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GENETIC DELETION OF MT_1/MT_2 MELATONIN RECEPTORS ENHANCES MURINE COGNITIVE AND MOTOR PERFORMANCE

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Abstract—Melatonin, an indoleamine hormone secreted into circulation at night primarily by the brain's pineal gland, has been shown to have a wide variety of actions on the development and physiology of neurons in the CNS. Acting via two G-protein-coupled membrane receptors (MT_1 and MT_2), melatonin modulates neurogenesis, synaptic functions, neuronal cytoskeleton and gene expression. In the present studies, we sought to characterize the behavior and neuronal biology of transgenic mice lacking both of these melatonin receptors as a way to understand the hormone's receptor versus non-receptor-mediated actions in CNS-dependent activities, such as learning and memory, anxiety, general motor performance and circadian rhythmicity. Assessment of these behaviors was complemented by molecular analyses of gene expression in the brain. Our results demonstrate mild behavioral hyperactivity and a lengthened circadian period of free-running motor activity in melatonin receptor-deficient mice as compared to receptor-intact control mice beginning at an early age. Significant improvement in cognitive performance was found using the Barnes Maze and the Y-Maze. No behavioral changes in anxiety levels were found. Electrophysiological measures in hippocampal slices revealed a clear enhancement of long-term potentiation in mice lacking melatonin receptors with no significant differences in paired-pulse facilitation. Quantitative analysis of brain protein expression levels of phosphoCREB and phosphoERK1/2 and key markers of synaptic activity (synapsin, glutamate receptor 1, spinophilin, and glutamic acid decarboxylase 1) revealed significant differences between the double-knockout and wild-type animals, consistent with the behavioral findings. Thus, genetic deletion of melatonin receptors produces mice with enhanced cognitive and motor performance, supporting the view that these receptors play an important role in neurobehavioral development.

Key words: melatonin, learning, memory, activity, LTP, mice.

INTRODUCTION

Knockout mice lacking both the MT_1 and the MT_2 (encoded by the MTNR1a and MTNR1b genes respectively) melatonin receptors have been used in a variety of studies to examine the mechanisms of melatonin action in specific disorders and conditions, including focal cerebral ischemia (Kilic et al., 2012), methamphetamine-induced locomotor sensitization (Hutchinson et al., 2012), sleep–wake characterization (Comai et al., 2013), nicotine sensitivity (Mexal et al., 2012), and blood glucose regulation (Mühlbauer et al., 2009); however, no comprehensive characterization of baseline differences between these transgenic animals and their wild-type counterparts has been published. Similarly, aspects of neurodevelopment have not been addressed before, despite the fact that these animals' brains develop not only without the receptor-mediated influence of melatonin, but they also lack the impact of the normally constituently active MT_1 receptor (Dubocovich et al., 2010).

The genetic absence of specific hormone receptors can result in a variety of neurobiological disorders, e.g., oxytocin receptors and social behavior (cf. Donaldson and Young, 2008), vasoactive intestinal peptide receptors and circadian behavior (Harmar et al., 2002), androgen receptors and reproductive systems (Chang et al., 2013). Functionally significant polymorphisms in the coding sequences of both the human MT_1 and the human MT_2 melatonin receptors have been identified and found to be associated with some diseases and disorders (Li et al., 2013; Comai and Gobbi, 2014). A few of the

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Abbreviations: aCSF, artificial cerebrospinal fluid; ADHD, attention deficit/hyperactivity disorder; ANOVA, analysis of variance; C3B6, mice with a combination C3H/He and C57BL/6 background; cDNA, complementary deoxyribonucleic acid; CAT, catalase; CREB, cAMP response element-binding protein; DBL-KO, double-knockout; DD, dark–dark or constant dark; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; EPM, elevated plus maze; EPSP, excitatory post-synaptic potential; ERK, extracellular signal-regulated kinases; GAD1, glutamic acid decarboxylase 1; GluR1, glutamate receptor subunit 1; GPx-1, glutathione peroxidase 1; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; HPC, hippocampus; LD, light–dark; LTP, long-term potentiation; MAPKs, mitogen-activated protein kinases; mRNA, messenger ribonucleic acid; MTNR, melatonin receptor; MT_1, melatonin receptor type 1; MT_2, melatonin receptor type 2; MTNR1a, gene encoding the MT_1 receptor; MTNR1b, gene encoding the MT_2 receptor; NIR, near-infrared; Nrf2, nuclear factor erythroid 2-related factor 2; NT, non-transgenic; PGK, phosphoglycerate kinase promoter; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; SCN, suprachiasmatic nuclei; SOD1, superoxide dismutase 1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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reported receptor polymorphism associated diseases include, but are not limited to, breast cancer (MT_1 and MT_2; Deming et al., 2012), type 2 diabetes mellitus (MT_2; Bonnefond et al., 2012) and multiple sclerosis (MT_2; Natarajan et al., 2012). As little is known about the consequences of complete melatonin membrane receptor deletion on brain function and behavior, our primary objective was to identify any behavioral, neurobiological or molecular differences in melatonin receptor-deficient mice as compared to mice expressing both receptors. Our findings point to a significant role for melatonin receptor signaling in brain development and in the regulation of behavioral activity through actions on major intracellular signal transduction pathways and on both excitatory and inhibitory synaptic communication.

