance without disrupting other functions associated with circadian rhythm (Zhdanova and Tucci., 2003; Sack et al., 1998). It is clear that melatonin is a key player in the regulation of the circadian rhythm and multiple physiological functions. In fact, evidence suggests that alternations in melatonin regulation may increase the risk for mood disorders and cognitive dysfunction (Kripke et al., 2009). One study showed that depressed patients exhibited clear circadian rhythm abnormalities that consisted mainly of melatonin amplitude reduction at night (Souetre et al., 1988). In addition, differences in photoperiods and the respective levels of melatonin were compared across generations in a maternal-fetal chronobiological study. This investigation found that reduced melatonin concentrations impaired in the animal's ability to regulate internal temperature and had detrimental effects on immature hippocampal neurons in animals (Schwartz, 2011).

NMDA Receptors

Mounting evidence suggests that detrimental effects of altered melatonin levels occur when circadian rhythms are disturbed. For example, schizophrenic patients, who have altered melatonin levels, also show impairments in memory and learning. Many of these changes have been correlated with changes in key proteins that mediate synaptic function. For example, levels of key postsynaptic receptors, subunits of a major glutamate receptor, the n-methyl-D-asparate (NMDA) receptor, have been shown to be differentially expressed in schizophrenic patients with right/left hemisphere differences (Vrajova et al., 2010). Studies show that melatonin alters NMDA subunit concentrations in a dose-dependent manner leading to the possibility that the

differences in concentration of NMDA subunits are linked to the alterations in NMDA receptor function (Sutcu et al., 2006). Since these receptors are key mediators of learning and memory, abnormalities in these functions are likely candidates for disturbances linked to mood disorder and schizophrenia.

GABA Receptors

Additional synaptic disturbances have been associated with other major synaptic receptors. For example, in contrast to glutamate's excitatory role, the neurotransmitter gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system. GABA receptors can be broken down into two groups of receptors, ionotropic ($GABA_A$) and metabotropic ($GABA_B$), and these receptors have been shown to influence circadian rhythm, primarily through regulation of cell activation or inactivation, and to contribute to jet-lag symptoms and phase-shifting (Golombek et al., 1996). With regard to the clock genes, the effects of GABA on individual clock cells have been studied with results indicating that GABA acting through its type A receptors is involved in phase shifting and synchronization of clock cells (Liu and Reppert, 2000). Although the interactions between GABA and other neurotransmitters in mood disorders such as schizophrenia are not entirely understood, abnormalities in GABA interactions are associated with schizophrenia (Wasserf et al., 2003). It has also been shown that GABAergic projection from the SCN to the dorsomedial hypothalamus controls the release of melatonin during the dark phase (Kalsbeek et al., 1996). It is possible that changes in melatonin levels may be coupled to the altered levels of GABA in schizophrenia.

In summary, the synthesis and release of melatonin during prenatal development, a period of time when circadian rhythms are being established, is central to an individual's health. Moreover, accurate regulation of melatonin levels is also critical during postnatal development. This is especially true in the hippocampus, a brain region where acquisition and consolidation of learning and memory take place. Hippocampal lesions have been used to demonstrate the importance of the hippocampus for short-term and temporal memories in humans (Scoville and Milner, 1957). In addition, hippocampal lesions result in behavioral disinhibition and reduced anxiety (Bannerman et al., 2004). An altered synaptic circuitry within the hippocampus and its extrinsic connections has also been associated with aberrant functional connectivity in schizophrenic individuals (Harrison, 2004). These observations suggest that melatonin may regulate neuronal plasticity (El-Sherif et al., 2003) and cell survival of new neurons (Ramirez-Rodriguez et al., 2009), which are required for hippocampal and cognitive function in adult rats.

Hypothesis

Altering maternal melatonin levels via exogenous melatonin treatment during pregnancy leads to differential mRNA gene expression in adult offspring for gene products mediating the proper control of the circadian rhythm.

MATERIALS AND METHODS

Animals and Tissue Collection

Eleven pregnant Sprague-Dawley dams from Hilltop Laboratories were randomly assigned to three treatment groups. The control group was injected with a vehicle containing 50%(volume/volume) sterile, filtered DMSO (Sigma Aldrich, St. Louis, MO) in distilled water. The melatonin group was injected with 5mg/kg melatonin (N-acetyl-5-methoxytryptamine) (Sigma Aldrich). The luzindole group was injected with 5mg/kg luzindole (N-acetyl-2-benzyltryptamine) (BA Chemicals, London, UK), a nonselective melatonin receptor antagonist (Dubocovich, 1998). All drugs were administered subcutaneously at a volume of 0.05 to 0.06mL prior to the dark cycle at 1600 to 1800 for 5 days from days 14 to 18 of gestation. Dams were housed individually, given food and water *ad libitum* and kept at a controlled temperature of 23°C under a strict 12hour light-dark cycle.

Offspring were weighed at birth, and on postnatal day 21, male offspring from the same treatment group were housed in pairs. Every 5 to 7 days, body mass per rat and food intake per cage were measured from weaning to sacrifice. After 120 days, half of the animals were sacrificed at 0000 hours and the rest were sacrificed at 1200 hours. Right and left hippocampi were dissected from each brain. (All animal protocols used were in accordance with the NIH Guide for Care and Use of Laboratory Animals and reviewed and approved by the Animal Care and Use Committee of Bucknell University).

Prior to reverse transcription, total RNA was extracted from the hippocampus of individual 120-day old male rats and purified using TRIzol (Invitrogen, Carlsbad, CA). Total RNA was then reverse transcribed using a RETROscript kit (Ambion, Austin, TX). Primers were

designed with the online NCBI database and confirmed with BLAST and then ordered from MWG Oligo Synthesis (High Point, NC). Real-time PCR was performed in 96-well optical plates with 0.05μM of forward and reverse primers, cDNA from 2μg RNA input (diluted 1:50) and 12.5μL Supermix (Bio-Rad, Hercules, CA). The assay was conducted using the iCycler iQ Real Time PCR Detection System (Bio-Rad, Hercules, CA).

Primer Design, RT-PCR and Analysis

Efficiency of the primers was determined using a serial dilution of RT-PCR products. Efficiencies were calculated using the following formula:

$$E = 10^{-1/slope}$$

Prior to analyzing experimental plates, control plates for each primer pair were analyzed by testing randomly selected samples, samples not containing cDNA, or dH_2O. Primer pairs that showed a high efficiency of amplification and "clean" control plates were then run on gel electrophoresis with 12% Boraid to ensure that there was no DNA contamination. Clean controls are defined such that: randomly selected samples, samples not containing cDNA, and samples with dH_2O only did not display a fluorescence peak before 35 cycles. Final genes of interest were selected only when the two criteria 1) clean controls confirmed by gel electrophoresis and 2) high efficiencies were met. RT-PCR on samples were conducted using a thermocycling

program of 3’ at 95^0C, 40x15’’ PCR cycles at 95^0C, 1’ at 60^0C , 1’ at 55^0C and concluded with 80x15’’ with a temperature ramp of 0.5^0C/repeat where dissociation curve data were collected to ensure that only target sequences were amplified.

The following genes were amplified from the hippocampus-derived cDNA: glutamate N-methyl-D-aspartate receptor (GRIN) subunits (GRIN1, GRIN2A, GRIN2B), gamma-aminobutyric acid (GABA) A receptor 2-2 (GABAA2-2), gamma-aminobutyric acid (GABA) B receptor 1 (GABAB1), growth associated protein 43 (GAP43), neural cell adhesion molecule 1 (NCAM1), aryl hydrocarbon receptor nuclear translocator-like (ARNTL) and nuclear receptor subfamily 1 member 1 (NR1D1). Each gene was co-amplified with succinate dehydrogenase complex subunit A (SDHA), a housekeeping gene to control for any pipetting errors.

Two negative controls were conducted to ensure that there was no contamination in both primers and samples. RT-PCR was conducted for each primer pair where cDNA samples were substituted with dH_2O to verify that there was no DNA contamination with the primers. Additionally, gel electrophoresis was conducted to reconfirm the lack of DNA contamination in the primers. RT-PCR was also conducted for each primer pair where cDNA was substituted with RNA to verify no DNA contamination of the primer samples. Efficiencies for each primer pair were conducted for those primer pairs with clear negative controls.

Triplicates of each sample were run and assigned cycle threshold (C_T) values. Differences in C_T (dC_T) were determined by subtracting the C_T SDHA from C_T for the genes of interest. The treatment/time group with the highest dC_T was set to zero and the dC_T for each group was subtracted from the group with the highest dC_T which was used to calibrate the others

(ddC_T). The ddC_T values were calculated as powers of 2 ($2^{\wedge ddCT}$) to account for exponential doubling during PCR and to provide a relative measure of gene expression for each gene.

Table 1: Primer Sequences used in real-time PCR (NCBI)

Primer ID	Primer Sequence 5'-3'	Accession No.	Length, nt
SDHA	Fwd: CGGGGTTGGCGCAGTTTCGA Rev: AAGTGAAAGCCACGAGTCGCCC	NM130428.1	134
GRIN1	Fwd: CTGCTGGACCGCTTCAGTCCC Rev: CCGGAGTTGAGCAGGACGCC	NM017010.1	93
GRIN2A	Fwd: TCAGTGCCTCCGTCTGGGTGA Rev: GCCCGTGGGGAGCTTCCCT	NM012573.3	113
GRIN2B	Fwd: ATGCAAGCGAGAAGAGGACCCTGG Rev: CTTCAGCTAGTCGGCTCTCTTGGT	NM012574.1	94
GABAA2-2	Fwd: GTGGAACCCAGCCAGGTTGGTG Rev: GCTGTCTCCCAGTCCTGGTCTAAGA	NM001135779.1	136
GABAB1	Fwd: GCCCCACTGCCAGGTGAATCG Rev: GCCCCCGCTCATGGGAAACA	NM031028.3	82
GAP43	Fwd: CCAAGCTGAGGAGGAGAAAGAAGCT Rev: GGGGCAACGTGGAAAGCCGT	NM017915.3	136
NCAM1	Fwd: CCAGCGCACCCAAGCTGGAA Rev: TCGGAGGCGAGCGCTCTGTA	NM031521.1	130
ARNTL	Fwd: GAGCGGTTTGCCAGTCGGA Rev: CCGTCGCCGCCGCTCTATTT	NM024362.2	94
NR1D1	Fwd: CATTGCCCACGGGGCGAGAG Rev: ACACCACCTGTGTTGTTGTTGGAGT	NM001113422.1	124

Final concentration of each primer was 1μM

RESULTS

Effects of Altered Prenatal Melatonin Signaling on Litter Size, Litter Composition, Growth and Feeding Patterns

The difference in effects of administration of vehicle, 5mg/kg melatonin, or 5mg/kg luzindole to pregnant dams from days 14-18 of gestation on litter size and birth male body mass were not significant (Table 2). Despite the lack of significance, the effect of treatment on litter size was approaching significance for a decrease in litter size for melatonin-treated dams compared to luzindole-treated dams, both in respect to the litter size for control dams (P=0.060 for melatonin, P=0.0805 for luzindole).

