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The Effects of Altered Prenatal Melatonin Signaling on Adult Behavior and Hippocampal Gene Expression of the Male Rat: A Circadioneuroendocrine-Axis Hypothesis of Psychopathology

Josh Ripple
Bucknell University

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by

Joshua A. Ripple

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Approved by:

Kathleen C Page

Adviser: Kathleen C. Page, PhD

Department Chairperson: David W. Evans, PhD

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ABSTRACT

Disturbances in melatonin—the neurohormone that signals environmental darkness as part of the circadian circuit of mammals—have been implicated in various psychopathologies in humans. At present, experimental evidence linking prenatal melatonin signaling to adult physiology, behavior, and gene expression is lacking. We hypothesized that administration of melatonin (5 mg/kg) or the melatonin receptor antagonist luzindole (5 mg/kg) to rats *in utero* would permanently alter the circadian circuit to produce differential growth, adult behavior, and hippocampal gene expression in the male rat. Prenatal treatment was found to increase growth in melatonin-treated animals. In addition, subjects exposed to melatonin prenatally displayed increased rearing in the open field test and an increased right turn preference in the elevated plus maze. Rats administered luzindole prenatally, however, displayed greater freezing and grooming behavior in the open field test and improved learning in the Morris water maze. Analysis of relative adult hippocampal gene expression with RT-PCR revealed increased expression of brain-derived neurotrophic factor (BDNF) with a trend toward increased expression of melatonin 1A (MEL1A) receptors in melatonin-exposed animals whereas overall prenatal treatment had a significant effect on microtubule-associated protein 2 (MAP2) expression. Our data support the conclusion that the manipulation of maternal melatonin levels alters brain development and leads to physiological and behavioral abnormalities in adult offspring. We designate the term circadioneuroendocrine (CNE) axis and propose the CNE-axis hypothesis of psychopathology.

INTRODUCTION

The Circadian Circuit

Melatonin, the hormone that encodes information about daily rhythms in mammals, has been implicated in various psychopathologies in humans. Changes in melatonin secretion have been found in depression (for a review see Srinivasan et al. 2006), Alzheimer's disease and Parkinson's disease (for a review see Srinivasan et al. 2006), schizophrenia (for a review see Sandyk et al. 1990), and other psychiatric illnesses (for a review see Pacchierotti et al. 2001). Despite indications that this neurohormone is important for proper brain function, the physiology of its impact on the brain is not clearly understood.

Melatonin release displays rhythmic behaviors with a period of approximately 24 hours in response to the circadian pacemaker (Figure 1) (Refinetti 2006). In mammals, the circadian pacemaker is controlled by clock genes in the suprachiasmatic nucleus (SCN) of the hypothalamus (Figure 2) (for a review see Okamura 2004). Daily fluctuations in the activity of the SCN can be altered by light from the environment via projections from retinal ganglion cells along the retinohypothalamic path. In addition, outputs from the SCN project indirectly to the pineal gland, the primary brain region responsible for the production of melatonin. The pineal releases melatonin with a nightly peak in response to input from the SCN to impact cells throughout the body. In addition, the SCN responds to melatonin secretion by the pineal via melatonin receptors in membranes of its cells. This feedback loop involving visual inputs and melatonin

Figure 1. Simplified schematic of the circadian circuit responsible for daily rhythm of activity in mammals (Adapted from Refinetti 2006). Light at the retina induces signaling at glutamatergic synapses by retinal ganglion cells projecting to the suprachiasmatic nucleus along the retinohypothalamic path. In addition, the intergeniculate leaflet of the thalamus receives signals from the retina and relays this information to the suprachiasmatic nucleus via neuropeptide Y synapses, and the raphe nuclei also communicate other environmental information to the suprachiasmatic nucleus via serotonergic synapses. The suprachiasmatic nucleus, which contains the primary circadian molecular clock, processes these inputs and elicits release of melatonin by the pineal gland in addition to projecting to other brain regions. Melatonin produced by the pineal gland is released into circulation in the blood and cerebrospinal fluid in response to this stimulation. In addition to feeding back to the suprachiasmatic nucleus, melatonin also modulates release of growth hormone by the pituitary gland by an as yet unidentified mechanism. Both melatonin and growth hormone affect the hippocampus and other brain regions in a circadian fashion.

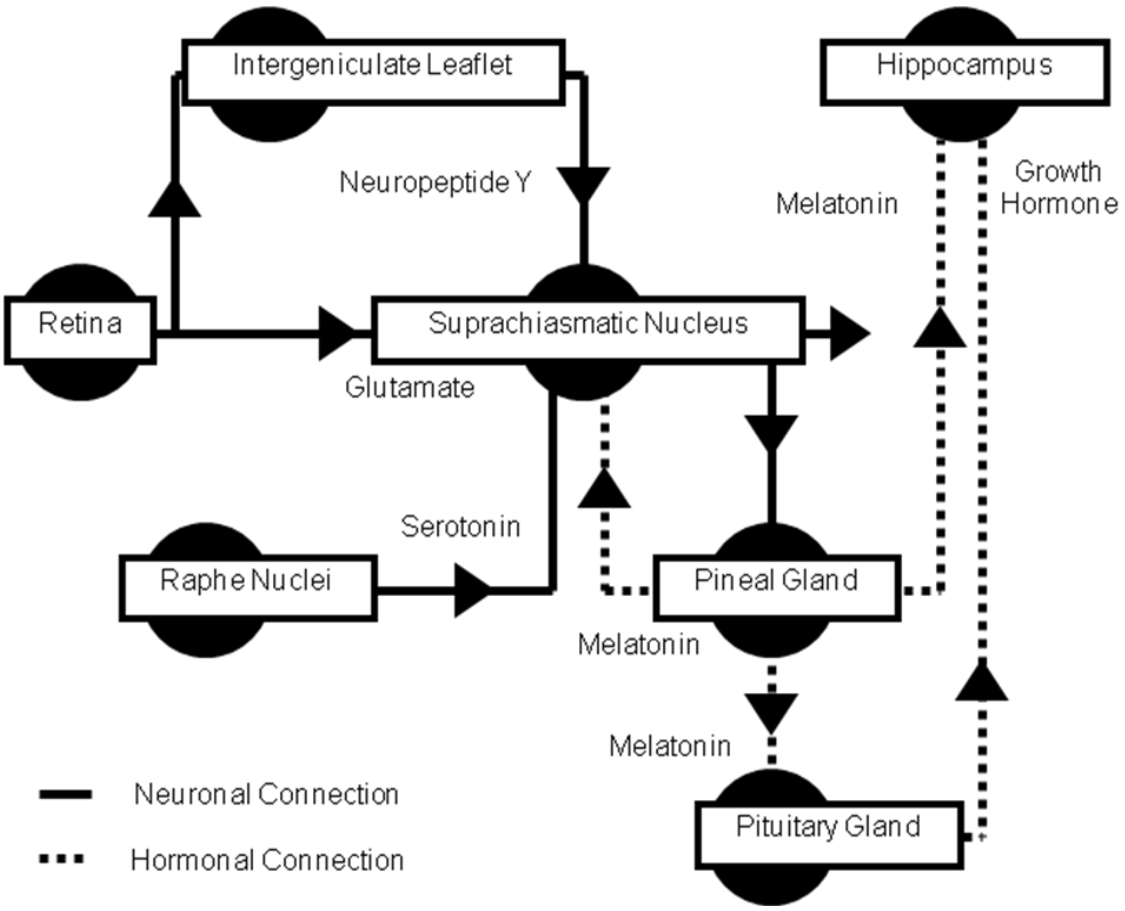
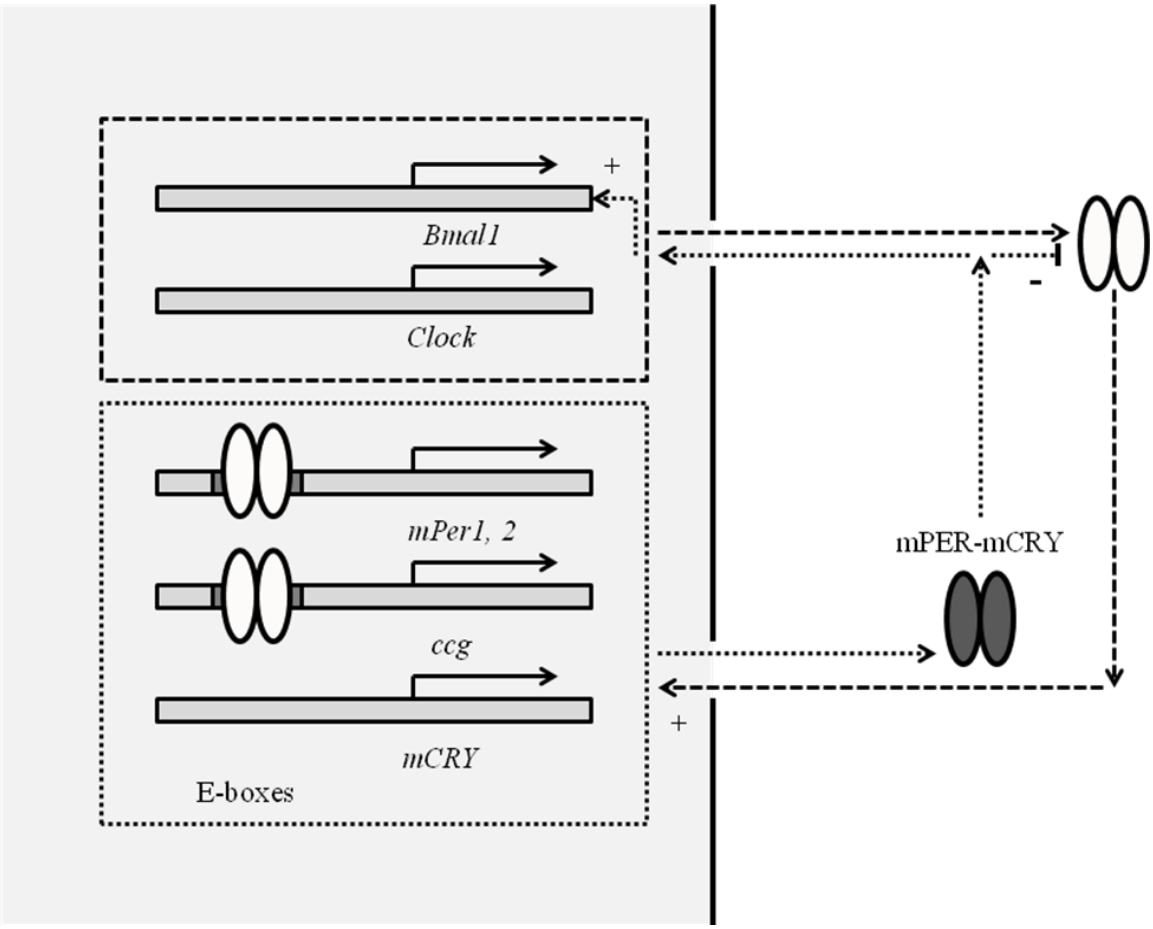

Intergeniculate Leaflet
Hippocampus
Neuropeptide Y
Melatonin
Growth Hormone
Retina
Suprachiasmatic Nucleus
Glutamate
Serotonin
Raphe Nuclei
Pineal Gland
Melatonin
Melatonin
Neuronal Connection
Hormonal Connection
Pituitary Gland

Figure 2. Simplified model of the oscillatory molecular clock gene network in cells of the suprachiasmatic nucleus of mammals (adapted from Okamura 2004). The *mPER1* and *mPER2* genes are expressed along with other *ccg* (*clock controlled genes*) to produce various proteins that dimerize. These mPER-mCRY dimers inhibit CLOCK-BMAL1 protein dimer function and indirectly promote expression of the *Bmal1* gene. Over several hours, *mPER1* and *mPER2* expression declines, but concentration of CLOCK-BMAL1 dimers gradually begins to rise again. These dimers bind E-boxes and promote expression of *mPER1*, *mPER2*, and *ccg* to complete the feedback loop.


+
Bmal1
Clock
mPer1, 2
ccg
mCRY
E-boxes
+
mPER-mCRY
-

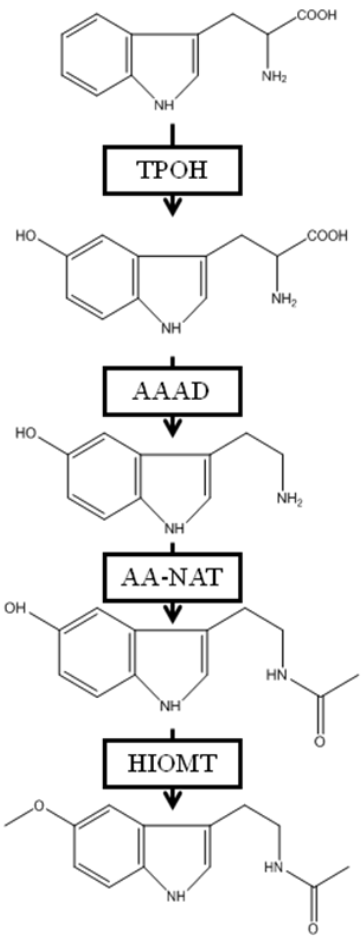
secretion entrains the sleep/wake cycle of mammals to their environment. We shall refer to the neurohormonal feedback loop involving the SCN, pineal, and associated structures as the circadian circuit.

Within cells of the pineal gland in mammals, melatonin is produced by a multi-step biosynthetic pathway (Figure 3) (for reviews see Axelrod 1970; Simonneuax et al. 2003). 5-Hyrdoyxytryptophan is synthesized from tryptophan by tryptophan hydroxylase (TPOH). Aromatic amino acid decarboxylase (AAAD) converts 5-hydroxytryptophan to serotonin, and acetylation of serotonin by arylalkylamine N-acetyltransferase (AA-NAT) produces N-acetylserotonin. Finally, hydroxyindole-O-methyltransferase (HIOMT) produces melatonin from N-acetylserotonin. Interestingly, children with autism spectrum disorder and their unaffected parents were more likely to have a mutation in the *HIOMT* gene and concomitant reduction in melatonin production compared to controls (Melke et al. 2008). The synthetic melatonin receptor antagonist luzindole has a structure similar to that of melatonin but contains an additional benzyl group (Dubocovich 1988).

Melatonin, Development, and Pathology

Melatonin produced by the circadian circuit has broad impacts throughout the body. There is evidence that melatonin may influence secretion of growth hormone by the pituitary by an as yet unidentified mechanism. The nightly peak in melatonin may be involved in the timing of growth hormone production (Brandenberger et al. 2004). Human subjects administered 10 mg of melatonin showed an increase in serum growth hormone in response to the drug (Valcalvi et al. 1993), however, earlier studies found

Figure 3. Structures of molecules in the melatonin biosynthetic pathway in the pineal gland (a) (adapted from Simonneaux et al. 2003) and of the melatonin receptor antagonist luzindole (b). Structures of all molecules shown contain indole groups composed of a pyrrole ring attached to a benzene ring. In pinealocytes, 5-hydroxytryptophan is synthesized from tryptophan by tryptophan hydroxylase (TPOH). Aromatic amino acid decarboxylase (AAAD) then converts 5-hydroxytryptophan to serotonin, and acetylation of serotonin by arylalkylamine N-acetyltransferase produces N-acetylserotonin. Finally, hydroxyindole-O-methyltransferase (HIOMT) produces melatonin from N-acetylserotonin. The synthetic melatonin receptor antagonist luzindole has a structure similar to that of melatonin but contains an additional benzyl group.