EXPERIMENTAL PROCEDURES

Ethics statement

All mice were housed and handled in accordance with Federal animal welfare guidelines and in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (2002) and the Guide for the Care and Use of Laboratory Animals (8th Edition). Experiments were reviewed and approved prior to being carried out by the Institutional Animal Use and Care Committee (IACUC) of the Florida State University (Protocols #1016, 1135; Association for Assessment and Accreditation of Laboratory Animal Care International accreditation unit #001031; Office of Laboratory Animal Welfare Assurance #A3854-01).

Animals

Mice in this study were progeny of a University of Massachusetts Medical School mouse colony, where the melatonin receptor knockout strains were generated by a genetic mutation introduced by homologous recombination (Liu et al., 1997; Jin et al., 2003). Sites within exon 1 of both the melatonin type 1 receptor gene (MT_1) and the melatonin type 2 receptor gene (MT_2) were replaced with phosphoglycerate kinase promoter (PGK-neomycin) cassettes. In each case, the clones carrying the targeted allele were injected into C57BL/6 mouse blastocytes. The resulting chimeric animals were bred to C57BL/6 animals to obtain offspring that were heterozygous for the knockout allele. These animals were then backcrossed for 10 generations to C3H/He mice because others have used the C3H strain extensively in examining circadian behavioral responses to melatonin. The C3H strain also has rhythmic melatonin production (Ebihara et al., 1986) unlike most other inbred strains of mice, including the C57BL/6 (Roseboom et al., 1998; Kasahara et al., 2010). The resultant C3B6 (C3H/He + C57BL/6) melatonin type 1 receptor-deficient homozygous mice ($MT_1^{-/-}$) were crossed with C3B6 melatonin type 2 receptor-deficient mice ($MT_2^{-/-}$) to obtain $MT_1^{-/-}/MT_2^{-/-}$ double-mutant mice, hereafter referred to as double-knockout (DBL-KO) mice, and their non-transgenic (NT) wild-type counterparts, hereafter referred to as NT mice.

Multiple cohorts of 6–10 male mice were randomly selected from breeders of the DBL-KO and NT lines for multiple aspects of this study: circadian, behavioral, a melatonin challenge, or serum collection. Some animals were sacrificed at specific ages for tissue analysis (e.g., long-term potentiation (LTP) at 6 months of age), while other cohorts were maintained for behavioral testing at 3, 6, 12, and 15 months of age. Prior to experimentation at each age, animal weights in grams were 24.2 ± 2.4, 29.8 ± 3.8, 33.6 ± 4.9, 32.9 ± 3.5 respectively, and there were no significant differences between genotype groups at any age. The animals were housed individually in a polycarbonate cage (Ancare, Bellmore, NY; 19 cm × 29 cm × 13 cm) with hardwood laboratory bedding chips (Nepco Beta Chip®, Warrensburg, NY), nesting material (Ancare Nestlet), and a polycarbonate mouse igloo (Bio-Serv, Flemington, NJ) for enrichment. They were maintained under a 12-h light–dark cycle (7 am to 7 pm) at 21.1 °C and given *ad libitum* access to LabDiet® 5001 Rodent Chow and water. Daily water intake was averaged from bottle weight measurements every 5–7 days for 4 weeks.

Genotyping by polymerase chain reaction (PCR)

At approximately 21 days of age, an IACUC and veterinarian recommended inhalation anesthetic, isoflurane (Butler Schein, Dublin, OH; 029405) was used to sedate the animal. Once anesthetized, 2-mm terminal segment of tail was removed with a sterile scissors. Hemostasis was achieved using a silver nitrate applicator stick (Butler Schein; 005383) and potential pain and discomfort were alleviated by applying bupivacaine hydrochloride in sterile isotonic solution (Sigma, St. Louis, MO; B5274; 2.5 μg/mL) locally to the excision site for long-acting pain management. Post-surgical excision site monitoring occurred for 10 days. The tail biopsy was placed in 250 μL of DirectPCR Tail lysis buffer (Viagen 101-T) with 10 μL Proteinase K solution (Viagen 501-K) and lysed for 6 h at 55 °C. The sample was then incubated at 85 °C for 45 min and briefly centrifuged. The supernatant containing the genetic material was collected and stored at −20 °C for subsequent PCR analysis. Primers used to amplify the MT_1 receptors included mMT1R-FW-WT (5′-GAAGTTTTCTCAGTGTC CCGCAATGG-3′), mMT1R-REV-WT (5′-GAGTCCAAGT TGCTGGGCAGTGGA-3′), and mMT1R-NEO-FW-KO (5′-CCAGCTCATTCCTCCACTCATGATCTA-3′). The genomic DNA (deoxyribonucleic acid) was subjected to a 3-min hot start at 94 °C and then 35 cycles of 45 s at 94 °C, 45 s at 60 °C, and then 3 min at 72 °C, with a final stage of 7 min at 72 °C. Primers used to detect the MT_2 receptor alleles included a common forward primer (mMT2R-FW-WT 5′-CCAGGCCCCCTGTGACTGCCCG GG-3′), a gene-specific reverse primer from intron 1 (mMT2R-REV-WT 5′-CCTGCCACTGAGGACAGAACAG GG-3′), and a reverse primer based on the PGK-neo cassette (mMT2R-NEO-REV-KO 5′-TGCCCCAAAGGC CTACCCGCTTCC-3′). These genomic DNA samples were subjected to a 3-min hot start at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 68 °C, and 1 min at 72 °C, with a final 7-min stage at 72 °C. The resulting samples were