Body mass and food intake were monitored for male rat litters treated prenatally with vehicle, melatonin, or luzindole (Figure 5). The body mass curve for litters treated prenatally with melatonin was significantly increased from those treated prenatally with vehicle or luzindole (P=0.00171 for reduced control and melatonin model to full control and melatonin model, P=.01084 for reduced melatonin and luzindole model to full melatonin and luzindole model, where growth data over time was averaged by litter, and linear regression analysis was performed by comparison of full and reduced models for body mass and food intake by treatment with family-wise Bonferroni correction). No significant effect on food intake was found in response to prenatal treatment.

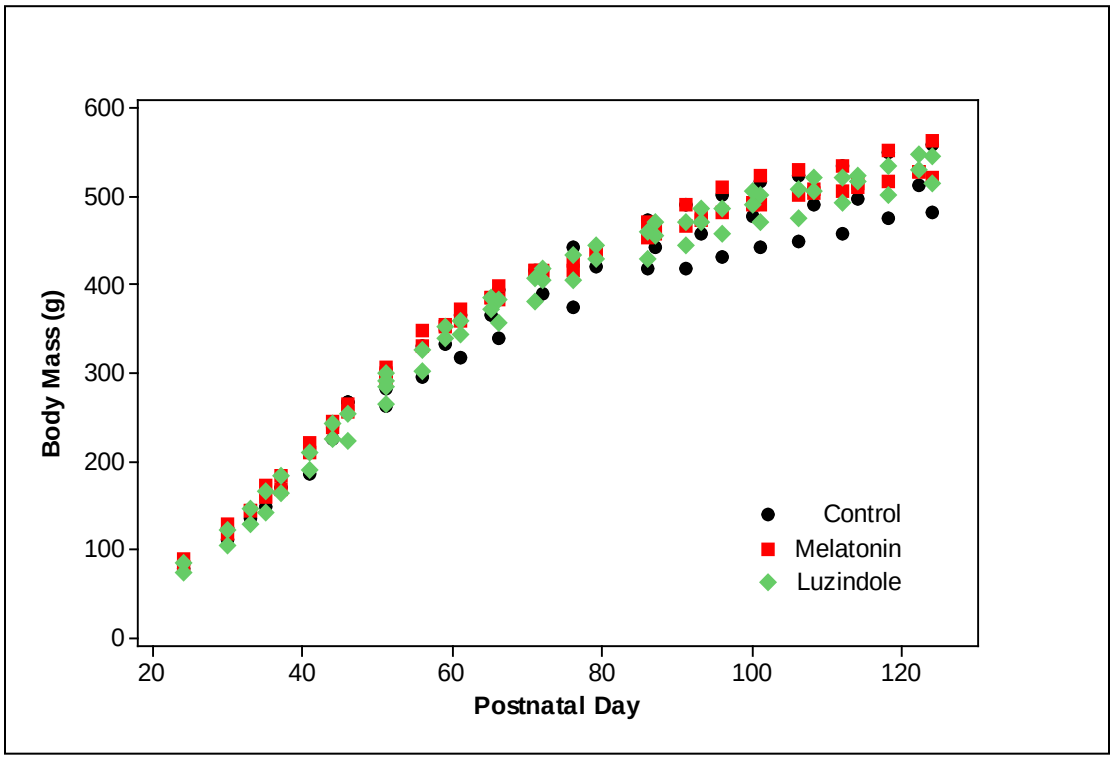
In addition, prenatal treatment did not have significant impact on percentage of body fat, left hippocampus mass, right hippocampus mass or left hippocampus mass to right hippocampus mass ratio.

Table 2: Litter size and average mass of male pups per litter for dams treated with vehicle (CON), 5mg/kg melatonin (MEL) or 5mg/kg Luzindole (LUZ) during days 14-18 of gestation

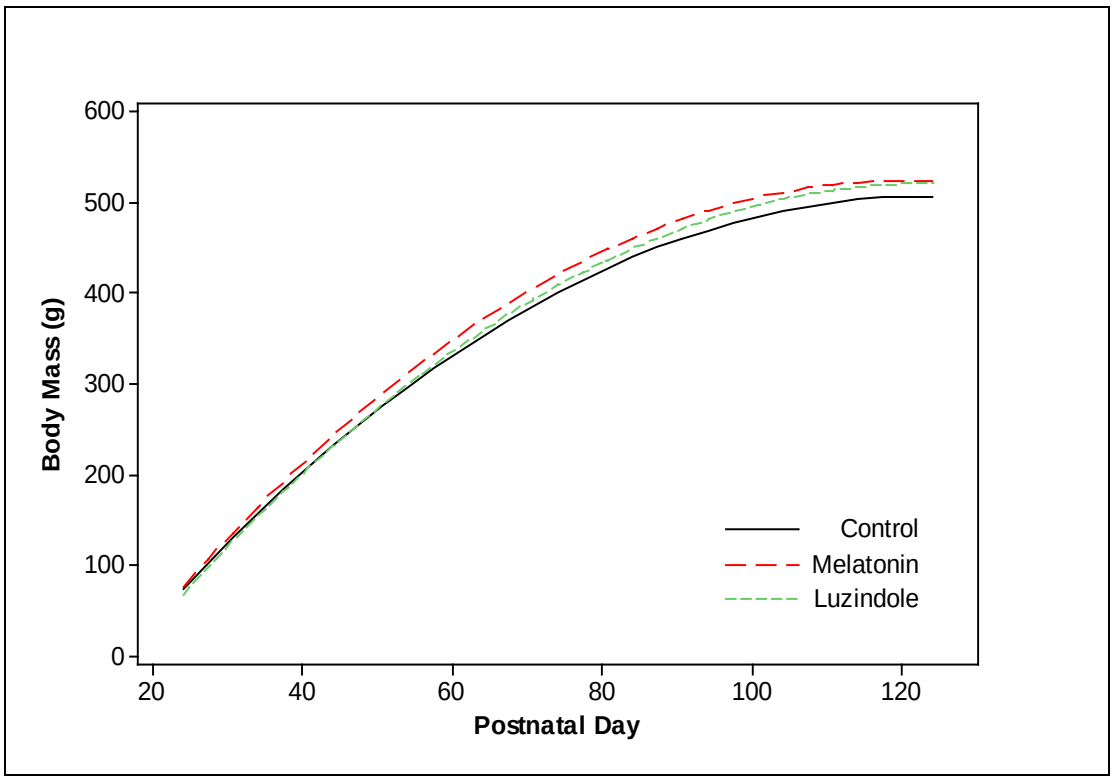
	Litter size (Pups)	**Average Male Mass (g/pup per litter)**
Control (CON)	14.3 ± 0.9	12.7 ± 1.0
Melatonin (MEL)	11.8 ± 0.6	13.5 ± 0.9
Luzindole (LUZ)	14.8 ± 0.9	11.8 ± 1.5
CON-MEL-LUZ	P=0.060	P=0.618
CON-MEL	P=0.197	P=1.00
CON-LUZ	P=1.00	P=1.00
MEL-LUZ	P=0.085	P=1.00

Figure 5. Average body weights and food intake of male rats from dams treated with vehicle, melatonin, or luzindole. Average body mass (a) and food intake (c) of 11 litters (Control n=3, Melatonin n=4, Luzindole n=3) measured from postnatal day (PD) 21 to PD 120. Litters treated with prenatal melatonin were found to have body mass curves significantly different from controls and Luzindole treated litters, (**P<0.01, *P<0.05) respectively. No significant effect of prenatal treatment on food intake was found.

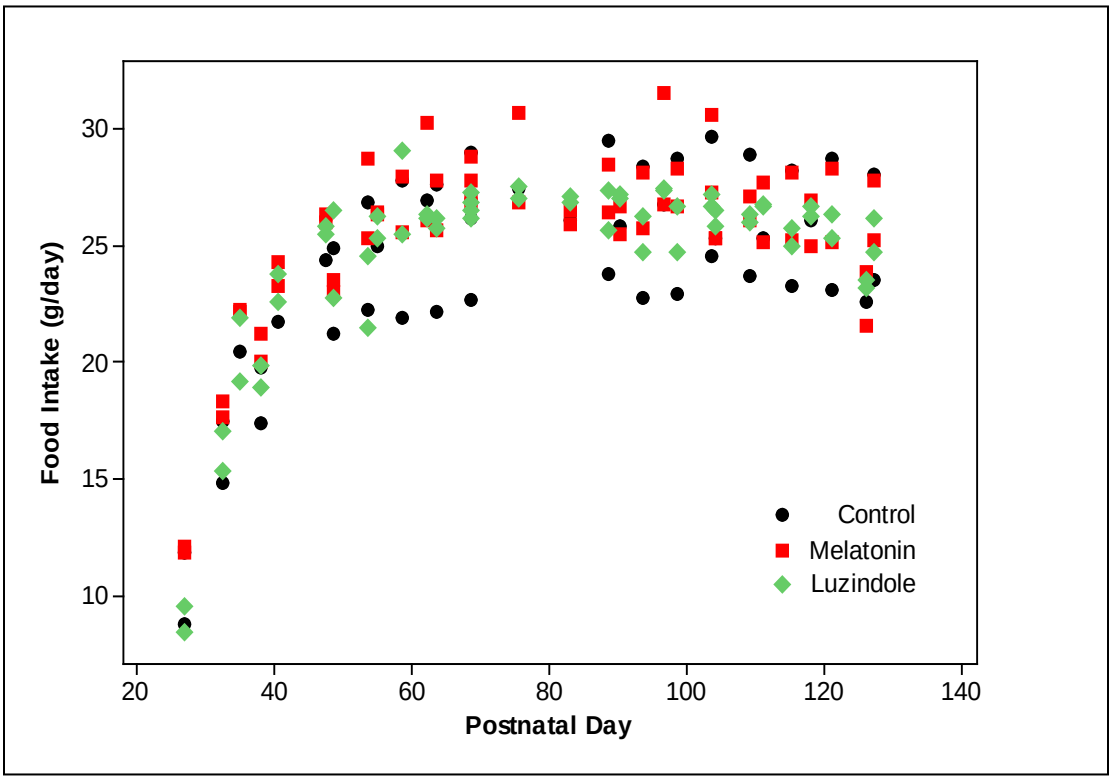
(a)