Tryptophan

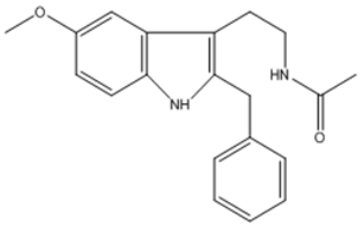
5-Hydroxytryptophan

Serotonin

N-Acetylserotonin

Melatonin

(a)



Luzindole

(b)

that melatonin has an inhibitory effect on growth hormone release in both humans (Smythe et al. 1974 a) and rats (Smythe et al. 1973). Insertion of melatonin implants in 3- to 4-month old heifers for 5 weeks in early summer induced precocious puberty in heifers (Tortonese et al. 1992), and pineal gland tumors that reduce melatonin secretion from the pineal gland during development elicited precocious puberty in humans (Cohen et al. 1964). Interestingly, precocious puberty was found to be a predictor for a subtype of schizophrenia in women (Cohen et al. 1999).

Because melatonin release by the circadian circuit is influenced by light, melatonin secretion is affected by seasonal variation in light patterns. Seasonal changes in melatonin profiles regulate reproduction in many mammals (for a review see Arendt 1998). Nightly peaks of melatonin in adult (Luboshitzky et al. 1998) and infant (Sivan et al. 2001) humans may be greatest when photoperiods are the longest. Moreover, risk of developing schizophrenia is greater for individuals born during winter and early spring at greater latitudes (Davies et al. 2003), when maternal nightly melatonin secretion during the third trimester is lowest. A seasonal correlation between birth date and adult disease has been found in various studies on psychiatric disorders in humans (for a review see Torrey et al. 1997). Explanations for this variation have been proposed, but the causes have yet to be identified.

Evidence in various animal models suggests a role for melatonin in brain development. Reduced prenatal melatonin by pinealectomy was associated with increased signs of depression in Siberian hamster offspring (Workman et al. 2008). In addition, studies using cell cultures suggest a possible role for melatonin during

embryonic development. Melatonin inhibits apoptosis in U973 human monocytic cells (Radogna et al. 2006), and it has been found to reduce damage to rat hepatoma cells by free radicals via its function as a free radical scavenger (Kimball et al. 2008). In addition to its effects on cell maintenance, melatonin has been found to affect cell growth. Melatonin promotes polymerization of tubulin and production of neurites in cells in vitro (Meléndez et al. 1996). Melatonin also increases the rate of cell proliferation in zebrafish embryos (Danilova 2004), and it promotes differentiation but not proliferation of adult hippocampal neurons in culture (Moriya et al. 2007).

Melatonin receptors are distributed in various brain regions in the human fetus, including the hypothalamus, thalamus, and meninges (Thomas et al. 2002) and are more widely distributed in the brains of fetal and neonatal rats compared to adults (Zitouni et al. 1995). Moreover, maternal pinealectomy reduces melatonin receptor number in the SCN, pituitary, and thalamic structures of rat neonates (Zitouni et al. 1995).

Signaling via these melatonin receptors in the fetal brain may cause permanent changes in the circadian circuit. Cocaine was found to cause long-term changes in expression of clock genes in zebrafish embryo, and this effect was reversed by administration of melatonin (Shang et al. 2007). In addition, prenatal melatonin reduction was found to alter the clock gene expression in the SCN of capuchin monkeys (Torres-Farfan et al. 2006) and treatment with prenatal melatonin altered postnatal photoperiodic traits of young meadow voles (Lee et al. 1989). These studies indicate that melatonin concentrations *in utero* may affect the function of the circadian circuit not only in the womb but also after birth.

Melatonin is also important in the brain postnatally, and melatonin receptors have been found in the adult rat hippocampus (Mushoff et al 2002). In fact, melatonin administration altered long-term potentiation and ultimately synaptic plasticity of hippocampal cells from CD-1 mice (El-Sherif et al 2003) and long-term treatment with melatonin increased NMDA receptor concentrations in the rat hippocampus (Sutcu et al 2006). Deletion of the gene for the MT2 melatonin receptor in mice produced memory deficits in habituation to the elevated plus maze (Larson et al 2006) and treatment with melatonin reduced hippocampal degeneration in a mouse model of Alzheimer's disease (Cheng et al 2008). Thus, regular secretion of melatonin in adulthood may contribute to maintenance of proper hippocampal function.

Hippocampus, Limbic System, and HPA Axis

Changes in hippocampal morphology have been found in various psychiatric disorders. For example, individuals with autism from ages 2 to 42 years were found to have reduced volume in the area dentata of the hippocampus (Saitoh et al. 2001), and three- to four-year-old children with autism have been found to have significant changes in hippocampal shape by large-deformation high-dimensional brain mapping (Dager et al. 2007). Moreover, enlarged volume of the left hippocampus was identified not only in children and adults with autism but also in parents of children with autism (Rojas et al. 2004), and changes in hippocampal structure have been found in individuals with schizophrenia. High-dimensional brain mapping revealed morphological deformations in regions of the hippocampus projecting to the prefrontal cortex in drug-naïve patients with

schizophrenia (Csernansky et al. 1998), whereas Rajarethinam et al. (2001) found no significant difference in hippocampal size between individuals with schizophrenia and controls but did find a correlation between shape of left hippocampal subregions and symptoms in male subjects. Meta-analyses reveal a general reduction in hippocampal volume in schizophrenia (McCarley et al. 1999; Nelson et al. 1998).

The hippocampus has been identified to have various roles in humans and other mammals. In elderly human subjects, hippocampal head volume was found to be negatively correlated with age (Chen et al. 2010). Specifically, right hippocampal tail volume was connected to spatial memory on the Groton Maze Learning Test, and left hippocampal volume was associated with deficits in verbal memory. In addition, adult males ages 18-31 who were more resistant to forgetting in a monetary incentive delay task displayed greater activation in the left posterior hippocampus via fMRI (Kuhl et al. 2010), and human subjects with amnesia resulting from bilateral hippocampal insult displayed greater difficulty imagining scenarios compared to controls (Hassabis et al. 2007). A recent review argued for roles of the hippocampus in imagination and prediction (Buckner 2010). Moreover, the hippocampus has been implicated in spatial learning across mammalian species (for a recent letter see Amrein et al. 2009; for a review see El Falougy et al. 2006).

More generally, the hippocampus is considered part of the limbic system, a brain circuit involved in emotion (for symposium see Nakano 1998; for review see Sapolsky 2003). The limbic system is capable of activating the hypothalamic-pituitary-adrenal (HPA) axis—the neuroendocrine mediator of the stress response in mammals—in

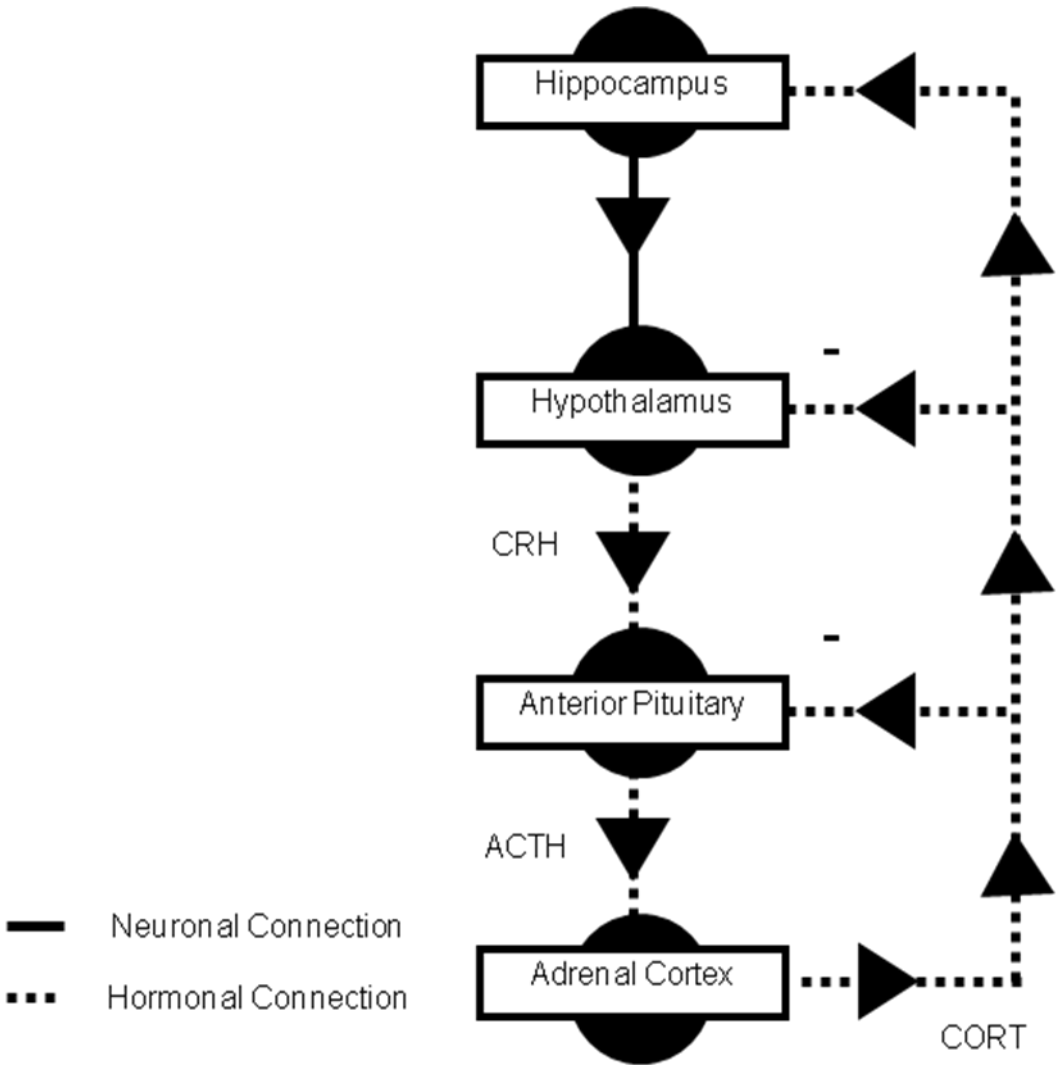
response to environmental stimuli (for a review see Engelmann et al. 2004). Within this circuit, the hypothalamus releases corticotrophin-releasing hormone (CRH) in response to signals from other brain regions (Figure 4) (for a review see Tsigos et al. 2002) and CRH elicits release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. ACTH promotes release of corticosteroids—including cortisol—from the adrenal cortices, and these corticosteroids inhibit further release of hormones by the hypothalamus and pituitary.

Dysregulation of the limbic system and HPA axis has been found in various psychiatric disorders. Patients with resistant schizophrenia were found to have a higher level of serum cortisol, and these levels were correlated with negative symptoms of the disorder (Zhang et al. 2005). Women with postpartum depression were found to have increased ACTH but reduced cortisol relative to controls (Jolley et al. 2007). Responsiveness of cortisol release to negative events was found to be blunted in individuals with major depressive disorder, but no basal difference in salivary cortisol levels was observed (Peeters et al. 2003). Moreover, children with autism showed greater variability in circadian salivary cortisol profiles relative to controls (Corbett et al. 2008). Thus, dysregulations of the HPA axis are connected with psychiatric disorders.

Serotonin Receptors

Evidence implicates the HPA axis in regulation of hippocampal serotonin (5-hydroxtrypatamine or 5HT) receptors (for a review see Leonard 2005). At present, 14 subtypes of serotonin receptors have been identified in mammals (for a review see

Figure 4. Simplified schematic of the hypothalamic-pituitary-adrenal (HPA) axis (adapted from Tsigos et al. 2002). The hippocampus and other regions of the limbic system elicit release of corticotropin-releasing hormone (CRH) from the hypothalamus in response to stress. CRH promotes release of adrenocorticotropic hormone (ACTH) by the anterior pituitary, and ACTH causes the adrenal cortices to release corticosteroids including cortisol (CORT) into the bloodstream. CORT inhibits further release of ACTH and CRH in addition to impacting the hippocampus and other brain regions.


Hippocampus
Hypothalamus
CRH
Anterior Pituitary
ACTH
Adrenal Cortex
CORT
Neuronal Connection
Hormonal Connection

Nichols et al. 2008). All serotonin receptor subtypes except for 5HT3 receptors are G-protein-coupled receptors (GPCRs). In particular, serotonin GPCRs are of the type A family of rhodopsin-like receptors (Fredriksson et al. 2003) while the 5HT3 receptor is a ligand-gated ion channel (Maricq et al. 1999) (for a review of 5HT receptors see Barnes et al. 1999).

Research also indicates potential differential involvement of serotonin (5-hydroxytryptamine or 5HT) receptor subtypes in psychopathology. Serotonin receptor concentrations have been found to be altered in the hippocampus of individuals with severe psychiatric illness (Knable et al. 2004). In particular, it was found that 5HT1A expression was lower in the hippocampus of individuals with major depressive disorder while subjects with schizophrenia and bipolar disorder had increased 5HT1B expression and decreased 5HT2A expression in the hippocampus (López-Figueroa et al. 2004). Since melatonin may also play a role in these psychopathologies, investigation of the effects of melatonin on the serotonergic system is warranted.

Corticosteroids have been found to be involved in the regulation of hippocampal 5HT1A receptor expression. In particular, adrenalectomy of Sprague-Dawley rats increased 5HT1A mRNA expression in the hippocampus 1 day and 7 days after surgery and this change could be prevented by administration of cortisol at surgery (Chalmers et al. 1993). Activation of 5HT1A receptors in the rat hippocampus inhibits adenylyl cyclase (Okada et al. 1989), and administration of serotonin and the 5HT1A receptor agonist NAN-190 to Wistar rat hippocampal CA1 cells in culture resulted in reduced excitatory post-synaptic potentials (EPSPs) (Schmitz et al. 1995). 5HT1A agonists were

unable to elicit hyperpolarization in mouse hippocampal neurons from mice lacking the gene for G protein-coupled inwardly rectifying K^+ channel 2 (GIRK2) (Luscher et al. 1997). In addition to their involvement in serotonergic transmission, 5HT1A receptors are also involved in noradrenergic transmission in the hippocampus. In particular, activation of hippocampal 5HT1A receptors elicited activation of noradrenergic pathways postsynaptically in rats (Hajós-Korcsok et al. 1999). In addition, the increase in acetylcholine release in response to administration of the 5HT1A agonist 8-OH-DPAT was prevented by administration of the 5HT1A receptor antagonist WAY-100135 (Nakai et al. 1998).