600
500
400
300
200
100
0
Body Mass (g)
20
40
60
80
100
120
Postnatal Day
Control
Melatonin
Luzindole

(b)


600
500
400
300
200
100
0
Body Mass (g)
20
40
60
80
100
120
Postnatal Day
Control
Melatonin
Luzindole

(c)


Food Intake (g/day)
30
25
20
15
10
20
40
60
80
100
120
140
Postnatal Day
Control
Melatonin
Luzindole

(d)

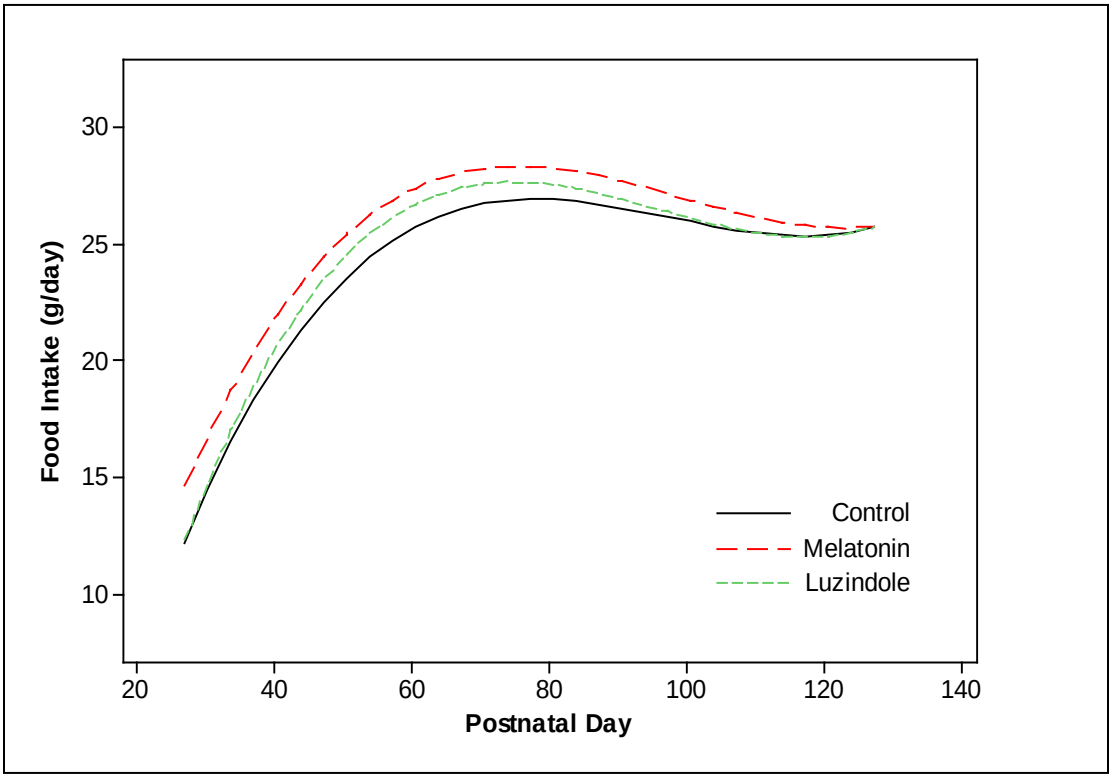

Food Intake (g/day)
30
25
20
15
10
20
40
60
80
100
120
140
Postnatal Day
Control
Melatonin
Luzindole

Table 3: Percent body fat, left and right hippocampus mass, and ratio of left hippocampus mass to right hippocampus mass at sacrifice for male rats from dams treated with vehicle, melatonin and luzindole.

	Percent Body Fat (fat/body mass)	**Left Hippocampus Mass (mg)**	**Right Hippocampus Mass (mg)**	**Left Hippocampus/ Right Hippocampus Mass**
Control (CON)	3.54 ± 0.16	75 ± 3	74.8 ± 1.8	1.01 ± 0.04
Melatonin (MEL)	3.4 ± 0.3	76 ± 3	75 ± 2	1.03 ± 0.03
Luzindole (LUZ)	3.46 ± 0.14	72.4 ± 1.3	72.8 ± 1.5	1.01 ± 0.03
CON-MEL-LUZ	P=0.855	P=0.411	P=0.651	P=0.901
CON-MEL	P=1.00	P=1.00	P=1.00	P=1.00
CON-LUZ	P=1.00	P=1.00	P=1.00	P=1.00
MEL-LUZ	P=1.00	P=0.606	P=1.00	P=1.00

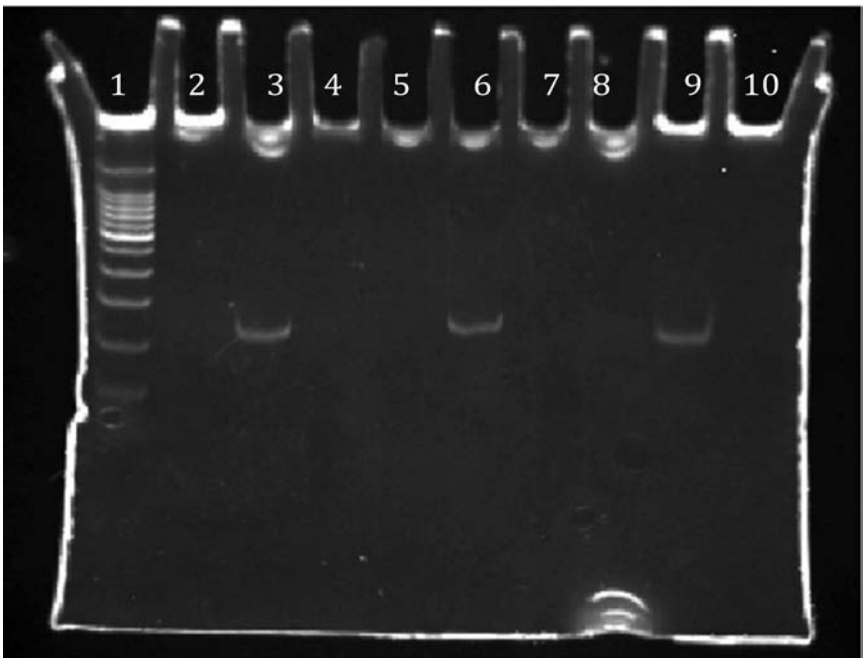
Effects of Altered Prenatal Melatonin Signaling on Hippocampal Gene Expression

Primers of targeted genes were tested for DNA contamination by running RT-PCR using selected cDNA samples, DNA-free samples, and dH_2O substituted for normal samples. Gel electrophoresis was conducted to insure the clarity of chosen primers (Figure 6). Primers that showed clarity by having single bands appear only on the RT+ lanes were then tested and efficiency was calculated using a small dilution of selected samples (Figure 7, Table 4). Nine primers with the highest efficiencies were chosen to be tested on experimental samples.

Hippocampal gene expression in the hippocampi of male rats prenatally treated with vehicle, melatonin and luzindole were analyzed (Figure 8, Figure 9). Analysis of gene expression between sides of the hippocampus (right vs. left) within two distinct time periods (noon vs. midnight) failed to produce significant difference in circadian expression (data not shown). Analysis of gene expression between two distinct time periods (noon vs. midnight) showed close to significant differences for the ARNTL gene (P=.09 for control vs. melatonin at noon and P=0.088 for noon vs. midnight for control group). Trends were observed in other genes albeit there was no significant difference amongst the groups or times for all genes, altered melatonin levels, whether they were increased with additional melatonin or decreased with luzindole administration.

Figure 6: Representative gel image photographed after gel electrophoresis was performed. a) Gel 1: 1) Ladder; 2) GRIN1A RT-; 3) GRIN1A RT+; 4) GRIN 1A S-; 5) GRIN2A RT-; 6) GRIN2A RT+; 7) GRIN2A S-; 8)GRIN2B RT-; 9)GRIN2B RT+; 10) GRIN2B S-; b). Gel 2: 1) SDHA S-; 2)SDHA RT+; 3)SDHA RT-; 4)GAP43 S-; 5)GAP43 RT+; 6)GAP43 RT-; 7) Ladder. RT- denotes DNA-free samples, RT+ denotes cDNA samples and S- denotes dH_2O samples.

(a)



(b)

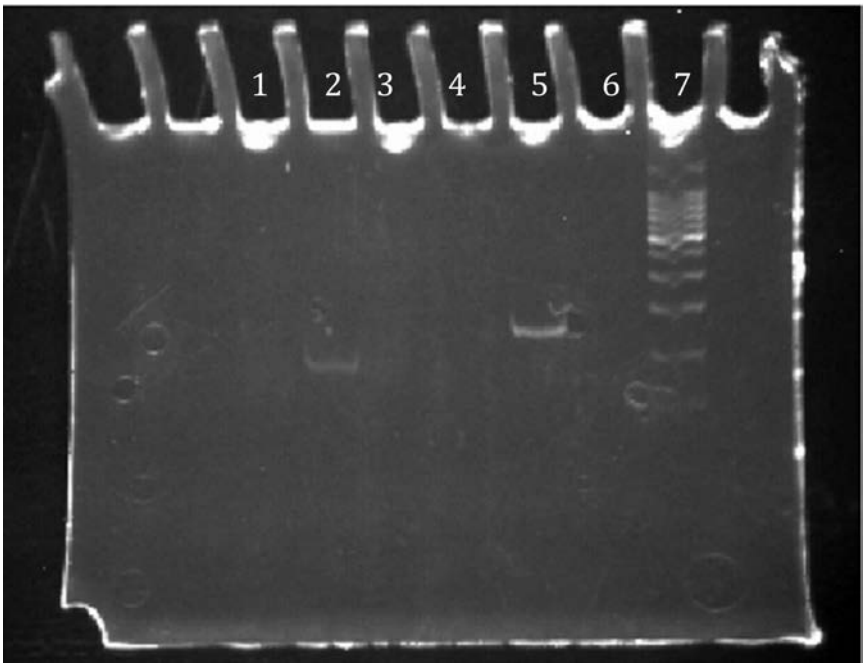


Figure 7. Representative Efficiency Curve.* Efficiency curves were determined through a serial dilution of selected samples that were run using the RT-PCR mix. Efficiency was calculated raising 10 by the negative reciprocal of the slope. *SDHA primer efficiency is displayed.