In contrast, corticosteroids were not found to regulate expression of 5HT1B mRNA in adrenalectomized male Sprague-Dawley rats (Neumaier et al. 2000). In CHO cells, 5HT1B receptors were found to inhibit adenylyl cyclase and increase activity of ERK2 (Mendez et al. 1999). Moreover, signaling via 5HT1B receptors reduced serotonin release in the rat hippocampus *in vivo* (Martin et al. 1992), suggesting a role for this receptor subtype as an autoreceptor. This receptor subtype has also been found to inhibit cholinergic pathways in the rat hippocampus (Maura et al. 1986).

5HT2A receptors have distinct roles in the stress response. NIH3T3 fibroblasts expressing rat 5HT2A receptors were found to release the endocannabinoid 2-arachidonoyl glycerol via activation of phospholipase C (PLC) (Parrish et al. 2006). In cultured astrocytes from newborn rat cerebral cortex, 5HT2A receptor activation elicited Ca^{2+} influx via ligand-gated ion channels (Hagberg et al. 1998). Moreover, hippocampal granule cells from male Sprague-Dawley rats administered serotonin induced

depolarization and this response was reduced by the 5HT2A receptor antagonist MDL 100,907 (Piguet et al. 1994). The reduction of BDNF expression in the hippocampus observed in response to immobilization stress in male Sprague-Dawley rats was also attenuated by administration of the 5HT2A receptor antagonist MDL, 100,907 prior to testing (Vaidya et al. 1999).

Brain Derived Neurotrophic Factor

Brain derived neurotrophic factor (BDNF) is a protein signaling molecule known for its promotion of growth and survival of neurons in the hippocampus (for a review see Binder et al. 2004). Activation of BDNF's tropomyosin-related kinase B receptor (TrkB) increased activity of synaptic proteins in rat cortical neurons in culture, and depletion of BDNF or inhibition of its receptor prevented this effect (Jia et al. 2008). Activation of TrkB was also found to be required for long-term synaptic facilitation in *Aplysia* (Sharma et al. 2006). Reduced levels of BDNF have been found in the serum of patients with schizophrenia (Toyooka et al. 2002), and BDNF has also been implicated in depression (for a review see Castrén et al. 2010).

Microtubule-Associated Protein 2

Microtubule-associated protein 2 is (MAP2) is one of several proteins involved in the cytoskeletal organization in dendrites (for a review see Farah et al. 2008), and increased expression of MAP2 has been found in the hippocampus of individuals with schizophrenia (Cotter et al. 2000). Dendritic size was found to be reduced in cerebellar

tissue from MAP2-knockout mice, and cells from these animals displayed reduced PKA signal transduction (Harada et al. 2002). Induction of long-term potentiation (LTP) in various hippocampal pathways increased expression of MAP2 mRNA (Roberts et al. 1998), and application of NMDA—a glutamate agonist mediating LTP induction—to hippocampal granule cells also elicited increased expression of MAP2 mRNA in dendrites (Johnston et al. 1994).

Spinophilin

Another dendritic protein associated with the cytoskeleton, spinophilin or neurabin 2, is localized in the heads of dendritic spines (for a review see Sarrouilhe et al. 2006). Phosphorylation of spinophilin by protein kinase A (PKA) *in vitro* elicits its dissociation from actin filaments (Hsieh-Wilson et al. 2003). Spinophilin also blocks a binding site of protein phosphatase 1 (PP1) without blocking its active site to direct the enzyme to particular substrates (Ragusa et al. 2010). Spinophilin-knockout mice showed reduced ability of PP1 to regulate AMPA and NMDA receptors resulting in inhibition of long-term depression in the caudotoputamen and reduced hippocampal size (Feng et al. 2000). This study also found increased numbers of dendritic spines in hippocampal cells from young spinophilin knockout mice relative to controls. Interestingly, patients with schizophrenia and mood disorders showed similar reductions in spinophilin mRNA in the hippocampus via *in situ* hybridization (Law et al. 2004).

Growth Hormone Receptor

Growth hormone receptor (GHR) is a cell membrane receptor (for a review see Postel-Vinay et al. 1995). Fusion of ligand to this receptor elicited receptor dimer formation and signal transduction in CHO cells (Wilkinson et al. 2007). Growth hormone administration enhanced excitatory post-synaptic potentials in slices of pyramidal neurons from the CA1 region of the hippocampus of male Sprague-Dawley rats (Mahmoud et al. 2006). Although circulating growth hormone is secreted from the anterior pituitary, growth hormone production has also been detected in the hippocampus of Sprague-Dawley rats of both sexes, and growth hormone mRNA levels in this brain region increase in response to stress (Donahue et al. 2006).

Melatonin Receptors

Two primary types of melatonin receptors have been identified in various mammalian tissues, including the hippocampus (for a review see Pandi-Perumal et al. 2008). Both MEL1A (Brydon et al. 1999) and MEL1B (Reppert et al. 1995) receptors are from the G-protein-coupled-receptor family and are coupled to inhibition of adenylyl cyclase. The C-terminal domain of both receptor subtypes is necessary for internalization (Sethi et al 2008). Evidence suggests a role for melatonin receptors in hippocampal pathology. For example, MEL1A receptor expression increased (Savaskan et al. 2002) and MEL1B receptor expression decreased (Savaskan et al. 2005) in the hippocampus of elderly patients with Alzheimer's disease relative to controls.

Hypothesis

Our study was designed to test the hypothesis that increasing or decreasing prenatal melatonin signaling in male *rattus norvegicus* affects growth, hippocampal mass, anxiety- and depression-related behaviors, spatial learning, and memory in adult rats. In addition, we hypothesized that prenatal treatment would produce differential adult hippocampal mRNA expression profiles for genes involved in regulating dendritic structure and function as well as neuron maintenance and cognitive function.

MATERIALS AND METHODS

Animals and Tissue Collection

Eleven pregnant Sprague-Dawley dams were obtained from Hilltop Laboratories in two cohorts and randomly assigned to three treatment groups. Three of the dams were injected with vehicle containing 50% (volume/volume) sterile, filtered DMSO (Sigma Aldrich, St. Louis, MO) in distilled water (i.e. 0.1 mL DMSO/kg dam per day), four were injected with 5 mg/kg melatonin (*N*-Acetyl-5-methoxytryptamine) (Sigma Aldrich), and four were injected with 5 mg/kg luzindole (*N*-Acetyl-2-benzyltryptamine) (BA Chemicals, London, UK), a nonselective melatonin receptor antagonist (Dubocovich 1988). All drugs were administered subcutaneously immediately before the dark cycle at 1600 to 1800 hours as this is the time of maximal response to the drugs (Golombek et al.

1993). Injections were given for 5 days from days 14 to 18 of gestation at a volume of 0.05 to 0.06 mL per day. Dams were housed individually.

All animals were kept under controlled lighting (0600 to 1800 hours) and temperature (23ºC) with food and water *ad libitum*. Food consisted of standard rat chow providing 3.85 kcal/g (dry wt), 19.2 gm% protein, 67.3 gm% carbohydrate, and 4.3 gm% fat (10 kcal% fat D12450B; Research Diets, New Brunswick NJ). At birth, offspring were weighed and litters were culled to 10 young per dam while maintaining all healthy males. At weaning on postnatal day 21, male offspring were housed in pairs with a conspecific from the same treatment group. Body mass per rat and food intake per cage were measured approximately every 5 to 7 days from weaning until sacrifice. After 120 days, half the animals were sacrificed at 0000 hours, and the rest were sacrificed at 1200 hours. The animals were momentarily placed in a pre-charged chamber of CO_2 and subsequently decapitated by guillotine. Immediately following sacrifice, right and left hippocampi were quickly dissected from each brain, weighed, and rapidly frozen with liquid nitrogen; and abdominal, retroperitoneal, and gonadal fat was excised. This research was conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all animal protocols used have been reviewed and approved by the Animal Care and Use Committee of Bucknell University.

Behavioral Measures

After postnatal day 90, a staggered behavioral testing regimen was begun on adult subjects. Animals from cohort one were subjected to tests in the following order: open

field test, forced swim test, elevated plus maze, and Morris water maze; cohort two subjects were tested as follows: Morris water maze, elevated plus maze, forced swim test, open field test. All behavioral tests were recorded, and data was measured by computer with tracking software (ANY-Maze, Stoelting, Wood Dale IL). Time inactive was found by subtracting time mobile, time rearing, and time grooming from total time. Turn preference was calculated as follows:

$$\text{Turn Preference} = \log\left(\frac{\text{Clockwise Turns} + 1}{\text{Counterclockwise Turns} + 1}\right) \quad (1)$$

Forced swim test. The forced swim test is a measure of learned helplessness. The learned helplessness hypothesis states that "when events are uncontrollable the organism learns that its behavior and outcomes are independent" (Maier et al. 1976). This behavioral construct includes "motivational," "cognitive," and "emotional" components. Porsolt et al. (1978) developed the forced swim test to measure learned helplessness. In the forced swim test, immobility in response to placement in an inescapable tank of water as evidenced by free floating is considered a sign of learned helplessness (West 1990). Although learned helplessness is a construct with respect to learning, changes in performance on learned helplessness tasks have been connected to depression (Sherman et al. 1982).

The protocol of Porsolt et al. (1978) was modified slightly for this study. Animals were placed in translucent white plastic cylinders (45 cm height 35 cm diameter) containing 30 cm water (25 ±2 °C) in 160 lux light for 15 minutes without recording, and the tank was emptied between subjects. Twenty-four hours later, animals were similarly

placed in tanks, and behavior was recorded for 5 minutes. Time immobile was determined by tracking software at 50% sensitivity, and total distance travelled was also used as an indicator of immobility (Hedou et al. 2001).

Open field test. The open field test was originally created by Calvin Hall as a measure of fearfulness (Walsh et al. 1976). In this test, the response of animal subjects to an open space from which they cannot escape is measured. The test has been considered a measure of both "emotionality" and "exploratory behavior", although other constructs have been developed (Maier et al. 1988). In addition, habituation of behavior in the testing environment occurs upon multiple trials, so learning is also involved (Bolivar et al. 2000). The open field test is frequently used in pharmacological studies for the measurement of anxiety (Choleris et al. 2001, Prut et al. 2003, Ramos et al. 1997), although measures of general locomotor activity can also be obtained from the test (Maier et al. 1988). The use of the test as a measure of anxiety is evidenced by the effects of anxiety drugs on behavior of rodents in the testing arena (Choleris et al. 2001). Rats spend more time in the center zone and less time in thigmotaxis (staying near the walls of the test arena) if they are less anxious (Prut et al. 2003). However, responses upon repeated trials can vary by rat strain (Bolivar et al. 2000).

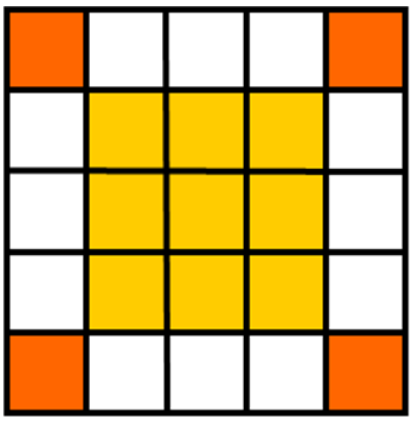
A black testing arena (100 cm x 100cm x 40 cm) with an open top was used (Ramos et al. 1997). Light reaching the arena was 65 lux at the center, 45 lux at the middle of the sides, and 30 lux in the corners. Although various colors ranging from white to black have been used for the arena (Walsh et al. 1976), black was chosen for greater contrast of the rat with background (Walsh et al. 1976). Although rats can be

placed in either the center or a corner of the arena at the start of the procedure, we placed them in the center (Ramos et al. 1997). The arena was divided by the software into 25 squares: 16 outer squares and 9 inner squares (Figure 5a). While behavior has been recorded for various lengths of time, we did so for 5 minutes (Ramos et al. 1997). While tracking software recorded most measures, human observers documented rearing and grooming behavior during testing using "keys" within the software package. Immobility detection was set at 75% sensitivity.

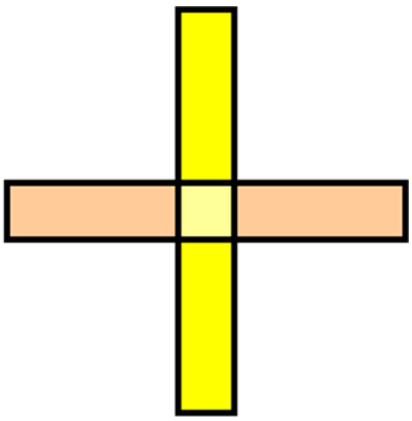
Elevated plus maze. The elevated plus maze is commonly used as a measure of anxiety in rodents (Hogg 1996). Anxiety is measured by decreased entries to the open arms of the apparatus. In particular, the decreased height or absence of walls on the open arms produces a fear response (Rodgers et al. 1997). Moreover, time on open arms increases in response to anxiolytic drugs (Rodgers et al. 1997). Others, however, have also included an approach/avoidance construct in measuring behavior in this test (Wall et al. 2001) by considering entries to the center of the apparatus.

The protocol of Walf et al. (2007) was used with modifications. Animals were placed in the center of the plus sign-shaped testing arena (arms 50cm long by 10 cm side with a 5 mm railing on the open arms) constructed of fiberboard coated with black acrylic paint and elevated 50 cm from the floor (Figure 5b). Light reaching the apparatus was 13 lux at the center, 25 lux on the open arms, and 3.5 lux in the closed arms. Behavior of animals was recorded for 5 minutes with human observer present in the room behind a curtain. Grooming and rearing behavior were recorded real-time by human observer using the tracking software package, and immobility detection was set at 75% sensitivity.

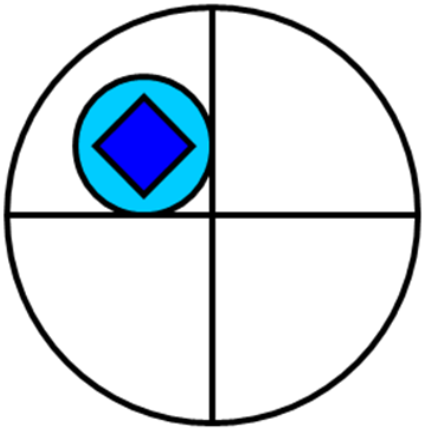
Figure 5. Diagrams of testing arenas used for the open field test (a), elevated plus maze (b), and Morris water maze (c). The open field testing arena was divided into 25 squares with center zone and corner regions (a). The elevated plus maze testing arena was divided into two closed arms with walls, two open arms without walls, and a central platform zone (b). The Morris water maze testing arena was divided into quadrants with cardinal directions, and a hidden platform was located at the center of a memory region zone (c).



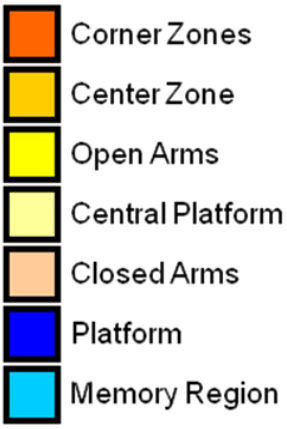
(a)



(b)



(c)


Corner Zones
Center Zone
Open Arms
Central Platform
Closed Arms
Platform
Memory Region

Animals that fell off the apparatus were returned to it and allowed to complete the testing, but data from these subjects was excluded from analysis (Walf et al. 2007). Before each trial, the maze was cleaned with water containing detergent and allowed to dry.