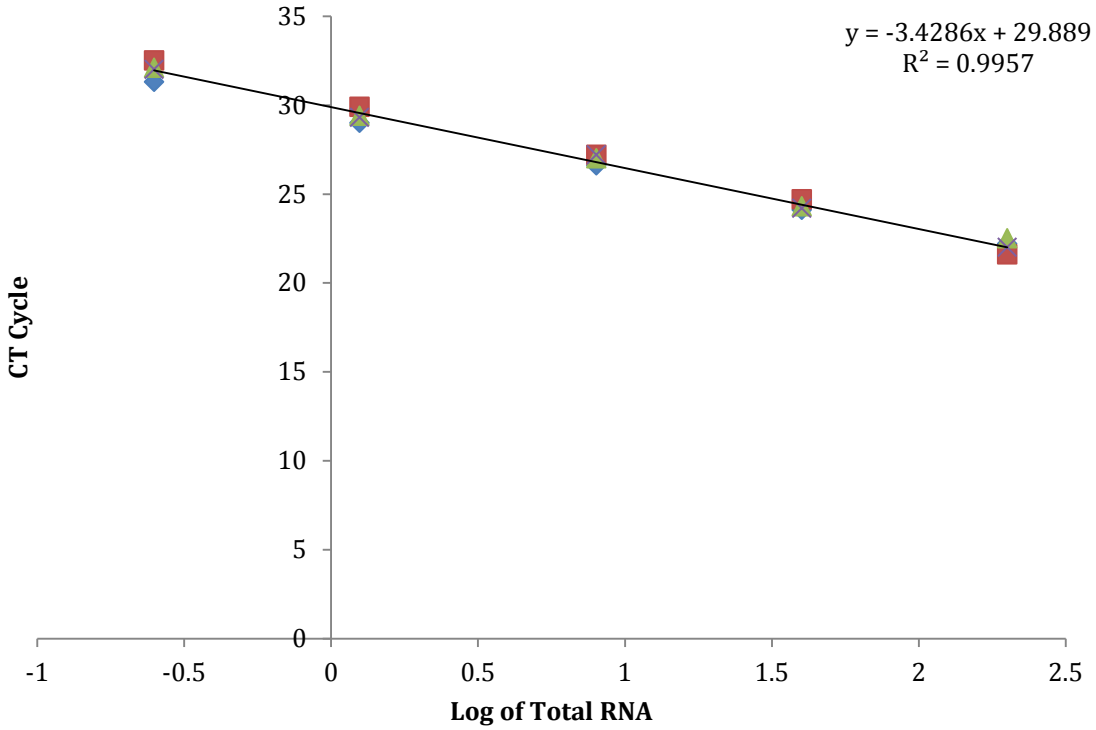
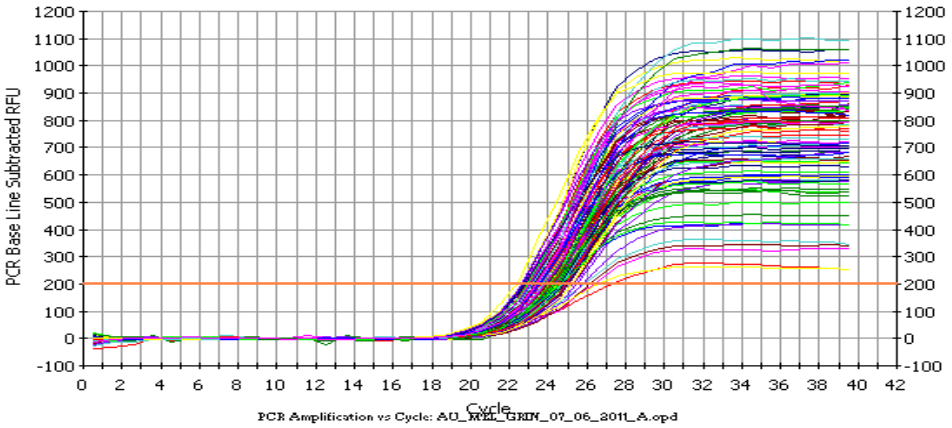

y = -3.4286x + 29.889
R² = 0.9957
CT Cycle
Log of Total RNA
35
30
25
20
15
10
5
0
-1
-0.5
0
0.5
1
1.5
2
2.5

Table 4: Efficiencies of primers.

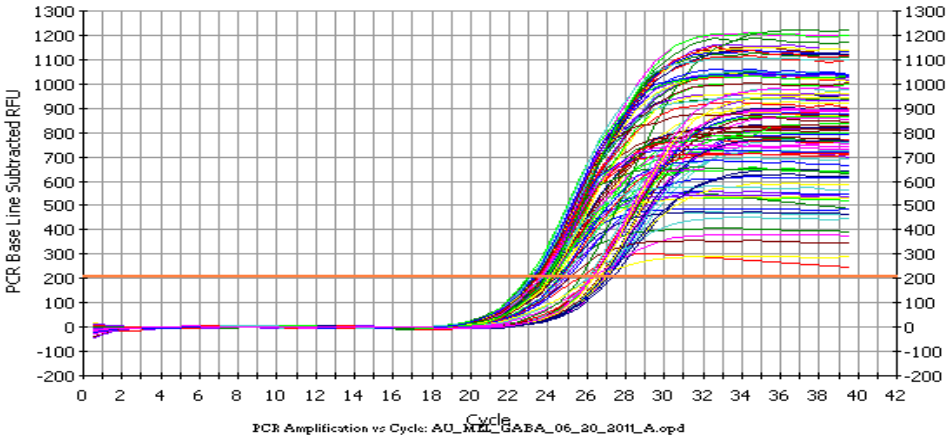
Gene	Intercept	Slope	R^2	Efficiency
ARNTL	33.724	-3.5614	0.98942	1.77
NR1D1	33.978	-3.6019	0.97755	1.78
GRIN1	29.047	-3.4022	0.9817	1.97
GRIN2A	31.442	-4.2103	0.9968	1.73
GRIN2B	30.576	-3.9476	0.9958	1.79
GABAA	32.0489	-4.8930	0.9670	1.60
GABAB	33.969	-4.1727	0.9971	1.74
GAP43	30.629	-4.1484	0.9834	1.74
NCAM	29.007	-3.5194	0.99381	1.95
SDHA	29.889	-3.4286	0.9957	1.96

Figure 8. The effects of prenatal melatonin or luzindole on adult male rat hippocampal gene expression. Relative cycle number of fluorescence for hippocampal cDNA during RT-PCR with primers for GRIN1, GRIN2A, GRIN2B (a); GABAA, GABAB, GAP43 (b); NCAM1, ARNTL, NR1D1 (c).

(a)


PCR Base Line Subtracted RFU
Cycle
PCR Amplification vs Cycle: AU_MIL_GRIN_07_06_2011_A.opd

(b)


PCR Base Line Subtracted RFU
Cycle
PCR Amplification vs Cycle: AU_MIL_GABA_06_20_2011_A.opd

(c)

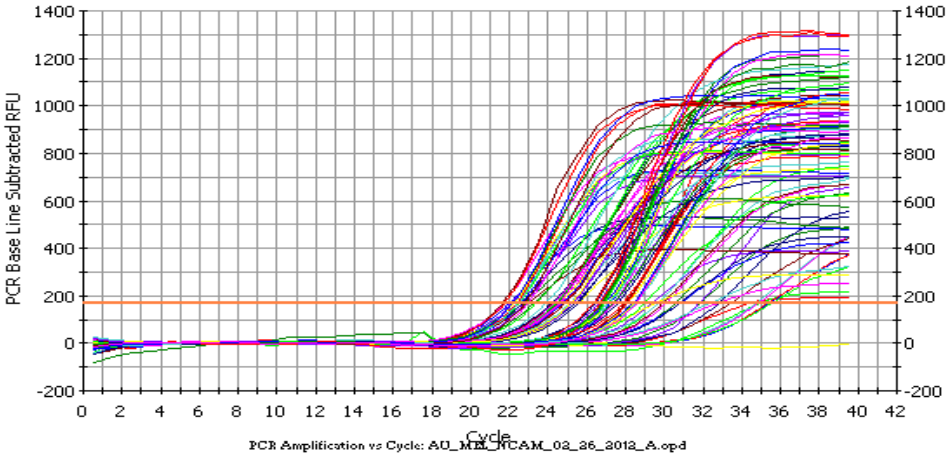
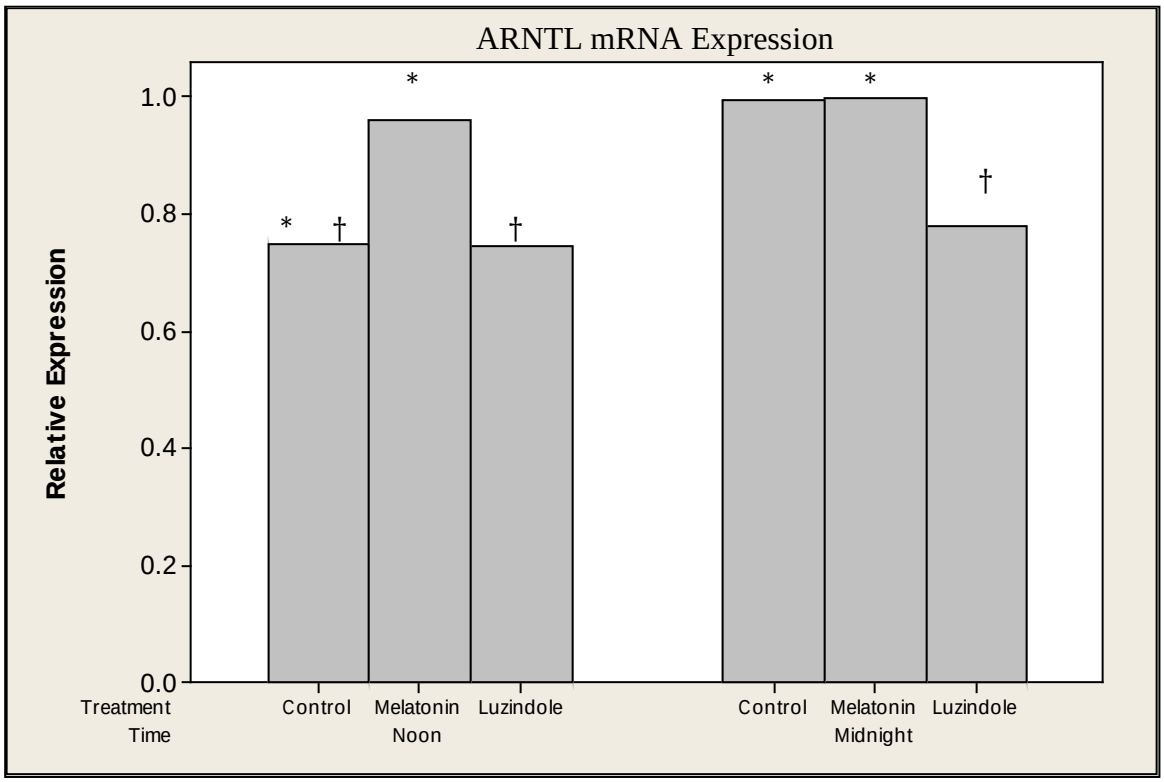

PCR Base Line Subtracted RFU
Cycle
PCR Amplification vs Cycle: AU_MIL_NCAM_02_26_2012_A.opd

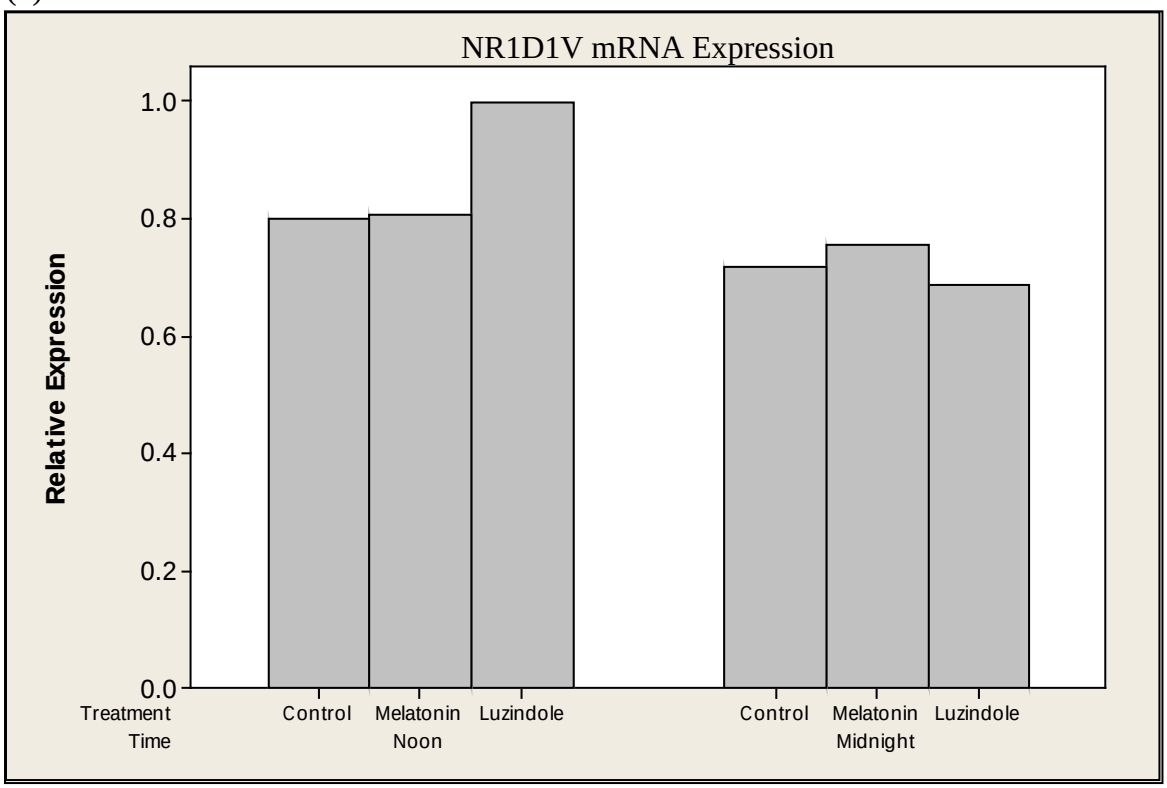
Figure 9. Relative gene expression of ARNTL (a), NR1D1V (b), GRIN1 (c), GRIN2A (d), GRIN2B(e), GABAA (f), GABAB (g), GAP43 (h), and NCAM1-1 (i), in hippocampal tissues from adult male rats exposed to melatonin or luzindole *in utero* compared to controls. Animals were sacrificed at noon or midnight. Values are expressed in relative expression with the group expressing the lowest level of each target gene evaluated set to 1.

(a)

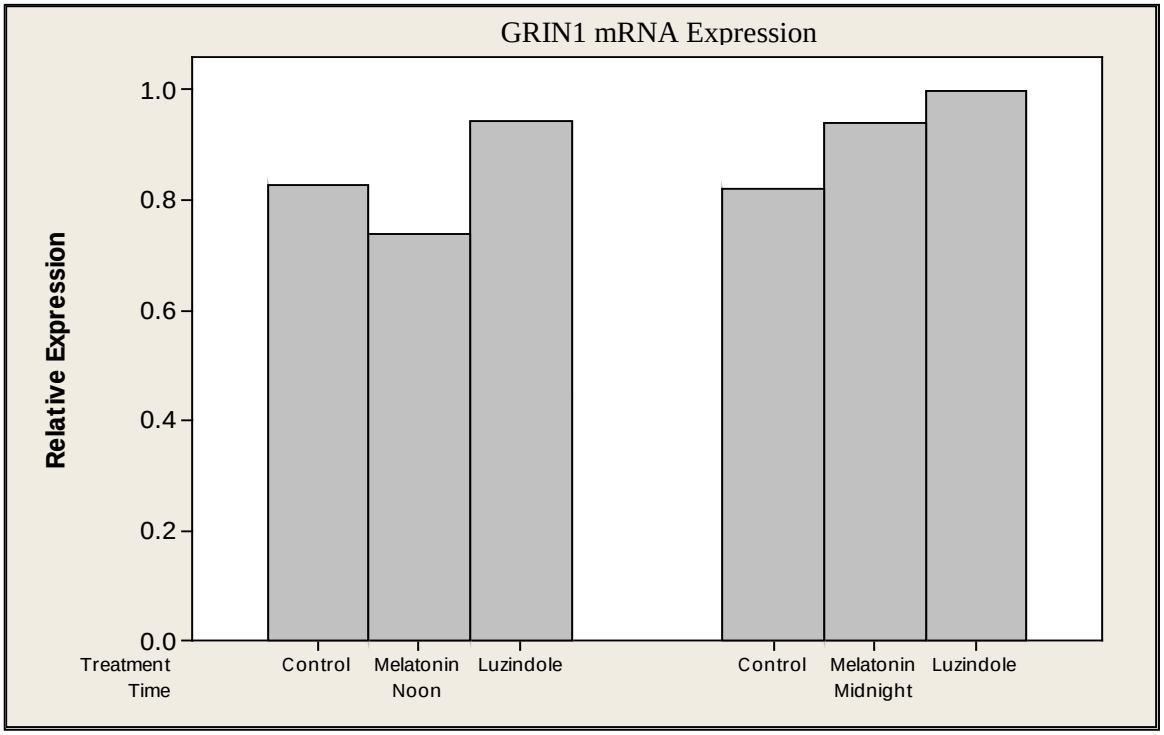


*P<0.1compared to Control at Noon. †no significant difference between Control at noon, Luzindole at noon and Luzindole at midnight.

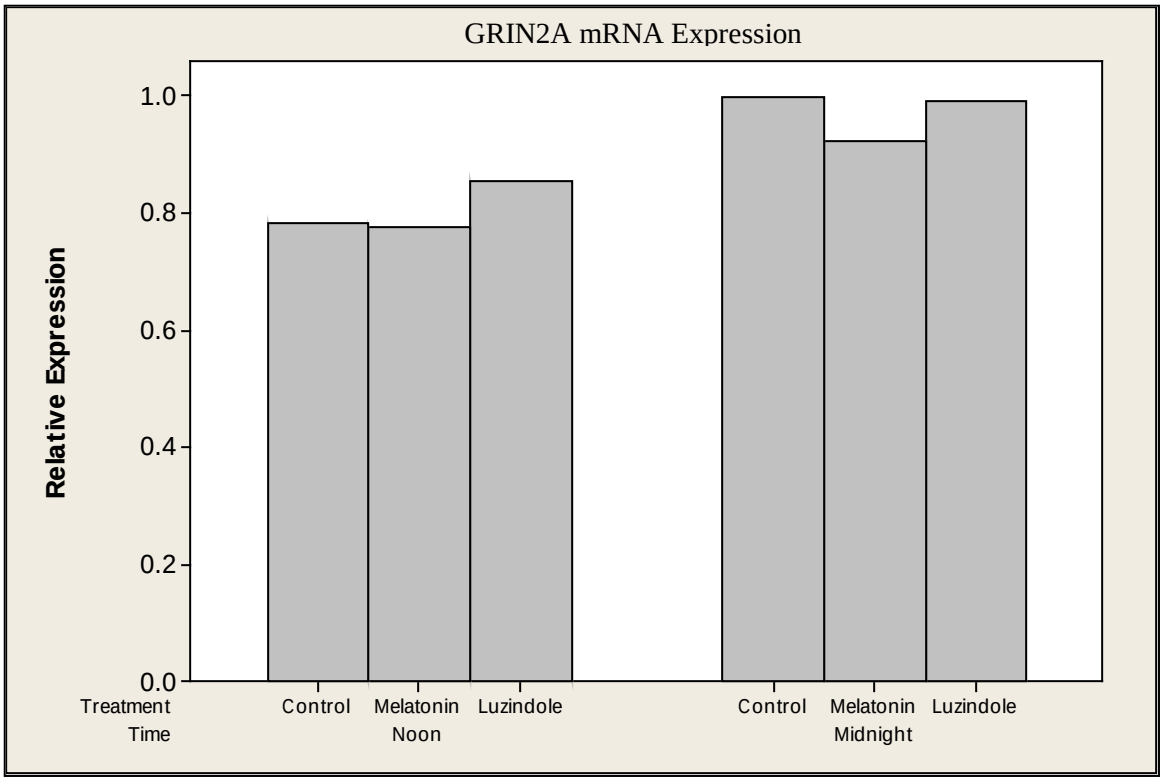
(b)