Morris water maze. The Morris water maze was developed by Richard G.M. Morris for the testing of spatial learning and memory in rats. In particular, lesions in the various brain regions—including the hippocampus—impair performance on this test (d'Hooge et al. 2001). In addition, the Morris water maze has also been used to measure cognitive deficits in models of a variety of neuropsychiatric disorders (d'Hooge et al. 2001).

The protocol of Vorhees et al. (2006) was used with the modifications. A platform (19.5cm x 19.5 cm x 19.5 cm black cinderblock) was placed in a tank (58 cm height, 168cm diameter) painted black (Figure 5c). At the center of the apparatus, light was 75 lux; at the walls, light was approximately 35 lux. Cardinal coordinates were arbitrarily chosen so that the platform was located in the NW quadrant of the tank. The tank was filled with water at 25 ±2 °C at the beginning of each testing day to 4 cm above the top of the cinderblock platform and emptied upon completion of all tests. Geometric patterns were placed on three walls of the testing room as visual cues. During the 5 days of the learning phase of the test, animals were placed in the tank facing the wall at one of each of the cardinal coordinates for 4 trials in a predetermined, randomly-selected order by cohort. Latency to platform was recorded by tracking software. A trial ended when the rat remained on the platform for 2 seconds or after 120 seconds. Animals that did not find the platform in 120 seconds were placed on the platform. Subjects were allowed to

rest on the platform for 15 seconds before the subsequent trial. If a rat did not remain on the platform, it was placed back on the platform repeatedly for the duration of 15 seconds. Latencies for the 4 trials for per day were averaged. Forty-eight hours after the completion of the learning phase of the test, animals were returned to the tank after removal of the platform for one trial. Preference for the region of the former location of the platform was recorded for one 30-second trial in order to test memory retention.

RNA Isolation, RT-PCR, and Data Analysis

Total RNA was extracted from the left hippocampus of each subject with TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, and after purification, total RNA was reverse transcribed with RETROscript (Ambion, Austin, TX) following producer's recommendations. Real-time PCR was effected with SYBR Green Supermix (Bio-Rad, Hercules, CA) on an iCycler iQ Real Time PCR Detection System (Bio-Rad, Hercules, CA). Primers were designed with the NCBI online database (www.ncbi.nlm.nih.gov) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primer pair sequence specificities were confirmed via BLAST (Table 1), and all primers were obtained from MWG Oligo Synthesis (High Point, NC). Real-time PCR was performed in 96-well optical plates with 0.05μM each of forward and reverse primers, cDNA from 2μg RNA input (diluted 1:50), and 12.5μL Supermix. Target genes were amplified through the following thermocycling program: 95ºC for 3', 40 X 15" PCR cycles at 95ºC, 60ºC for 1', and 55ºC for 1'. At the end of the program, 80 repeats of 15" each accompanied by a temperature ramp of 0.5ºC/repeat

Table 1. ***Primer sequences (GenBank/NCBI) used in real-time PCR***

Primer ID	Primer Sequence 5'—3'	Accession No.	Length, nt
18S	Fwd: AAACGGCTACCACATCCAAG Rev: GGCCTCGAAAGAGTCCTGTA	M11188	76
5HT1A	Fwd: TGTTGCTCATGCTGGTTCTC Rev: CCGACGAAGTTCCTAAGCTG	NM 012585	76
5HT1B	Fwd: CTGGTGTGGGTCTTCTCCAT Rev: GTTCACAAAGCAGTCCAGCA	NM 022225	84
5HT2A	Fwd: CTGCTGGGTTTCCTTGTCAT Rev: ATCCAGATCGCACAGAGCTT	NM 017254	93
BDNF	Fwd: AGAGATCCAGCCCTGAGACA Rev: TCACCACATGGAAGGGTCTT	NM012513.3	98
MAP2	Fwd: ACGTTCGCTTGAAAAGGAGA Rev: CATGACGTTGATTCGTTTGG	NM013066.1	89
SPN	Fwd: GCAGGATCCAAGTGAACGAT Rev: CGGTTTGGTGTTCCTAAGCA	NM	96
GHR	Fwd: CCTGATCCGCCCATTGGCCT Rev: GCGGTGGCTGCCAACTCACT	NM017094	76
MEL1A	Fwd: GAGCGGGGTAAGGGGCAGGA Rev: AGTGCCCAGCTCTGCTTGCG	NM053676	82
MEL1B	Fwd: CCGAGCCTGCAGTCAGTGGC Rev: GGGCACCAAGGCCACCAGAG	NM001100641	76

Final concentration of each primer was 1μM

were performed during which dissociation curve data were collected to verify that only target sequences were amplified.

The following genes were amplified from hippocampus-derived cDNA: three serotonin (5-hyroxytryptamine or 5HT) receptor subtypes (5HT1A, 5HT1B, and 5HT2A), brain-derived neurotrophic factor (BDNF), microtubule-associated protein 2 (MAP2), spinophilin (SPN), melatonin receptor subtypes 1A and 1B (MEL1A and MEL1B), and growth hormone receptor (GHR). Each gene was co-amplified with a standard housekeeping gene, 18S ribosomal RNA, to control for pipetting error.

A real-time PCR was conducted for each primer pair in which cDNA samples were substituted with dH_20 to verify that exogenous DNA was not present. Additionally, 2μg of RNA isolated by the procedure described above was substituted for cDNA in a real time PCR reaction to confirm that there were no genomic DNA contaminants in the RNA samples. Both negative controls showed no amplification after 35 cycles.

Computer-calculated cycle numbers at which amplified DNA surpassed fluorescence threshold were normalized and contrasted to determine relative gene expression across treatment groups. Low cycle numbers signified higher initial amounts of cDNA or greater gene expression. All samples were run in triplicate, and threshold values were averaged to determine cycle threshold (C_T) values. Change in threshold (dC_T) was calculated for each sample by subtracting co-amplified 18s C_T values from the C_T values for genes of interest.

For graphs, the group with the highest mean dC_T value (lowest gene expression) per amplified gene target was set to zero and the mean dC_T values of the other three

groups were set relative to this calibrator (ddC_T). The ddC_T values were calculated as powers of 2 (2^{ddCT}), to account for the exponential doubling of the polymerase chain reaction.

Statistical Analysis

Growth data over time were 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 (Minitab 15 Statistical Software, State College PA). Comparisons of group-by-group reduced and full models were also performed with family-wise and *post hoc* Bonferroni corrections.

Anatomical measures and most behavioral measures were compared first using one-way ANOVA for control, melatonin, and luzindole animals followed by multiple comparisons with Bonferonni *post hoc* analysis (SPSS 16.0, Chicago, IL). Morris water maze data were analyzed with nonlinear mixed effects modeling via the method of Young et al. (2008) (R, R Foundation for Statistical Computing, Vienna, Austria). Gene expression data were compared by Kruskal-Wallis analysis followed by group-by-group Wilcoxon analysis with Bonferroni correction (SPSS). Ideally, studies examining prenatal effects on litters should use litter averages as the sample unit (Krinke 2000). Given that the present data include only 3-4 litters per treatment group, it was decided to use individual young as sample units for most measures in this report. Our final study for publication from this data will include at least 8 litters per treatment group. For all measures, statistical significance was set to $P < 0.05$.

RESULTS

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

The effects of administration of vehicle, 5 mg/kg melatonin, or 5mg/kg luzindole to pregnant dams from days 14-18 of gestation on litter size, litter sex composition, and birth body mass of male and female pups are indicated in Table 2. Treatment had no significant effect on litter size, although this measure was approaching significance [$F_{(2,8)}$=4.078, P=0.060] for a reduction in litter size for the melatonin-treated dams compared to luzindole-treated dams [P=0.085]. No significant difference was found between litters for male to female pup ratio [$F_{(2,8)}$=0.333, P=0.726] or for average male body mass [$F_{(2,8)}$=0.511, P=0.618] and average female body mass [$F_{(2,8)}$=0.332, P=0.727] per litter.

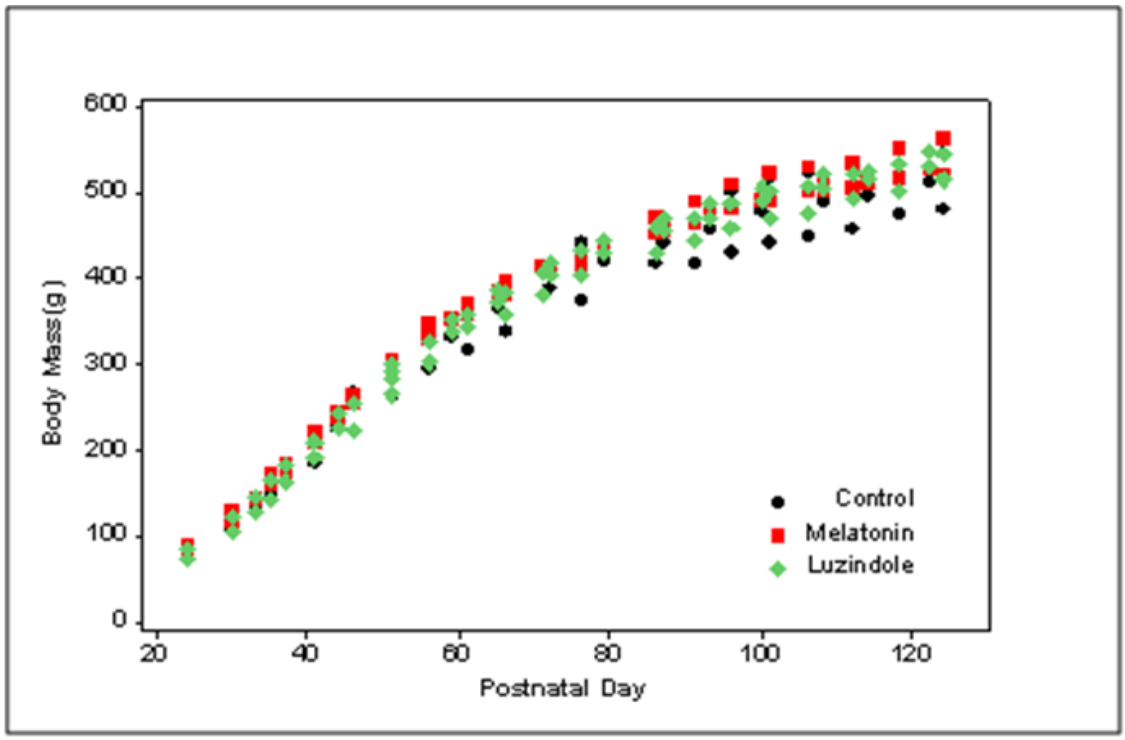
Body mass gain and food intake for male rat litters treated prenatally with vehicle, melatonin, or luzindole is shown in Figure 6. The body mass curve for litters treated prenatally with melatonin was significantly different from litters treated prenatally with vehicle or luzindole [Overall reduced model to overall full model [$F_{(6,174)}$=5.0564, P=0.00075]; reduced control and melatonin model to full control and melatonin model [P=0.00171]; reduced control and luzindole model to full control and luzindole model [P=1.00]; reduced melatonin and luzindole model to full melatonin and luzindole model [P=0.01084]]. In addition, prenatal treatment was not found to have a significant effect

Table 2. ***Litter size, ratio of male pups to female pups per litter, and average body mass of male and female pups per litter for dams treated with vehicle, 5 mg/kg melatonin, or 5 mg/kg luzindole during days 14-18 of gestation***

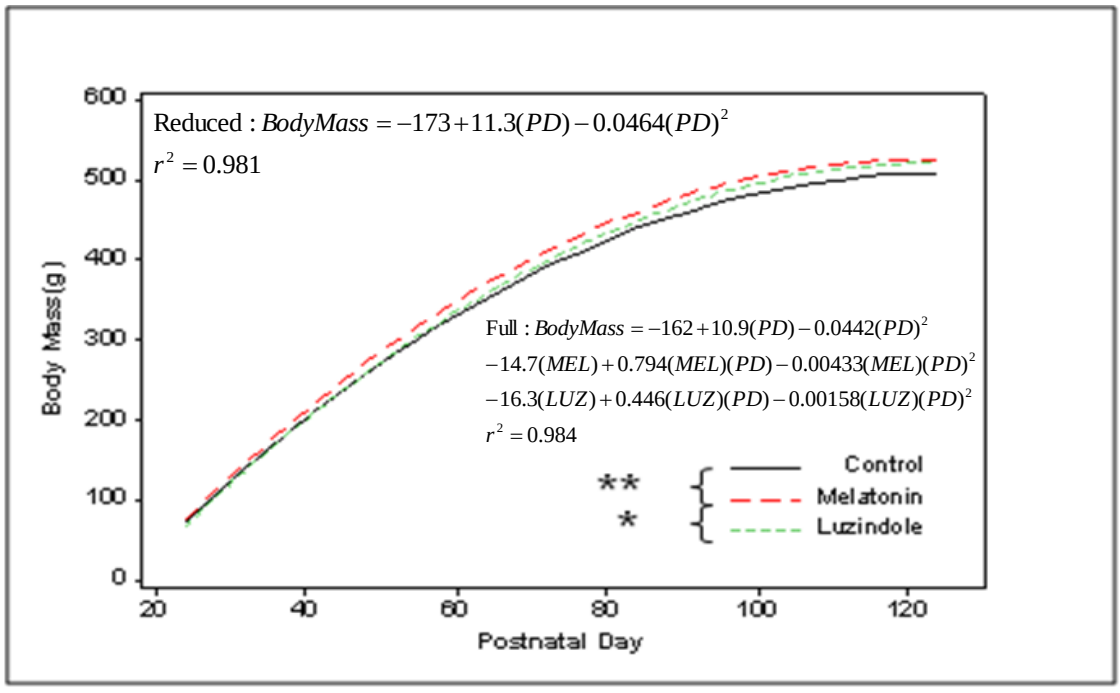
	Litter Size (Pups)	Male Pups/ Female Pups	Average Male Mass (g/pup per litter)	Average Female Mass (g/pup per litter)
Control (CON)	14.3 ± 0.9	0.88 ± 0.07	12.7 ± 1.0	12.3± 1.2
Melatonin (MEL)	11.8 ± 0.6	0.9 ± 0.2	13.5 ± 0.9	12.8 ± 1.0
Luzindole (LUZ)	14.8 ± 0.9	1.1 ± 0.2	11.8 ± 1.5	11.5 ± 1.3
CON-MEL-LUZ	*P=0.060*	*P=0.726*	*P=0.618*	*P=0.727*
CON-MEL	*P=0.197*	*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=0.085*	*P=1.00*	*P=1.00*	*P=1.00*

Values are means ± SEM. Differences between groups (control-melatonin-luzindole) were determined using one-way ANOVA. Significant differences were further analyzed using multiple comparison tests and Bonferroni *post hoc* analysis. Values for litters from dams treated with vehicle, 5 mg/kg melatonin, or 5 mg/kg luzindole during days 14-18 of gestation were compared. *** P < 0.001, ** P < 0.01, * P < 0.05 means are significantly different from control.