(c)


GRIN1 mRNA Expression
Relative Expression
1.0
0.8
0.6
0.4
0.2
0.0
Treatment
Time
Control
Melatonin
Luzindole
Noon
Control
Melatonin
Luzindole
Midnight

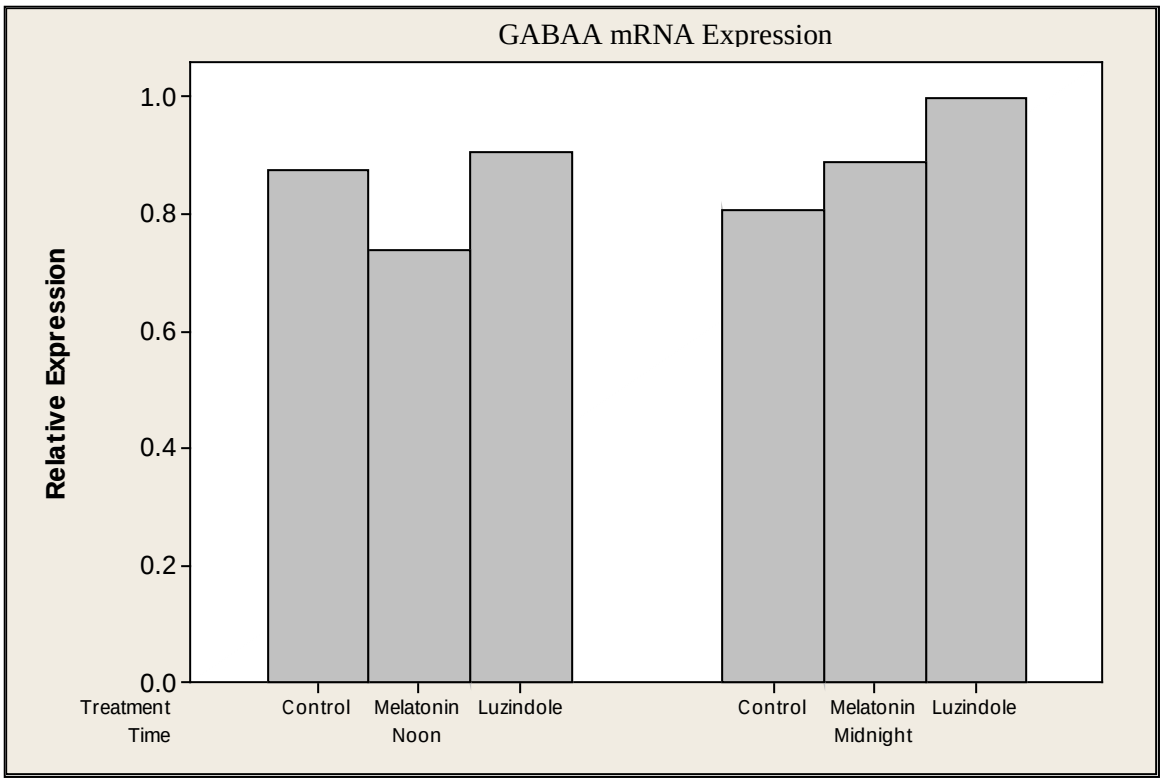
(d)


GRIN2A mRNA Expression
Relative Expression
1.0
0.8
0.6
0.4
0.2
0.0
Treatment
Time
Control
Melatonin
Luzindole
Noon
Control
Melatonin
Luzindole
Midnight

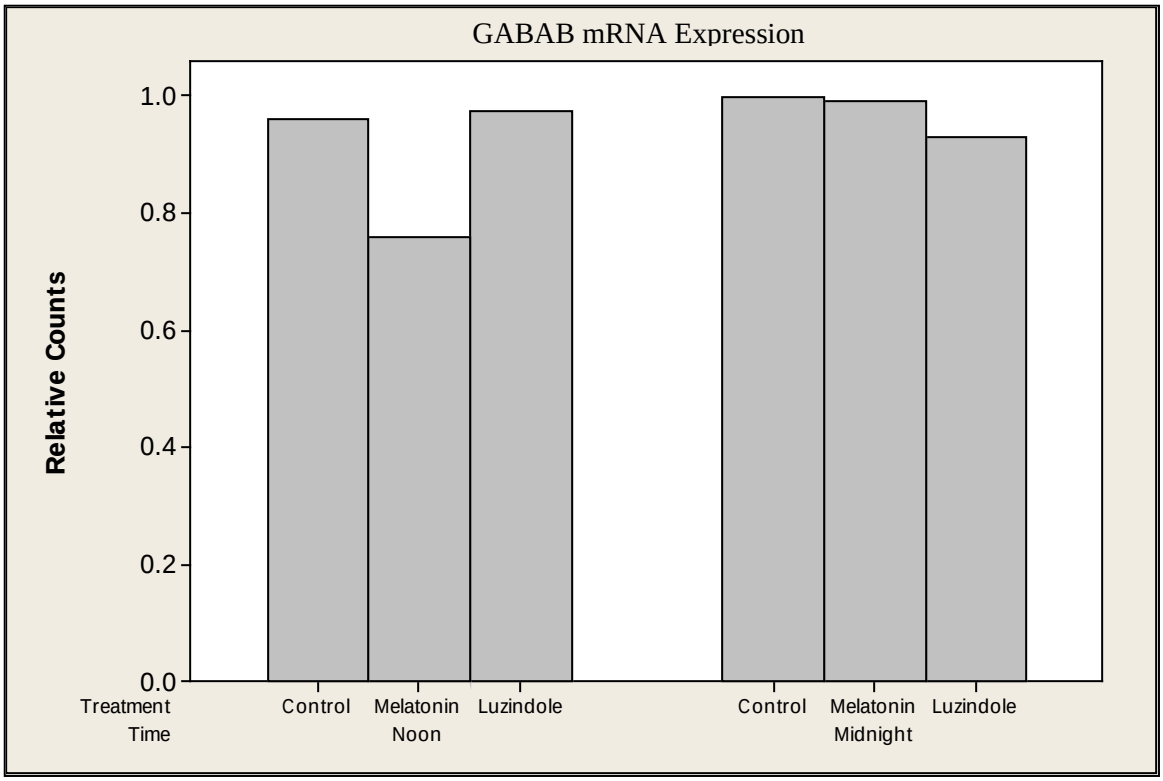
(e)


GRIN2B mRNA Expression
Relative Expression
1.0
0.8
0.6
0.4
0.2
0.0
Treatment
Time
Control
Melatonin
Luzindole
Noon
Control
Melatonin
Luzindole
Midnight

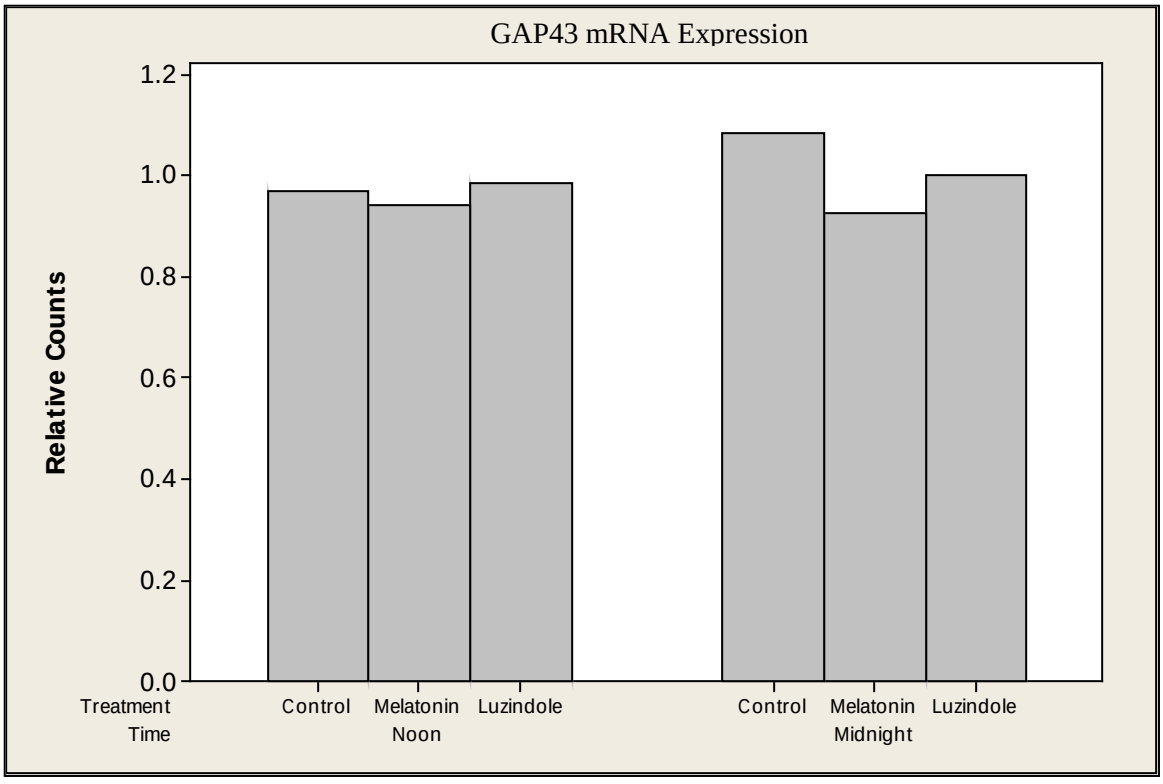
(f)


GABAA mRNA Expression
Relative Expression
1.0
0.8
0.6
0.4
0.2
0.0
Treatment
Time
Control
Melatonin
Luzindole
Noon
Control
Melatonin
Luzindole
Midnight

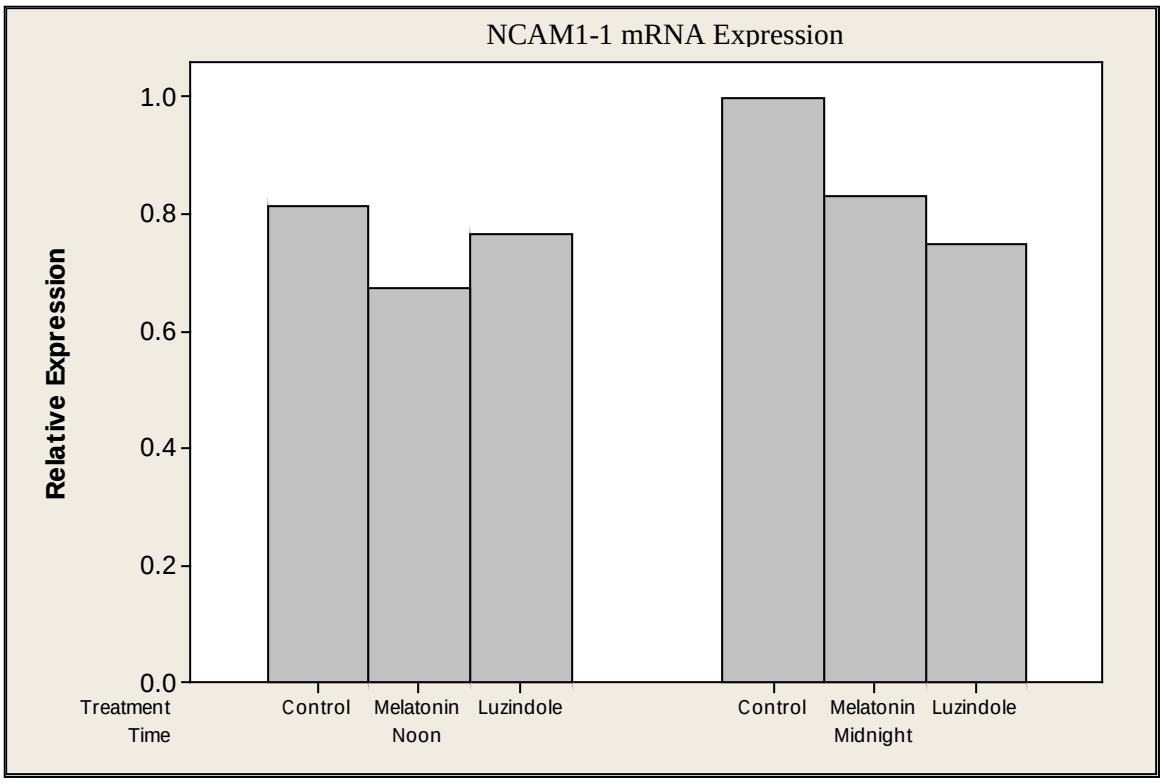
(g)


GABAB mRNA Expression
Relative Counts
1.0
0.8
0.6
0.4
0.2
0.0
Treatment
Time
Control
Melatonin
Luzindole
Noon
Control
Melatonin
Luzindole
Midnight

(h)


GAP43 mRNA Expression
Relative Counts
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Treatment
Time
Control
Melatonin
Luzindole
Noon
Control
Melatonin
Luzindole
Midnight

(i)


NCAM1-1 mRNA Expression
Relative Expression
1.0
0.8
0.6
0.4
0.2
0.0
Treatment
Time
Control
Melatonin
Luzindole
Noon
Control
Melatonin
Luzindole
Midnight

DISCUSSION

This study was designed to test the effects of altered maternal melatonin levels on hippocampal gene expression in adult male rat offspring. Existing research in this field is very limited, however, we are adding our data to the literature and were able to develop an explanation for our observations.

Melatonin and the Hippocampus

Melatonin has two main receptors MT_1 and MT_2, both of which have been found in various regions within the hippocampus, including the dentate gyrus, CA3 and CA1 regions (Musshoff et al., 2002). In a previous study conducted by my fellow student, Joshua Ripple, behavior experiments including the open field test, Morris water maze, and elevated plus maze were conducted. Rats that were exposed to higher prenatal melatonin levels showed increase in rearing in the open field test and an increased right turn preference in the elevated plus maze. On the other hand, rats exposed to prenatal luzindole displayed greater freezing and grooming behavior in the open field test and improved learning in the Morris water maze. Using the same samples used in this study, gene expression for both melatonin receptors was measured in a similar fashion using rt-PCR. For the offspring whose mother received the exogenous melatonin, MT_1 gene expression was significantly higher than in the control and luzindole group. In addition, MT_1 gene expression was significantly higher compared to MT_2 gene expression for all three experimental groups (Ripple, 2010). The alterations in circadian rhythm may, in part, be explained by this increase in MT_1 gene expression associated with the increased melatonin levels and ARNTL gene expression found in our study.

Melatonin has also been studied as a contributor to learning, memory, and psychopathological disorders that depend on neuronal plasticity processes in the hippocmpus. Alterations in melatonin receptor concentration have been observed in various psychopathologies. For instance in Alzheimer's disease (AD), a significant increase in MT_1 receptors have been found, possibly as a regulatory response to the impaired melatonin levels in AD individuals (Savaskan et al., 2001). Additionally, MT_2 receptors are significantly decreased in AD patients, suggesting that MT_2 receptors are mediated by melatonin in the hippocampi of humans (Savaskan et al., 2005). Such findings have led to possible therapeutic approaches that involve supplementing antidepressant treatment with some type of melatonin receptor agonist. It is thought that the ratio of MT_1/MT_2 receptors may contribute to improving antidepressant effects (Hirsch-Rodriguez et al., 2007).

Unlike MT_1, MT_2 receptors have been shown to have inhibitory effects on long-term potentiation (LTP) using the elevated plus-maze as observed in mice where inhibitory actions of melatonin were lost in those deficient in MT_2 but not in MT_1 (Larson et al., 2006). Furthermore, administration of luzindole was seen to prevent the inhibition of LTP by melatonin via the MT_2 receptors (Wang et al., 2005). However, melatonin's inhibitory characteristics were not seen in the MT_1 receptors thereby suggesting opposing receptor activity for the two melatonin receptors (Musshoff et al., 2002).

Previously, it was found that the lasting effects of overexpressed melatonin levels *in utero* show an increase in MT_1 receptors but not in MT_2 receptors; thus the increased melatonin receptor ratio is similar to that shown in AD individuals. In addition, it has been shown that altered maternal melatonin levels significantly affect brain function in offspring. For example, at postnatal day 9, offspring of pinealectomized Wistar rat dams had reduced melatonin receptor

gene expression in their brains (Zitouni et al., 1995). In addition, exogenous melatonin administration in pinealectomized Swiss albino rats showed inhibition of harmful effects of epilepsy, thereby allowing proper hippocampal development in the offspring (Turgut et al., 2006).

Melatonin and Clock Gene Expression

In addition to the melatonin receptors, the aryl hydrocarbon receptor nuclear translocator-like (ARNTL) gene in the rat contributes to circadian rhythm generation. This gene is considered to be an ortholog to the brain and muscle aryl hydrocarbon receptor nuclear translocator 1 (BMAL1) protein in humans, which forms a heterodimer with the circadian locomotor output cycles kaput (CLOCK) protein. The BMAL1/CLOCK heterodimer then is a critical regulatory component since it binds to a promotor region, the E-box, which allows for the transcription of many genes including those associated with circadian rhythm, the period (PER) and cryptochrome (CRY) genes (Gekakis et al., 1998; Sangoram et al., 1998). PER and CRY, in their protein form, also form a dimer. PER is known to be a positive regulator of the circadian loop, but CRY is known to be a negative regulator (Shearmen et al., 2000). Hence, the increase in CRY concentration would lead to an inhibition of the BMAL1/CLOCK dimer, which then decreases the rate of transcription of PER and CRY. As PER/CRY protein levels increase, they form a dimer that exerts negative feedback in this loop. When concentrations of PER and CRY are low, BMAL1/CLOCK form a dimer to allow transcription. This fluctuation of protein production creates the circadian rhythm within the SCN.

Irregularities in the oscillation of clock gene expression lead to arrhythmic circadian systems that have been associated with physiological dysfunction. For example, synchronized

circadian cycle was absent in Wistar rats following a 30-day exposure to continuous light. This disruption was especially apparent for BMAL1, which was no longer expressed in synchrony with other clock genes (Novakova et al, 2011). Moreover, low amplitude PER gene expression was observed using in situ hybridization and was associated with the lack of intercellular synchrony, which is critical for generating circadian rhythm. In our study, the high BMAL1 expression detected in the offspring from treated dams exposed to melatonin at noon may increase the risk of arrhythmic circadian cycles, as the BMAL1 expression at noon did not differ from the BMAL1 expression at midnight in these animals. In contrast, the offspring from control rats exhibited a significantly higher expression of BMAL1 at midnight. In contrast to both of these groups, Luzindole decreased the expression of BMAL1 at both time points.

Our finding is interesting considering that melatonin is an important regulator of rhythmic clock gene expression in mice. For example, loss of melatonin receptors and the effect this loss had on clock gene expression was studied using melatonin 1-receptor knockout and melatonin 2-receptor knockout mice (Von Gall, 2005). Using in situ hybridization and western blotting, gene expression was analyzed in the hypophyseal *pars tuberalis*, one of the main target regions for melatonin. In this study, they found that gene expression of PER1, CRY1, CLOCK and BMAL1 were dramatically reduced only in the melatonin 1-receptor knockout mice. This indicates that melatonin, through its melatonin 1-receptor, has regulatory effects on critical clock genes such as PER and CRY, and most likely BMAL1, thereby playing a crucial role in regulating and maintaining circadian rhythm.

Another study that directly relates to our study is one in which maternal melatonin levels were manipulated in pregnant Capucin monkeys. Suppression of maternal melatonin and addition of maternal melatonin resulted in offspring that exhibited lowered clock gene expression

in the fetal SCN (Torres Farfan et al., 2006). Hence, altering melatonin levels *in utero* has significant effects on circadian rhythm development and clock gene expression, which include the expression of BMAL1, PER2, CRY2 and CLOCK. Since fetuses rely on their mother's hormonal levels to establish bodily functions like circadian rhythm and endocrine modulation (Tamura et al., 2008), altering the melatonin levels during gestation can lead to critical changes in the fetal clock gene expression.