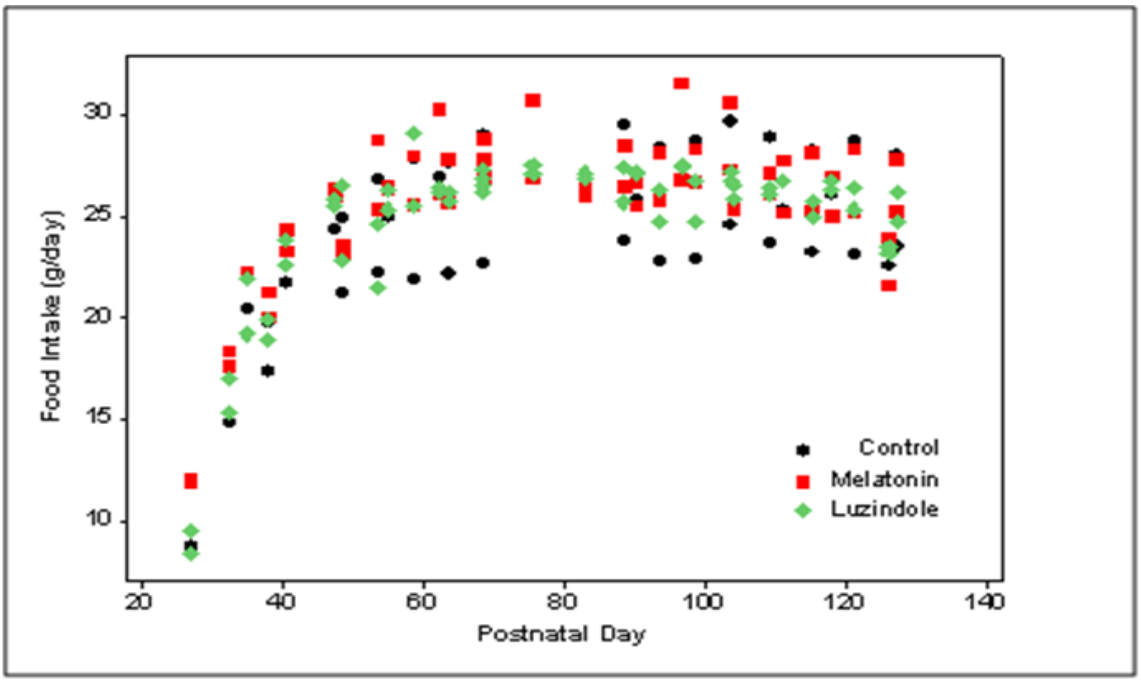
Figure 6. Average body weights (a) and food intake (c) of male rats treated prenatally with vehicle, melatonin, or luzindole. Weight and food intake of 11 litters [Control n=3, Melatonin n=4, Luzindole n=3] was averaged from weaning at postnatal day (PD) 21 to after PD120. Overall reduced and full regression models with family-wise Bonferroni corrections were fit to data and compared for significance followed by group-by-group comparisons with additional group-by-group Bonferroni corrections (b,d). Litters treated with prenatal melatonin were found to have body mass curves significantly different from controls and luzindole-treated litters [[** $P<0.01$], [*$P<0.05$]]; however, prenatal treatment was not found to have a significant effect on food intake.


Body Mass (g)
Postnatal Day
Control
Melatonin
Luzindole

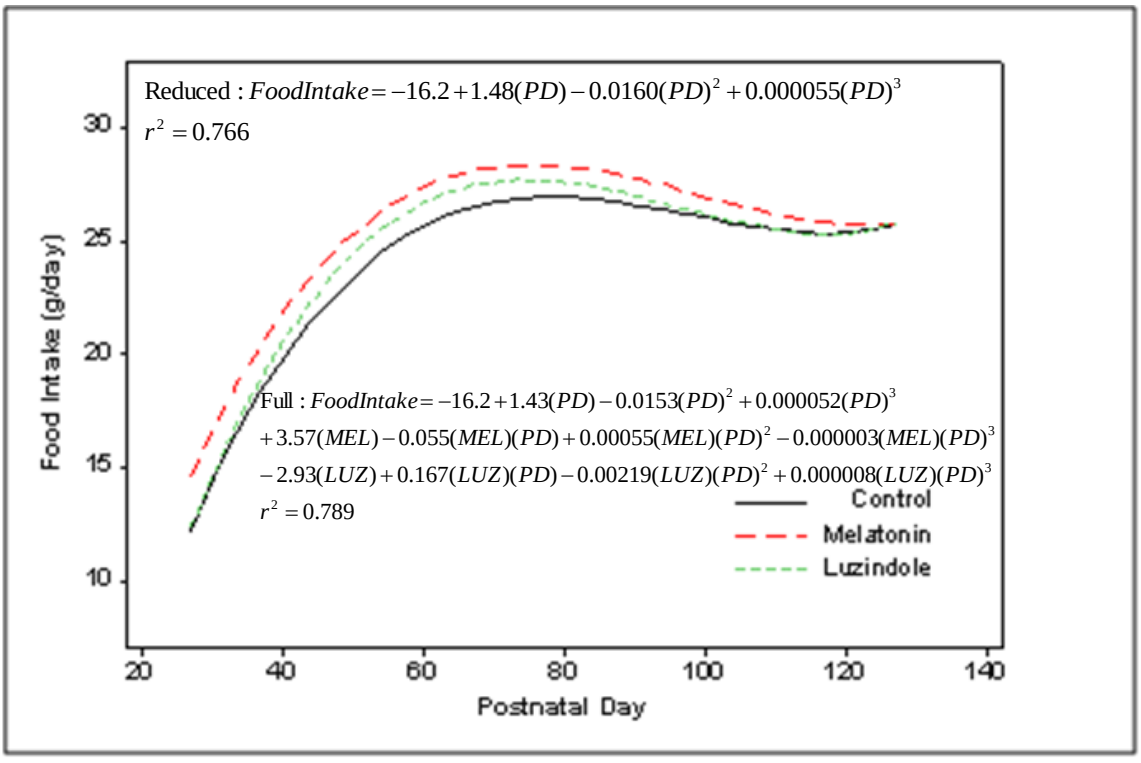
(a)


Reduced : $BodyMass = -173 + 11.3(PD) - 0.0464(PD)^2$
$r^2 = 0.981$
Full : $BodyMass = -162 + 10.9(PD) - 0.0442(PD)^2$
$-14.7(MEL) + 0.794(MEL)(PD) - 0.00433(MEL)(PD)^2$
$-16.3(LUZ) + 0.446(LUZ)(PD) - 0.00158(LUZ)(PD)^2$
$r^2 = 0.984$
**
*
Control
Melatonin
Luzindole
Body Mass (g)
Postnatal Day

(b)


Food Intake (g/day)
Postnatal Day
Control
Melatonin
Luzindole

(c)


Reduced : $FoodIntake = -16.2 + 1.48(PD) - 0.0160(PD)^2 + 0.000055(PD)^3$
$r^2 = 0.766$
Full : $FoodIntake = -16.2 + 1.43(PD) - 0.0153(PD)^2 + 0.000052(PD)^3$
$+ 3.57(MEL) - 0.055(MEL)(PD) + 0.00055(MEL)(PD)^2 - 0.000003(MEL)(PD)^3$
$- 2.93(LUZ) + 0.167(LUZ)(PD) - 0.00219(LUZ)(PD)^2 + 0.000008(LUZ)(PD)^3$
$r^2 = 0.789$
Control
Melatonin
Luzindole
Food Intake (g/day)
Postnatal Day

(d)

on food intake curves of litters [Overall reduced model to overall full model [$F_{(8,154)}$=2.0693, P=0.3832].

As summarized in table 3, prenatal treatment had no significant impact on percentage body fat [$F_{(2,61)}$=0.157, P=0.855], left hippocampus mass [$F_{(2,60)}$=0.902, P=0.411], right hippocampus mass [$F_{(2,60)}$=0.432, P=0.651], or left hippocampus mass to right hippocampus mass ratio [$F_{(2,60)}$=0.105, P=0.901].

Effects of Altered Prenatal Melatonin Signaling on Adult Behavior

Exposure to melatonin or luzindole *in utero* did not have a significant impact on adult male rat behavior in the forced swim test (Figure 7). Prenatal treatment did not affect total distance traveled [$F_{(2,58)}$=0.887, P=0.418], number of fecal boli produced [$F_{(2,58)}$=0.593, P=0.556], time spent immobile [$F_{(2,58)}$=0.570, P=0.569], or number of immobile episodes [$F_{(2,58)}$=0.1864, P=0.427]. Although the results for the effects of prenatal treatment on turn preference were not significant, a trend was indicated [$F_{(2,58)}$=2.826, P=0.067] for a greater right turn preference for male rats exposed to melatonin prenatally [p=0.066].

The impact of prenatal treatment on adult male rat behavior in the open field test was dramatic (Figure 8). Although treatment did not have a significant impact on time spent in the center of the arena [$F_{(2,61)}$=0.769, P=0.468] or turn preference [$F_{(2,61)}$=0.681, P=0.510], significant effects were observed for total distance traveled [$F_{(2,61)}$=4.586, P=0.014], time inactive [$F_{(2,61)}$=4.920, P=0.010], time rearing [$F_{(2,61)}$=4.175, P=0.020], time grooming [$F_{(2,61)}$=5.470, P=0.007], number of corner entries [$F_{(2,61)}$=4.938,

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 treated prenatally with vehicle, melatonin, or 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*

Values are means ± SEM. Differences between groups (control-melatonin-luzindole) were determined using one-way ANOVA. Significant differences were further analyzed using multiple comparison tests and Bonferroni *post hoc* analysis. Values at sacrifice for male rats treated with vehicle, melatonin, or luzindole prenatally were compared. Percent body fat was found by summing masses of abdominal, gonadal, and retroperitoneal fat deposits in grams and dividing by total body mass in grams.*** P < 0.001, ** P < 0.01, * P < 0.05 means are significantly different from control.

Figure 7. The effects of prenatal melatonin or luzindole on adult male rat behavior in the forced swim test. Prenatal treatments were not found to have a significant effect on total distance traveled (a), fecal boli (b), immobile time (c), immobile episodes (d), and turn preference (e); however, turn preference was approaching significance [$F_{(2,58)}$=2.826, P=0.067] with rats exposed prenatally to melatonin treatment showing a trend toward increased preference for turning right compared to controls [P=0.066]. Data are expressed as means $\pm$ SE [Control n=16, Melatonin n=17, luzindole n=28]. Statistical differences were determined using one-way ANOVA for the effects of prenatal treatment followed by multiple comparison testing using Bonferroni *post hoc* analysis.

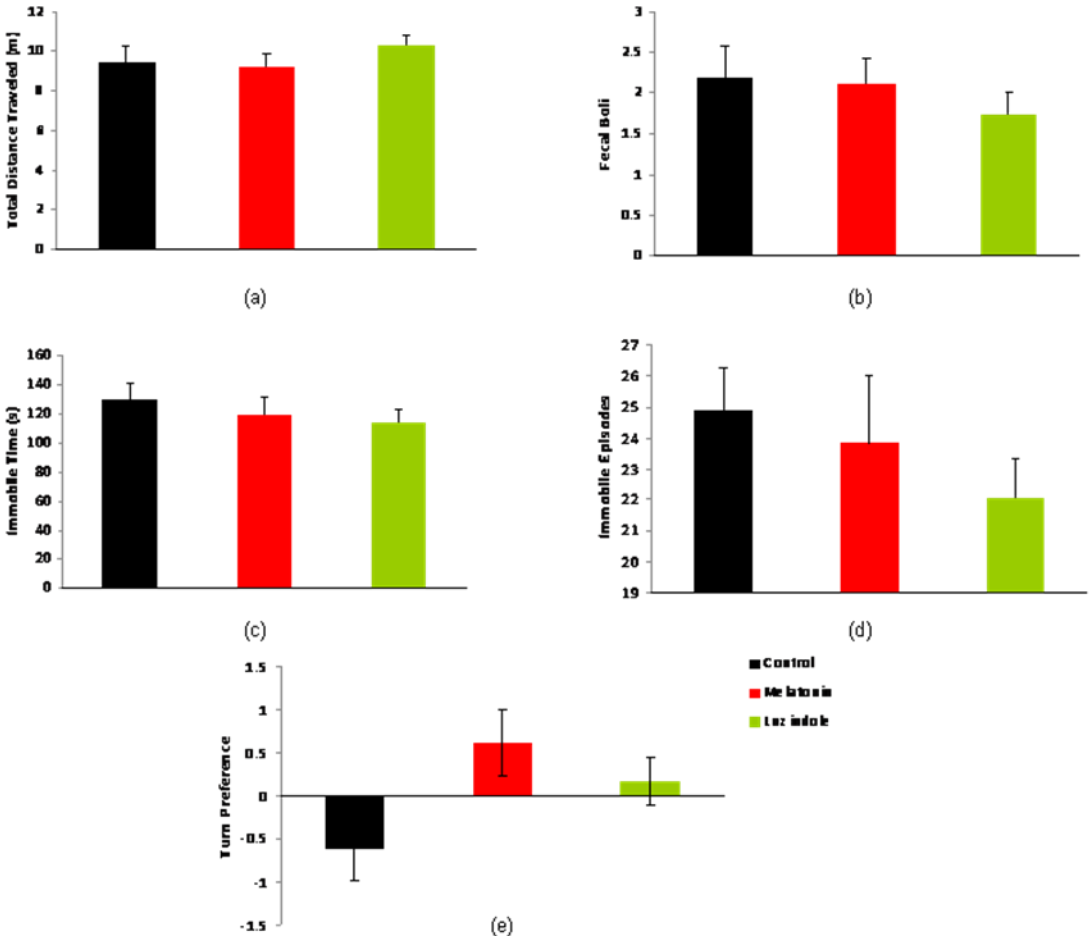
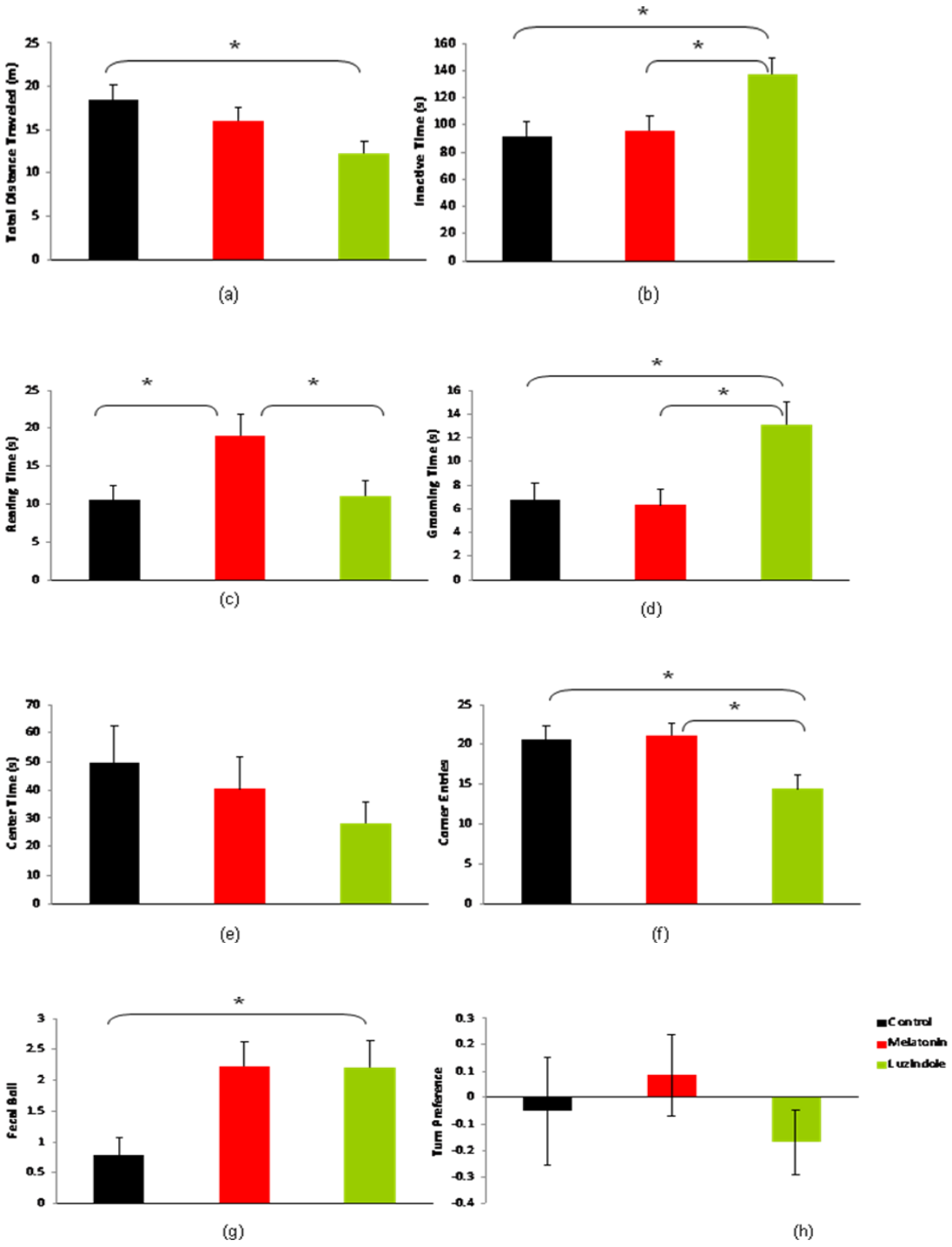