The full consequences of altered fetal clock gene expression and changes in circadian rhythms are unknown. However, it is known that BMAL1 and PER proteins have other properties apart from their function as the circadian oscillator. For example, they participate in essential non-redundant regulatory roles during tissue homeostasis and aging (Kondratov, 2007). Not only is there a loss of circadian rhythm if BMAL1 is underexpressed or if there are irregularities in BMAL1 availability, but, it has been reported that such mice also exhibit premature aging (Bunger et al., 2005; Kondratov et al., 2006; Nadon, 2006). Characteristics of progeria, such as disruptions in control of glucose and fat metabolism, homeostasis and modulation of genotoxic stress have all been seen in BMAL1 knockout mice (Rudic et al., 2004; Shimba et al., 2005).

Decreased melatonin levels are often found in individuals with depression or related psychopathological disorder, like bipolar disorder (Kennedy 1996). In a small sample of US individuals with psychopathological disorders, such as bipolar 1 disorder, schizoaffective disorder, and schizophrenia, a significant association was found between the reduction in BMAL1, *Timeless* and PER3 concentrations compared to CLOCK (Mansour, 2005). Moreover, in a circadian clock gene study that evaluated ten genes, haplotypes in BMAL1 and PER3 were assessed to have significant association with bipolar affective disorder (Nievergelt 2006). Hence,

it can be said that bipolar disorder is very closely associated with the malfunctioning of the circadian rhythm, in part due to abnormal melatonin levels, even at the molecular level.

The nuclear receptor subfamily 1, group D, member 1 (NR1D1), is the gene for Rev-ErbA, a protein that regulates cellular proliferation and differentiation that is coupled to the circadian rhythm through associations with BMAL1. NR1D1 has been identified as an orphan nuclear receptor and is the major regulator of cyclic BMAL1 transcription via the negative feedback that is exerted by PER/CRY dimer (Preitner et al., 2002). More specifically, NR1D1 has been found to act as a transcriptional inhibitor at the E-box DNA sequence that functions as response elements for the core circadian-clock components of CLOCK and BMAL1 (Triqueneaux et al., 2004). Most importantly, however, it has been determined that NR1D1 transcription is rhythmic within the hippocampus of rats, primarily due to fluctuations in CRY concentration, which is controlled by the BMAL1/CLOCK protein (Valnegri et al., 2011). The importance of NR1D1 in development has been seen in the cerebellum of mice lacking the NR1D1 gene where unexpected abnormalities including alterations of the Purkinje cell development and delays in proliferation and migration of granule cells were observed (Chomez et al., 2000). NR1D1 is also a target of interest for drug treatment for certain psychopathological disorders. In bipolar disorder, lithium is commonly used to alter the circadian rhythm by inhibiting the glycogen synthase kinase 3 (GSK3) which leads to degradation of NR1D1 and thereby activation of BMAL1 (Yin et al., 2006). Altered levels of NR1D1 have been seen in patients who had disturbed sleep and therefore altered circadian rhythms, which are undoubtedly impacted by abnormal levels of melatonin (Wulff et al., 2009). In our study, the lack of significant difference in the NR1D1 gene expression observed by alteration of melatonin levels *in utero* seems to indicate that the effects on BMAL1 were not large enough to significantly alter

the production of CRY proteins that would then affect NR1D1 expression, or that some other component critical to BMAL1 transcriptional regulation is involved. Although clear associations between melatonin levels and NR1D1 have not been seen as of yet, alterations in external melatonin levels during development will most likely have an effect on fetal development as NR1D1 has already been seen to have effects with altered melatonin levels.

Melatonin and Cognitive Function

Long-term plasticity has been associated with the activity of the glutamate NMDA receptors which are present in high concentration in the hippocampus (Collingridge and Bliss, 1987; Herron et al., 1986; Bashir et al., 1993). These metabotropic glutamate receptors have been shown to be required for hippocampal long-term potentiation to occur within the hippocampus (Malenka and Nicoll, 1993). The NMDA receptor is a heterotetramer made up of two GluNR1 and two GluNR2 subunits (either GluNR2A or GluNR2B). In situ hybridization has shown that the three subunits are expressed in different concentrations throughout the various hippocampal subfields with GRIN1 having the most even expression throughout the hippocampus, possibly due to the fact that GRIN1 must be present to form the NMDA heterodimer (Xu et al., 2002).

Studies have also shown that melatonin has different effects on LTP, as melatonin in the CA1 region blocked LTP while melatonin injected into the CA3 region barely reduced LTP (Ozcan et al., 2006). In relation to melatonin, in a study where melatonin was administered for four weeks, concentrations of NMDA receptor subunits 2A and 2B were found to increase within the rat hippocampus (Sutcu et al., 2005). Furthermore, melatonin exhibited partial protective effects on the NMDA receptor subunits 2A and 2B on rats given ochratoxin A, a mycotoxin that

accumulates in the brain and reduces NMDA receptor concentration (Delibas et al., 2003). The protective capacity of melatonin was also shown in response to a challenge from quinolinic acid degeneration of rat hippocampal neurons (Southgate et al., 1998).

In contrast to the excitatory effects of glutamate and its NMDA receptors on learning and memory, GABA is the major inhibitory neurotransmitter with its receptor GABAR. There are two main classes of GABA receptors - the ionotropic receptor $GABA_A$ and the metabotropic receptor $GABA_B$; both of the $GABA_A$ and $GABA_B$ receptors mediate inhibitory postsynaptic transmission in the hippocampus (Kuffler and Edwards, 1958; Isaacson et al., 1993). Clinical data seems to indicate that psychopathological disorders oftentimes involve decreased GABA function where treatments have targeted an upregulation of $GABA_B$ receptor (Petty, 1995). While there is little data on melatonin's effect on GABA receptors, a connection between melatonin's ability to control excitability and GABA-containing neurons' ability to inhibit neuronal excitability has been proposed (Golombek et al., 1998; Acufla-Castroviejo et al., 2007). Studies have shown that melatonin seems to participate in the regulation of the GABA-benzodiazepine receptor complex, but does not directly act on the complexes (Nilcs et al., 1987; Rosenstein and Cardinali, 1990).

As previously mentioned, the exact interactions between altered melatonin and behavior is scarce, but melatonin has been shown to be correlated with significant differences in pro-exploratory activity in rats that were tested using a plus-maze (Golombek et al, 1993). In addition, a clinical study reported that melatonin co-administered during withdrawal periods of hypnotic drugs have eased the discontinuation (Garfinkel et al., 1999). Moreover, forced swimming induced behavioral despair tests have shown similar behavioral response from the rats that underwent melatonin treatment and GABA-benzodiazepine receptor agonists (Raghavendra

et al., 2000). This suggests that the antidepressant-like effect of melatonin is very similar to the effect of GABA-benzodiazepine receptor agonists. If melatonin is having similar inhibitory effects on neurons as GABA, this may account for the slight decrease in $GABA_A$ and $GABA_B$ in gene expression, observed in the Melatonin-Noon groups in our study although this data did not reach significance.

Synaptic plasticity, learning and memory also involve molecules like the neural cell adhesion molecule (NCAM), which are members of the immunoglobulin superfamily and are expressed on the surface of neuronal cells. Of the three NCAM forms, NCAM140 and NCAM180 have shown a decrease in Wistar rats exposed to constant light for 7 days (Baydas et al., 2002). In contrast, the rats that were administered melatonin for 7 days had increased levels of NCAM140 and NCAM180 expression. Furthermore, injection of 10mg/kg of melatonin, twice the amount used compared to our study, into the experimental rats as opposed to the dams showed significant alteration in the gene expressions of different NCAM isoforms (Nedzvetsky et al., 2003). In our study, altered maternal melatonin levels, on the other hand, do not seem to alter NCAM140 expression in the offspring. However, the importance of melatonin was supported in our study since the NCAM expression levels in the Luzindole-Noon and Luzindole-Midnight did not change at all whereas the controls exhibited a significant difference at these two time points. These data shows the need for melatonin in the regulation of NCAM expression.

The growth-associated protein 43 (GAP43) is another important protein for neural plasticity, especially during neuronal development. However, GAP43 has also been seen to play critical roles in synaptic remodeling in adults, especially after lesions. Using in situ hybridization, an increase in GAP43 mRNA expression was seen in the granule cells of hippocampus in rats that underwent experimental lesions (Bendotti et al., 1994). Although there

has been no direct study on the effects of melatonin on GAP43 gene expression, studies have shown that melatonin has protective effects against neuronal degeneration by maintaining key synaptic molecules, such as tyrosine hydroxylase, synaptophysic and GAP43 (Kaewsuk et al., 2009). Morevoer, alterations in GAP43 have been detected in the brains of individuals with psychopathological disorders. In a study on the hippocampi of schizophrenic patients, quantitative immunoblots revealed that GAP43 is increased in association cortices as well as in the hippocampi, as if it is one manifestation of the brain trying to compensate for the perturbations of synaptic organization that schizophrenia is associated with (Perrone-Bizzozero et al., 1996; Blennow et al., 1999). This evidence suggested that there might be a connection between altered prenatal melatonin and GAP43 expression; however, we did not detect any significant differences.

Circadian Rhythm During Development

Our study was designed to examine the importance of the maternal melatonin during fetal brain development, in particular, the programming of "set points" for the expression of key genes mediating hippocampal control of circadian rhythm. As fetuses are only exposed to their mother's hormone levels while *in utero*, the maternal circadian circuit becomes the basis for the fetal circadian circuit. Hence, when the maternal circadian circuit was disturbed by the presence of excess melatonin following its exogenous administration, or, suppression of melatonin following the administration of luzindole, a significant change was observed in the offspring's expression of BMAL1, one of the key clock genes that control the circadian cycle. It is important to note, however, that the altered levels of melatonin and consequent changes in hippocampal BMAL1 gene expression did not have any significant effects on the expression of other genes that have been reported to be changed by alteration in melatonin or circadian rhythm

disturbances, including those for subunits of NMDA receptors as well as GABA receptors. This could suggest that there are protective mechanisms in the mother or fetus that override the influence of the particular dose of melatonin and luzinolde that were used in our study.

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