Total Distance Traveled [m]
12
10
8
6
4
2
0
(a)
Fecal Boli
3
2.5
2
1.5
1
0.5
0
(b)
Immobile Time (s)
160
140
120
100
80
60
40
20
0
(c)
Immobile Episodes
27
26
25
24
23
22
21
20
19
(d)
Turn Preference
1.5
1
0.5
0
-0.5
-1
-1.5
(e)
Control
Melatonin
Luzindole

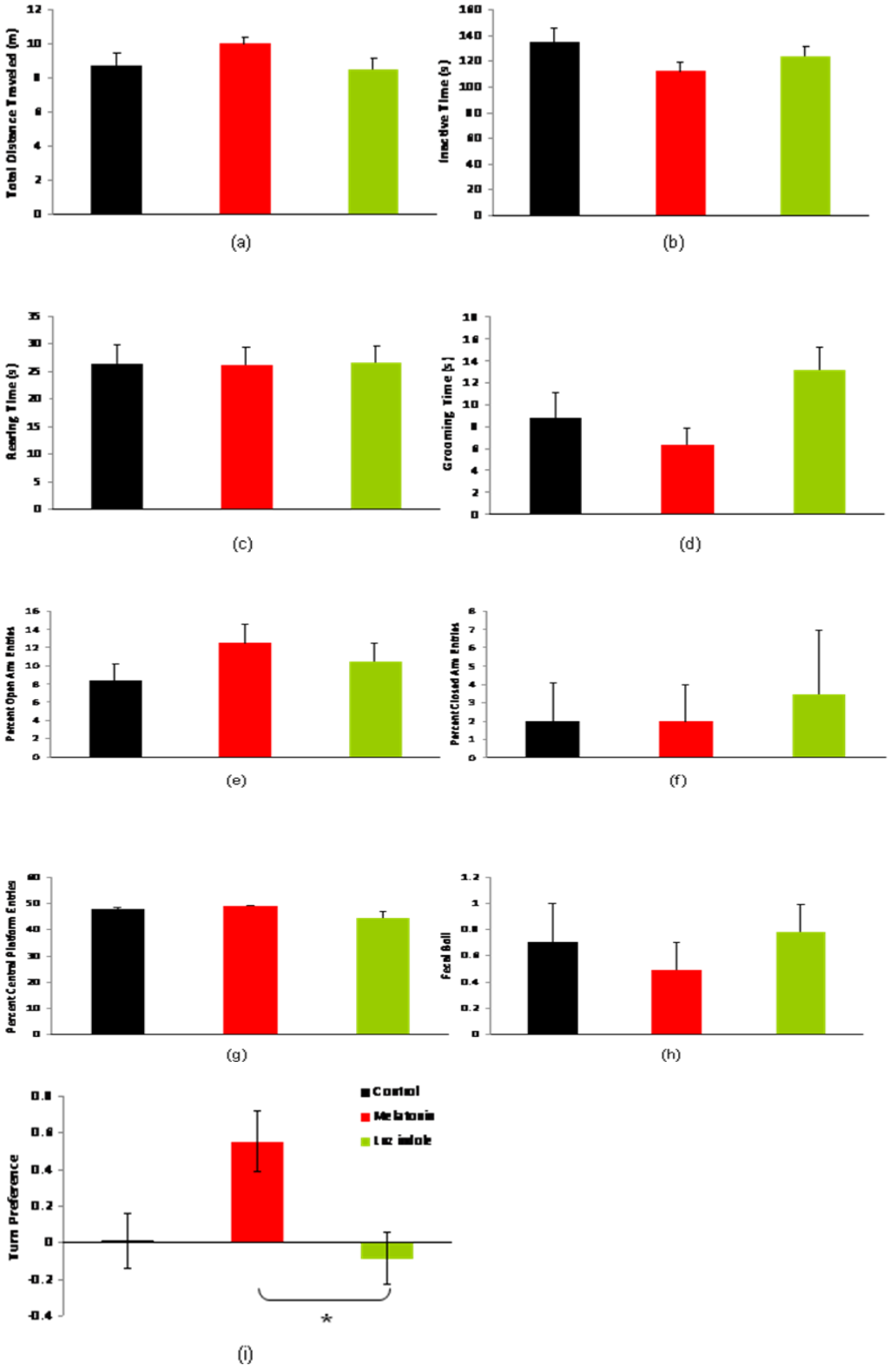
Figure 8. The effects of prenatal melatonin or luzindole on adult male rat behavior in the open field test. Prenatal treatment did not have a significant impact on time spent in center of arena (e) or turn preference (h). Prenatal treatment did significantly affect total distance traveled (a), inactive time (b), rearing time (c), number of corner entries (f), and fecal boli (g) [*P<0.05] in addition to grooming time (d) [**P<0.01]. Data are expressed as means ± SE [Control n=18, Melatonin n=18, luzindole n=28]. Statistical differences were determined using one-way ANOVA for the effects of prenatal treatment followed by multiple comparison testing using Bonferroni *post hoc* analysis.


Total Distance Traveled (m)
Inactive Time (s)
(a)
(b)
Rearing Time (s)
Grooming Time (s)
(c)
(d)
Center Time (s)
Corner Entries
(e)
(f)
Fecal Ball
Turn Preference
(g)
(h)
Control
Melatonin
Luzindole

P=0.010], and number of fecal boli produced [$F_{(2,61)}$=4.070, P=0.022]. In particular, rats exposed to luzindole traveled significantly less distance than controls [P=0.013] and produced significantly more fecal boli [P=0.033]. In addition, rats treated with luzindole prenatally spent significantly more time inactive [P=0.023 control, P=0.050 melatonin] and grooming [P=0.029 control, P=0.018 melatonin] and made significantly fewer entries to the corners [P=0.042 control, P=0.026 melatonin] compared to controls and melatonin-exposed animals. Moreover, animals subjected to melatonin prenatally exhibited rearing behavior for a significantly greater duration than control [P=0.045] and luzindole rodents [P=0.036].

In the elevated plus maze, adult male rat behavior was differentially altered by prenatal treatment (Figure 9). Prenatal treatments had no significant effects on total distance traveled [$F_{(2,60)}$=1.467, P=0.239], time inactive [$F_{(2,60)}$=1.625, P=0.205], time rearing [$F_{(2,60)}$=0.005, P=0.995], percentage of open arm entries number [$F_{(2,60)}$=0.0822, P=0.444], percentage of closed arm entries [$F_{(2,60)}$=1.407, P=0.253], percentage of central platform entries [$F_{(2,60)}$=2.000, P=0.144], and number of fecal boli [$F_{(2,60)}$=0.396, P=0.675]. However, rats exposed to prenatal melatonin showed a significantly stronger right turn preference [$F_{(2,60)}$=4.761, P=0.012] than luzindole rodents [P=0.012] with a trend toward an augmented right turn preference relative to controls [P=0.080]. In addition, a trend was observed for grooming time [$F_{(2,60)}$=3.101, P=0.052] such that luzindole-treated animals approached increased grooming time [P=0.058] in relation to melatonin-exposed subjects.

Figure 9. The effects of prenatal melatonin or luzindole on adult male rat behavior in the elevated plus maze. Prenatal treatment did not have a significant impact on total distance traveled (a), time spent inactive (b), time spent rearing (c), percentage open arm entries (e), percentage closed arm entries (f), percentage central platform entries (g) or number of fecal boli produced (h). Grooming time [$F_{(2,60)}$=3.101, P=0.052] was approaching a significant effect for prenatal treatment such that luzindole animals displayed a trend toward increased grooming time [P=0.058] relative to melatonin subjects. Rats exposed to melatonin prenatally displayed a significantly stronger right turn preference than luzindole rodents [*P<0.05] (i). Data are expressed as means $\pm$ SE [Control n=17, Melatonin n=18, luzindole n=28]. Statistical differences were determined using one-way ANOVA for the effects of prenatal treatment followed by multiple comparison testing using Bonferroni *post hoc* analysis.


Total Distance Traveled (m)
(a)
Inactive Time (s)
(b)
Rearing Time (s)
(c)
Grooming Time (s)
(d)
Percent Open Arm Entries
(e)
Percent Closed Arm Entries
(f)
Percent Central Platform Entries
(g)
Fecal Ball
(h)
Turn Preference
Control
Melatonin
Luz indole
*
(i)

Prenatal treatment also significantly changed performance in the learning phase of the Morris water maze but not in the memory phase (Figure 10). Overall nonlinear mixed effects modeling revealed a significant difference for the slope of the learning curve [$F_{(2,263)}$=12.52689, P<0.0001] but not for the intercept [$F_{(2,263)}$=2.07505, P=0.1276] as a result of prenatal treatment. T-test comparison by treatment groups with Bonferroni corrections revealed that rats administered luzindole prenatally had learning curves with slopes significantly reduced compared to controls [P=0.0015] and melatonin-treated animals [P<0.0003]. No significant differences were found for latency to platform [$F_{(2,61)}$=0.231, P=0.794], mean distance from platform [$F_{(2,61)}$=0.153, P=0.858], time spent in memory region [$F_{(2,61)}$=0.405, P=0.669], total distance traveled [$F_{(2,61)}$=0.775, P=0.465], or turn preference [$F_{(2,61)}$=0.857, P=0.429].

Effects of Altered Prenatal Melatonin Signaling on Hippocampal Gene Expression

Due to outliers and observable skew in the data sets, nonparametric Kruskal-Wallis tests were used to analyze gene expression data (Figure 11). Analysis of gene expression during two distinct time periods (0000 hr vs. 1200 hr) failed to produce a significant difference in circadian expression of the genes examined (data not shown), although MAP2 expression displayed circadian behavior closest to significance [P= 0.1547]. In contrast, Kruskal-Wallis analysis revealed a significant effect of prenatal treatment on expression of BDNF [P=0.043] and MAP2 [P=0.048]. Group-by-group Wilcoxon comparisons with Bonferroni corrections found a significant increase in expression of BDNF in melatonin rats compared to controls [P=0.0425], but the increase

Figure 10. The effects of prenatal melatonin or luzindole on adult male rat behavior in the Morris water maze. Linear mixed effects modeling showed that prenatal treatment produced a decrease in slope of the learning curve for luzindole-treated animals relative to controls [**P<0.01] and melatonin-treated animals [***P<0.001] but had no significant impact on intercept of the learning curve (a). No significant effects of prenatal treatment were observed in the memory phase of the test (b-f). Data are expressed as means $\pm$ SE [Control n=18, Melatonin n=18, luzindole n=28]. Statistical differences for the learning phase were determined by nonlinear mixed effects modeling. Statistical differences for the memory phase were determined using one-way ANOVA for the effects of prenatal treatment followed by multiple comparison testing using Bonferroni *post hoc* analysis.

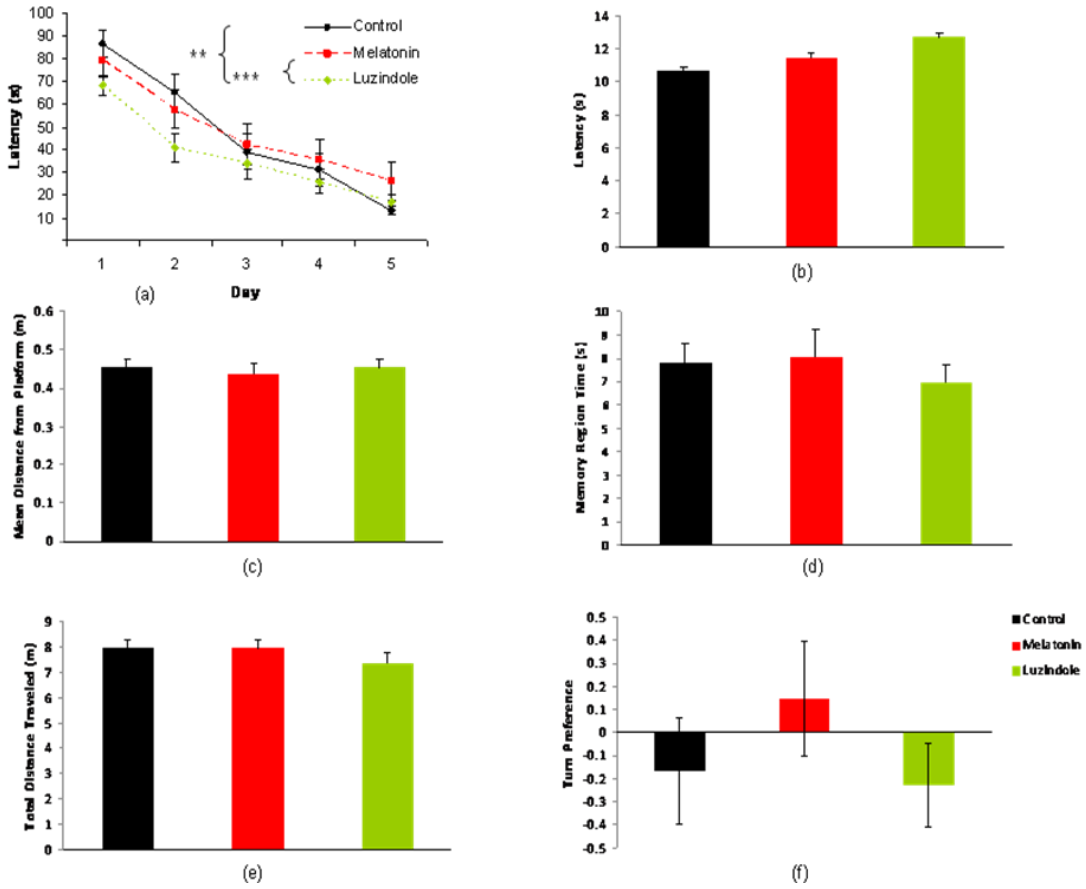
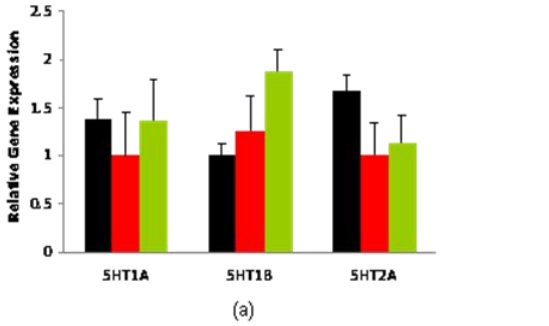
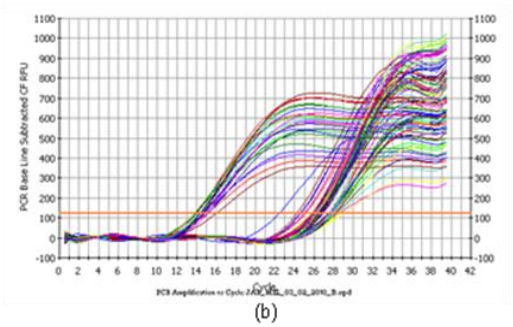

Latency (s)
Day
Control
Melatonin
Luzindole
**
***
(a)
Latency (s)
(b)
Mean Distance from Platform (m)
(c)
Memory Region Time [s]
(d)
Total Distance Traveled (m)
(e)
Turn Preference
Control
Melatonin
Luzindole
(f)

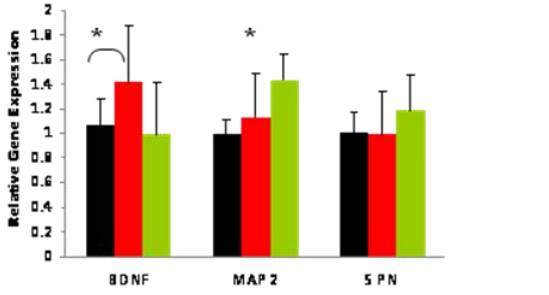
Figure 11. The effects of prenatal melatonin or luzindole on adult male rat hippocampal gene expression. Relative cycle number to fluorescence for hippocampal cDNA during real-time PCR with primers for 5HT1A, 5HT1B, 5HT2A (b); BDNF, MAP2, spinophilin (SPN) (d); and growth hormone receptor (GHR), MEL1A, MEL1B (f); with 18s is indicated in representative kinetics curves. Relative gene expression for all genes assayed is also indicated (a,c,e). Prenatal treatment had a significant effect on expression of BDNF and MAP2 [*$P<0.05$] and produced a trend toward significance for MEL1A expression [$P=0.078$]. In particular, a significant increase in expression of BDNF in rats treated prenatally with melatonin relative to controls [*$P<0.05$] was found. Data are expressed as means $\pm$ SE [Control n=18, Melatonin n=18, luzindole n=28]. Statistical differences were determined using Kruskal-Wallis for the effects of prenatal treatment followed by group-by-group Wilcoxon comparisons with Bonferonni corrections.


Relative Gene Expression
5HT1A
5HT1B
5HT2A

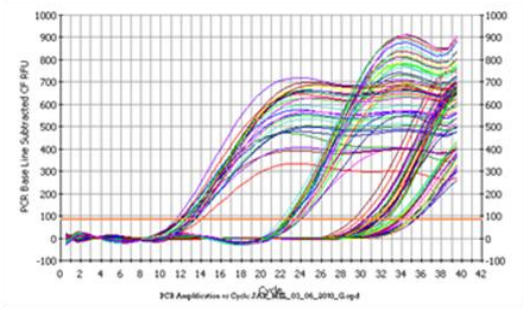
(a)



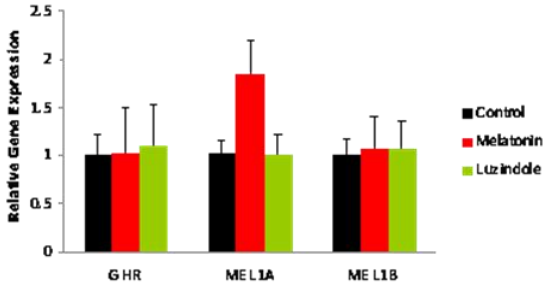
(b)


Relative Gene Expression
*
*
BDNF
MAP 2
SYN

(c)

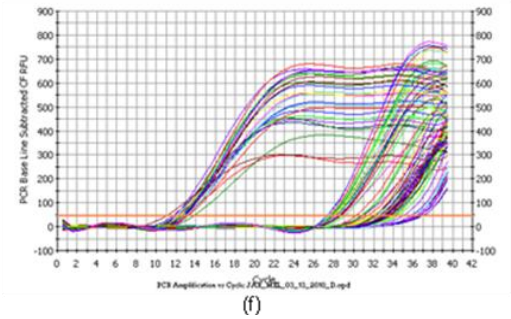


(d)


Relative Gene Expression
GHR
MEL1A
MEL1B

(e)

Control
Melatonin
Luzindole



(f)

in expression of BDNF in melatonin animals compared to luzindole animals was not significant [P=0.1317]. In addition, group-by-group comparison revealed a trend toward increased expression of MAP2 in luzindole rats compared to controls [P=0.0526]. Finally, a trend for a treatment effect on MEL1A expression [P=0.078] was also observed.

DISCUSSION

Our study was designed to test the effects of altered prenatal melatonin signaling on growth patterns, behavior, and hippocampal gene expression in adult male rats. The limited existing research on the postnatal effects of altered prenatal melatonin signaling precludes a definitive conclusion; however, data from a series of diverse studies on the functions of melatonin in general provide a context for our findings.

Melatonin and Body Growth

As predicted, prenatal melatonin had a significant impact on growth patterns of offspring. An upward shift in growth curve and accelerated growth was observed for melatonin-treated animals compared to luzindole-treated animals and controls. These alterations were not accompanied by a change in body fat at sacrifice which suggests that this difference was not the result of increased fat deposition. Growth hormone has been shown to increase muscle and skeleton mass and to reduce fat mass in female pigs (Sørensen et al. 1996), and growth hormone administration to 19- to 21-month-old

Sprague-Dawley rats induced greater protein synthesis (Sonntag et al. 1985). Thus, it is possible that the growth changes measured in our animals may be due to alterations in growth hormone secretion.

The direction of the change in growth patterns, however, is difficult to interpret given the contradictory findings in the literature. Administration of melatonin (4 μg/mL) in drinking water for 12 weeks to adult Sprague-Dawley rats produced sex-dependent metabolic effects (Bojková et al. 2008). In particular, in adult males, exposure to excess melatonin reduced body weight, liver glycogen, and serum insulin and increased concentrations of corticosterone, serum and heart muscle cholesterol, heart muscle phospholipids, and indicators of oxidative stress. Interestingly, the increase in growth hormone secretion elicited by administration of serotonin (10 mg/kg) to 80-day-old Wistar rats was blocked by concomitant administration of melatonin (80 mg/kg) (Smythe et al. 1973). Similar antagonistic effects for both melatonin and serotonin on growth hormone secretion were also observed in 21-28-year-old human male subjects (Smythe et al. 1974 b). Thus, it appears that melatonin may mediate reduction in growth hormone secretion and body mass in adult rats.

However, melatonin's effect on growth may be different when administered during prenatal development or lactation. Injection of meadow vole *Microtus pennsylvanicus* dams with 10 μg melatonin per animal daily during pregnancy reduced offspring weight in both sexes and delayed sexual development only in male offspring (Lee et al. 1989). In contrast, melatonin treatment during lactation increased body growth from postnatal day 21 until sacrifice at postnatal day 49 for males and females. In

the male rat, pineal and serum melatonin levels at the middle of the dark phase of the circadian cycle were found to rise during postnatal days 1 to 9, remain high during postnatal days 11 to 17, and lower after day postnatal day 21 (Tang et al. 1988). Moreover, the drop in melatonin levels after postnatal day 21 was found to be independent of weaning. Although we altered only prenatal melatonin levels in our experimental animals, we had hypothesized that treatments would permanently alter function of the circadian circuit causing increased endogenous melatonin production postnatally in melatonin-treated animals and reduced melatonin secretion postnatally in luzindole treated animals. The observed augmentation of growth in our melatonin-treated rats may not be consistent with immediate effects of prenatal treatment on growth patterns of offspring but may be consistent with augmented postnatal function of the circadian circuit. In particular, our data may suggest increased melatonin levels in offspring during the period of lactation as a result of prenatal melatonin treatment.

Melatonin and Behavior

As predicted, prenatal treatments in our study produced differential behaviors in adult offspring. These differences are not fully consistent with existing literature since increases or decreases in melatonin levels in adult rodents produce differential behaviors based on experimental design. For example, depression of locomotor activity in adult male golden hamsters was observed after intraperitoneal administration of at least 30 μg melatonin/kg for 5 days (Golombeck et al. 1991). This effect on behavior was found to be lowest at midnight and could be blunted by administration of a benzodiazepine. A

reduction in locomotor activity, rears, and entries into the unfamiliar zone of the free exploratory test was also observed in adult C3H/He mice (Kopp et al. 1999). However, this change in behavior was measured in animals treated with 10 mg melatonin/kg per day at the beginning of the dark phase during and immediately after a 3-week stress period and then exposed to a chronic mild stress procedure.

Adult melatonin treatment has also been shown to elicit differential behavioral patterns by sex. Administration of melatonin (4 μg/mL) in drinking water for two weeks was associated with an increase in climbing in the forced swim test in thirteen-month-old Long Evans male rats (Brotto et al. 2000). However, both treated and control females displayed augmented activity in both the forced swim test and open field test compared to males. Among the females studied, melatonin-exposed animals exhibited increased immobility on the first day of the forced swim test. This study also showed that melatonin exposure was associated with increased ambulation in the open field test and reduced swimming in the forced swim test in both sexes. No significant change in behavior in the forced swim test was observed for either treatment in our study compared to the significant changes observed by Brotto et al. Moreover, the significant change in behavior of adult male animals exposed prenatally to melatonin and tested in the open field test in our study is different from the changes in response to adult melatonin treatment in the Golombek et al., Kopp et al., and Brotto et al. studies. More specifically, our melatonin-exposed animals displayed augmented rearing compared to animals treated with luzindole *in utero* or controls but not increased distance traveled relative to controls. Given the increased ambulation but not rearing in the open field test of animals treated

with melatonin in adulthood in the Brotto et al. study and the reduction in locomotor activity by Golombek et al. and Kopp et al., we suggest that prenatal and postnatal melatonin changes may elicit differential behavioral effects in adult animals.

Melatonin not only produces differential effects by sex but also by the receptor subtype via which it elicits signaling. MEL1A receptor knockout mice of both sexes displayed impaired sensorimotor gating as evidenced by the prepulse inhibition test (Weil et al. 2005). These mice also showed decreased preference for the center of the arena in the open field test. In addition, they spent more time immobile in the forced swim test. Female wild-type mice showed greater locomotor activity than males in the open field test, but this difference between sexes did not exist in knockout mice. In another study, 4- to 5-week-old C3H/HeN mice administered melatonin (30 mg/kg) did not show a significant change in behavior in the forced swim test while luzindole (30 mg/kg) reduced immobility time in the forced swim test with a greater effect at midnight than at noon (Dubocovich et al. 1990). Moreover, administration of 30 mg/kg melatonin with 10 mg/kg luzindole blunted the effects of luzindole on behavior. In subsequent study using MEL1A-knockout and MEL1B-knockout C3H/HeN mice, it was found that this effect of luzindole on forced swim test behavior was mediated via the MEL1B receptor (Sumaya et al. 2005). Since no effects of treatment were observed in the forced swim test for our subjects, our data suggest that activity of MEL1B receptors are not significantly affected by treatment. In contrast, the augmented fear response of luzindole animals in the open field test as indicated by increased time inactive suggest a possible role for MEL1A receptor dysregulation in these animals. Interestingly, rats exposed prenatally to

luzindole in our study outperformed both melatonin-treated and control animals in the learning phase of the forced swim test by finding the platform more quickly. Since MEL1B receptor knockout mice displayed impaired spatial learning in the Morris water maze (Larson et al. 2006), our data from luzindole-exposed offspring in the learning phase of the forced swim test may also preclude involvement of MEL1B receptors in the behavioral differences observed in our subjects.

In addition to postnatal changes in melatonin signaling via administration of melatonin agonists or antagonists in adulthood and lifelong changes in melatonin signaling by receptor knockout, prenatal alterations of melatonin signaling have also been found to impact adult behavior. Maternal pinealectomy of Siberian hamsters produced increased immobility in the forced swim test and rearing behavior in the open field test of adult male offspring (Workman et al. 2008). Moreover, this study found that postnatal pinealectomy of adult males augmented locomotor activity in the open field test. Our results, however, are inconsistent with this study. No significant differences in forced swim test behavior in our prenatally animals were observed compared to the increased learned helplessness in response to reduced prenatal melatonin signaling in the Workman et al. study. In addition, melatonin-treated animals in our study displayed increased rearing and locomotor activity in the open field test relative to luzindole-treated animals. Luzindole treatment would be expected to have reduced effectiveness of melatonin signaling during early development of our rats. Since the subjects in the Workman et al. study showed increased locomotor activity in response to reduced melatonin signaling following postnatal pinealectomy, it is possible that prenatal and postnatal effects of

melatonin on signaling are significantly different. Moreover, the use of pinealectomy or removal of endogenous melatonin in their study versus exogenous hormone or hormone antagonist treatment in ours may have produced differential effects. Species differences may also be important in this case.

Additional studies designed to alter the intrauterine environment included melatonin administration to spontaneously hypertensive rat (SHR) dams. SHR animals are considered a model of attention deficit hyperactivity disorder (Sagvolden 2000) which is associated with heightened locomotor activity. SHR dams given water containing melatonin (20 μg/mL) during gestation and lactation were found to have offspring that displayed reduced locomotor activity and rearing behavior as compared to control SHR offspring (Kim et al. 2002). Our findings differ from the Kim et al. study in that prenatal melatonin treatment reduced locomotor activity in the forced swim test in their animals while our animals exposed to melatonin in utero showed no significant difference from controls. In addition, their animals also displayed reduced locomotor activity in the open field test in response to prenatal treatment with melatonin while our melatonin animals showed increased locomotor activity. However, since they used a strain of rats considered to be a model of a psychiatric disorder, it is possible that their animals responded differently to melatonin treatment than wild type rats.

Animals treated prenatally with luzindole in our study displayed significantly greater grooming than melatonin-treated animals and controls. Previous studies suggest that increased grooming behavior may be a result of increased stress response in these animals. For example, administration of the corticotrophin releasing factor 1 (CRF1)

receptor agonist MJL-1-109-2 (1 nM) to adult male Wistar rats produced increased freezing, grooming, and mounting behaviors (Zhao et al. 2007). Administration of CRF1 to adult male Wistar rats also induced an increase in hippocampal free corticosterone levels and a reduction in serotonin (de Groote et al. 2005). Further data collection will include radioimmunoassay analysis of serum for basal corticosterone levels in our animals.

In contrast to our luzindole-exposed adult offspring, animals treated prenatally with melatonin in our study displayed significantly increased rearing behavior. A first review of evidence for a role of the septo-hippocampal system in rearing behavior concluded, however, that the role of the hippocampus in exploratory rearing behavior is still poorly understood (Lever et al. 2006). The authors cite various lines of evidence implicating the involvement of projections from the dentate gyrus to the CA3 region of the hippocampus and also importance of the CA1 region in rearing behavior in reference to spatial learning, but admit that the specific functions of these regions are highly context dependent. Thus, the increased rearing behavior of animals exposed to melatonin prenatally in our study suggests a potential role for prenatal melatonin in adult hippocampal function.

Interestingly, in our melatonin-exposed offspring, a significant right turn preference was observed in the elevated plus maze with a trend toward significance in the forced swim test. A stronger right-turn preference was also found in adult Long-Evans hooded rats in response to neonatal novelty (Tang et al. 2003). Moreover, these animals showed increased social memory and lower basal plasma corticosterone levels compared

to controls after neonatal novelty exposure. Abnormal brain lateralization has also been associated with autism (Escalante-Mead et al. 2003) and schizophrenia (Sommer et al. 2001) in humans. In our study, measurements of left hippocampal mass compared to right hippocampal mass suggest a slight increase in animals treated prenatally with melatonin; however, the method of measurement may have been too imprecise to detect significant mass differences. In addition, other studies suggest that volume measurements are preferred to those of mass. Thus, additional procedures, such as staining and sectioning of brain tissue, may be required to observe possible lateralization differences in additional cohorts of rats reserved for this purpose. Given the potential importance of brain lateralization in adult psychopathology, further research to confirm a role for melatonin in brain lateralization is warranted.

Melatonin and Gene Expression

Our molecular data suggest a connection between prenatal melatonin signaling and adult hippocampal gene expression. Moreover, evidence in the literature also supports a significant molecular impact of maternal melatonin levels on offspring. For example, pups of pinealectomized Wistar rat dams displayed reduced expression of melatonin receptors in their brains at postnatal day 9 (Zitouni et al. 1995). MEL1A receptor concentrations were reduced and clock gene expression oscillations were altered in the SCN of capuchin monkey fetuses of pinealectomized mothers, and the effect of pinealectomy on these variables could be ameliorated by melatonin administration (Torres-Farfan et al. 2006).

In our study, augmentation of prenatal melatonin signaling significantly increased adult hippocampal BDNF expression in adult offspring. Recent studies from other *in vivo* models have also found links between melatonin receptor signaling and BDNF expression. Cerebellar granule cells from 5-day old MEL1B knockout CH3 mice showed increased expression of BDNF in response to melatonin administration *in vivo* and *in vitro* compared to those from wild-type and MEL1A-knockout mice (Imbesi et al. 2008 a). In addition, nanomolar concentrations of the melatonin receptor agonist, ramelteon, increased expression of BDNF protein without increasing mRNA levels in cerebellar granule cells from all MEL1A-knockout, MEL1B-knockout, and control mice (Imbesi et al. 2008 b).

Results from in vitro cell culture studies suggest that melatonin regulates the expression of its own receptors. For example, in CHO cell lines expressing high levels of MEL1A receptors, a significant reduction in these receptors at the cell surface was detected 1 hour after treatment with melatonin, but total cellular concentration of the MEL1A receptor was unaffected (Kokkol et al. 2007). In contrast, this same study found that CHO cell lines expressing high levels of melatonin MEL1A receptors decreased total cellular MEL1A receptor concentrations in response to administration of luzindole at both 1 and 72 hours after treatment, and expression at the cell surface was increased at 72 hour after treatment. Additional studies have shown that MEL1A receptors are also expressed in the immortalized C17.2 dopaminergic neural stem cell line, and administration of melatonin to these cells increased release of glial-cell derived neurotrophic factor (Niles et al. 2004). A later study showed that melatonin treatment of

these cells—which do not express MEL1B receptors—promoted neurite outgrowth, increased expression of the neural stem cell marker nestin, histone deacetylases HDAC 3, HDAC 5, and HDAC 7, and augmented histone H3 acetylation (Sharma et al. 2008). Administration of valproic acid, a mood stabilizer, to rat C6 glioma cells for 24 hours increased expression of MEL1A receptor mRNA and protein in a dose-dependent manner and this increase in MEL1A receptor was associated with an increase in BDNF, GDNF, and histone deacetylase (HDAC) mRNA levels (Castro 2005). In addition to influencing the expression of neurotrophic factors, melatonin been found to inhibit apoptosis at the mitochondrial level via MEL1A and MEL1B receptor signaling in U937 cells (Radogna et al. 2008). Melatonin supplementation has also been shown to affect hippocampal cells directly. For example, melatonin promoted survival of neural precursor cells from the hippocampi of adult female C57BL/6 mice, and this effect could be diminished by administration of luzindole (Ramírez-Rodríguez et al. 2009).

In addition to these cell culture studies, *in vivo* evidence suggests a role for melatonin in hippocampal cell maintenance. Ramírez-Rodríguez et al. (2009) found that administration of 8 mg/kg melatonin intraperitoneally to adult mice promoted survival of neural precursor cells, but maturation and proliferation of these cells was unaffected. The hippocampi of rats treated prenatally with melatonin in our study approached significance for increased MEL1A expression relative to controls and luzindole-treated animals while simultaneously displaying a significant increase in BDNF expression. Thus, our data suggest an *in vivo* connection between MEL1A receptor signaling and BDNF expression in the hippocampus and support a role for melatonin in hippocampal cell maintenance.

Melatonin has been found to have various antioxidative properties that are independent of its receptor-mediated effects (for reviews see Hardeland 2005; Ortiz et al. 2008). Pretreatment with 2 mg/kg melatonin subcutaneously was found to counteract oxidative damage by methamphetamine in the neonatal Wistar rat prefrontal cortex, nucleus accumbens, and dorsal striatum (Kaewsuk et al. 2009). These antioxidant properties also extend to melatonergic drugs. Melatonin and related compounds have been found to protect rat synaptosomal membranes from oxidative damage (Millán-Plano et al. 2010). In the ABTS assay, both melatonin and luzindole at the concentration of 10 μM showed greater antioxidant capability compared to a similar concentration of ascorbic acid (Mathes et al. 2008). Luzindole at a dose of 800 μM prevented lipid peroxidation induced by iron and LPS in F344N rat brain tissue homogenates (Requintina et al. 2007).

The antioxidant properties of both melatonin and luzindole, however, suggest a possible complication with our design. Given that the DMSO vehicle used has been found to produce apoptosis of cells in the developing mouse brain at 0.3 mL/kg, the lowest dose examined (Hanslick et al. 2009), it is possible that any deficits produced by the vehicle can be ameliorated by either melatonin or luzindole. In our study, we used DMSO at a concentration of 0.1 mL/kg per day. Although this dose is less than that used by Hanslick et al. (2009), use of DMSO as a vehicle allows for the possibility that some predicted deficits in either treatment group relative to controls may be hidden by the antioxidant properties of the treatment.

Prenatal treatment in our study also altered mRNA expression for MAP2. Previous studies suggest a role for MAP2 in the structure and function of hippocampal

neurons. For example, hippocampal cells from MAP2 knockout mice cultured for 3 weeks displayed reduced dendritic length compared to cells from wild-type animals (Harada et al. 2002). MAP2 expression in the hippocampus has been found to change in response to learning tasks. In particular, MAP2 was down-regulated in the hippocampus of adult male Wistar rats after exposure to the Morris water maze relative to controls exposed to swimming or no testing 1 or 24 hours before sacrifice (Cavallaro et al. 2002). Conversely, MAP2 protein and mRNA expression in the hippocampus was positively correlated with learning and memory performance in the Morris water maze in male Wistar rats exposed to cerebral hypoperfusion for 20 weeks (Liu et al. 2005). Our data indicate that rats treated prenatally with luzindole displayed the greatest relative expression of MAP2 mRNA and augmented performance in the learning phase of the Morris water maze. Since the MAP2 protein is considered a marker of dendritic density, a link between this protein and learning is logical. Given that the direction of change in MAP2 expression is the same in both melatonin and luzindole-treatment groups, it is possible that some of the treatment effects may be due to the antioxidant properties of the drugs. It is also possible that hippocampal cell plasticity is increased in both groups for different reasons. Examination of relative mRNA levels for additional genes—including the marker of hippocampal neurogenesis, *doublecortin* (DCX) (Brown et al. 2003)—would help to further explain these learning differences and is underway in our lab. It is worth noting, however, that increased expression of MAP2 has been found in the hippocampus of individuals with schizophrenia (Cotter et al. 2000) and thus can be a marker of psychopathology.

Although indications of an increase in anxiety-related behavior such as increased inactive time and grooming in the open field test were identified in animals exposed to luzindole prenatally, it is surprising that none of the 5HT receptor genes showed differential expression in response to treatment. It is possible that the expression of these serotonin receptors is altered only after a stressful challenge, and this possibility will be studied in a future cohort of our prenatally exposed animals. Future research will also entail radioimmunoassay of serum melatonin, growth hormone, and cortisol levels to examine the effects of prenatal treatment on adult hormone secretion. Moreover, despite a significant effect of prenatal treatment on MAP2 expression, no change in expression of SPN was observed. Thus, our present data suggest that the prenatal treatments had an impact on overall dendrite structure but not the fine structure of dendritic spines given the localization of spinophilin to dendritic spines but MAP2 to entire dendrites. Also, despite the effects of melatonin on growth curve shape, no significant differences in GHR expression were observed.

Given that we have only extracted mRNA from the left hippocampi of our subjects, we cannot make any conclusions regarding lateralization at the molecular level at this time. In other studies, however, the hippocampus of rats showed differential norepinephrine, serotonin, and choline uptake on the side opposite the direction of turn preference in the T maze (Valdes et al. 1981). Comparisons of gene expression between right and left hippocampi will be conducted.

Model Incorporating the Circadian Circuit in Development

This study was based on four related hypotheses, although additional research beyond this study will be necessary to validate all of them. First, maternal melatonin secretion helps drive the growth and maintenance of neurons of the fetus. Some of this effect results from cell signaling via cell membrane receptors that specifically bind melatonin. Blockade of these receptors or increased binding of ligand to these receptors results in greater or lesser growth of neurons. This altered neuron growth affects the construction of the meshwork of circuits in the developing fetal brain. In addition to this immediate effect on early brain development, changes to the fetal circuit meshwork impact future brain growth by providing an abnormal scaffold for later cell migration, growth, and connectivity.

Second, since melatonin receptors are found in high concentrations in the fetal hypothalamus, early growth of the SCN is more markedly affected by prenatal melatonin concentrations compared to other brain regions. Given that the SCN functions as the circadian pacemaker, altering the development of this brain region permanently affects the function of the circadian circuit of an organism. These permanent changes in the circadian circuit result in physiological and behavioral changes in adult organisms

Third, since the hippocampus develops rapidly during a specific period in the third trimester of pregnancy while other brain regions develop more gradually, this brain region shows larger changes in response to altered prenatal melatonin levels in late gestation than other brain regions. Because this brain region is particularly plastic throughout development and adulthood, improper function of the circadian circuit

resulting in aberrant melatonin secretion compounds existing developmental aberrations by influencing further postnatal growth, maintenance, and plasticity. As a result this summation of prenatal and postnatal effects of prenatal melatonin on hippocampal development, initial pathology of this brain region becomes more pronounced as the brain develops through puberty and early adulthood.

Fourth, since circadian melatonin secretion regulates the release of other hormones, permanently altering the circadian circuit causes various changes in circadian physiology of neuroendocrine circuits. In particular, melatonin influences growth hormone release. Since growth hormone is important in various aspects of growth, metabolism, and brain function, changes in circadian growth hormone signaling may cause subtle aberrations in these processes. In addition, because the circadian circuit and the hippocampus also interact with the HPA axis, changes in these brain regions in response to alterations of prenatal melatonin signaling will permanently alter HPA-axis function.

Although these hypotheses are broad, they provide a framework for a model involving the circadian circuit in brain development. Given the numerous interactions of the circadian circuit and the HPA axis of mammals and the importance of variation in function of both circuits over a 24-hour period, we designate the term circadioneuroendocrine axis (CNE) axis. The CNE axis includes structures of both the circadian circuit and HPA axis in addition to hormones released by the pituitary, pineal, and other neuroendocrine glands which directly impact brain function and organismal behavior in response to environmental stimuli. Use of this term stresses the necessity of

investigating these circuits as a function of time. Such a framework is required for integration of the litany of varied and often contradictory research already conducted in the field of neuroscience. Moreover, future research will need to be conducted precisely within such a framework to connect it to existing literature if a holistic understanding of brain development and function is to be obtained.

From this terminology follows a name for our hypothesis regarding the role of the CNE axis in brain development and psychopathology: the CNE-axis hypothesis of psychopathology. According to this hypothesis, the magnitude, direction, timing, and duration of disturbances of the CNE axis during development determine the resulting psychiatric disorder. This hypothesis does not preclude genetic causation but does provide for the incorporation of not only environmental stimuli but also the timing of those stimuli relative to endogenous rhythms. Future research will be necessary, however, to discover potential new and integrate existing known critical periods and critical disturbances capable of producing outlying developmental trajectories.

PERSPECTIVES AND SIGNFICANCE

Although our study does not provide definitive support for any of the broad hypotheses of our proposed model, our data do support the hypothesis that melatonin signaling *in utero* disturbs neuroendocrine setpoints to produce differential growth, behavior, and gene expression in a region of the brain in the male rat that is important in human psychopathology. Because nocturnal rats (Mandera et al. 2003) secrete melatonin in profiles that are comparable to those of diurnal organisms, signaling via melatonin

receptors in rats is likely to produce different cellular responses compared to those found in diurnal mammals. Comparative studies show that while melatonin exerts certain differential effects between nocturnal and diurnal organisms, some of its actions may be similar across species (López-Olmeda et al. 2006). Studies of melatonin functions in multiple organisms allow for possible mechanisms in other species to be elucidated and for the formulation of further research questions. Moreover, as the body of literature regarding the developmental impacts of melatonin is built, current theoretical frameworks of the circadian circuit and its effectors can be further amended to incorporate interspecies variations.

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