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MELATONIN IN THE MAMMALIAN OLFACTORY BULB

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Abstract—Melatonin is a neurohormone associated with circadian rhythms. A diurnal rhythm in olfactory sensitivity has been previously reported and melatonin receptor mRNAs have been observed in the olfactory bulb, but the effects of melatonin in the olfactory bulb have not been explored. First, we corroborated data from a previous study that identified melatonin receptor messenger RNAs in the olfactory bulb. We then investigated whether melatonin treatment would affect cells in the olfactory bulbs of rats. Using a combination of polymerase chain reaction (PCR), quantitative PCR (qPCR), cell culture, and electrophysiology, we discovered that melatonin receptors and melatonin synthesis enzymes were present in the olfactory bulb and we observed changes in connexin43 protein, GluR1 mRNA, GluR2 mRNA, Per1 mRNA, Cry2 mRNA, and K^+ currents in response to 2-iodomelatonin. Via qPCR, we observed that messenger RNAs encoding melatonin receptors and melatonin biosynthesis enzymes fluctuated in the olfactory bulb across 24 h. Together, these data show that melatonin receptors are present in the olfactory bulb and likely affect olfactory function. Additionally, these data suggest that melatonin may be locally synthesized in the olfactory bulb.
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Key words: melatonin, receptor, olfaction, GluR2, juxtaglomerular, potassium.

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Abbreviations: AADC, aromatic L-amino acid decarboxylase; AANAT, arylalkylamine-N-acetyltransferase; ASMT, acetylserotonin methyltransferase; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; EPL, external plexiform layer; ET, external tufted cell; FIHC, fluorescent IHC; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCL, granule cell layer; GL, glomerular layer; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; HIOMT, hydroxyindole-O-methyltransferase; IHC, immunohistochemistry; I-mel, 2-iodomelatonin; JG, juxtaglomerular cell; K+, potassium; MCL, mitral cell layer; MT1R, melatonin receptor 1; MT1R-KO, melatonin receptor 1 knock-out mouse; MT2R, melatonin receptor 2; MT2R-KO, melatonin receptor 2 knock-out mouse; Ni-DAB, nickel-diaminobenzidine; OB, olfactory bulb; ONL, olfactory nerve layer; PCR, polymerase chain reaction; PG, periglomerular cell; qPCR, quantitative PCR; SA, short-axon cell; SCN, suprachiasmatic nucleus of the hypothalamus; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPH1, tryptophan hydroxylase isoform 1; TPH2, tryptophan hydroxylase isoform 2; TTX, tetrodotoxin.

INTRODUCTION

Melatonin is a lipophilic neurohormone that signals the onset of darkness. Melatonin affects circadian rhythms in animals that generate melatonin (Hunt et al., 2001; reviewed in Pandi-Perumal et al., 2006, and Zawilska et al., 2009). A previous study (Granados-Fuentes et al., 2011) reported a diurnal rhythm in olfactory discrimination behavior that was sensitive to the knockout of some clock genes. Melatonin can affect different clock genes, and melatonin receptor mRNAs have been previously reported in the olfactory bulb (OB; Ishii et al., 2009). We wanted to determine if melatonin administration could affect the olfactory system. However, melatonin can act via direct binding to intracellular proteins (Nosjean et al., 2000) or membrane-bound G-protein-coupled receptors. Much more is known about the effects of melatonin binding to its receptors, and we chose to focus our investigations there.

Membrane-bound melatonin receptors, in mammals, come in two isoforms: melatonin receptor 1 (MT1R; also called MTNR1a) and melatonin receptor 2 (MT2R; also called MTNR1b). A third putative isoform, melatonin receptor 3, was revealed to be the intracellular protein quinone reductase 2 (Nosjean et al., 2000). Melatonin receptors (reviewed by Dubocovich et al., 2010) are 7-transmembrane domain proteins, attached to G-proteins (G_i/G_o) that interact with adenylyl cyclase, leading to a dephosphorylation of cAMP response element-binding protein and/or changes in mitogen-activated protein kinase or mitogen-activated protein kinase kinase, and therefore changes in transcription and translation of different genes, including entrainment of the SCN clock (Lee et al., 2010). Melatonin receptors can also indirectly interact with K^+ channels in the suprachiasmatic nucleus of the hypothalamus (SCN; Inyushkin et al., 2007) and K^+ channels and glycine receptors in the retina (Zhao et al., 2010; Yang et al., 2011). Melatonin receptors are involved in the circadian timing of some behaviors in different species, mostly via receptors expressed by SCN cells. Messenger RNAs encoding MT1R and MT2R were previously reported in the OB of rats (Ishii et al., 2009), but these data, to date, have not been corroborated or further explored. The OB is similar to the retina by virtue of its laminar organization and function in initial sensory processing, while the OB is

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similar to the SCN and the retina because the OB has circadian rhythms in gene expression and electrical activity that continue without outside input (Granados-Fuentes et al., 2004); due to these similarities, we chose to focus our investigation on known actions of melatonin in the SCN and the retina and to examine if melatonin's actions in the OB were similar.

Odorant processing begins in the mammalian OB after odorants bind to receptors in the olfactory mucosa of the nose. A message from the nose is sent by olfactory sensory neuron axons, which form the olfactory nerve layer (ONL) of the OB, and project to structures called glomeruli in the glomerular layer (GL) of the OB. Juxtaglomerular (JG) cells surround glomeruli and can be subdivided into periglomerular (PG), short-axon (SA), and external tufted (ET) cells, along with some histologically unidentified cell types (Kosaka and Kosaka, 2011). The principal output neurons of the OB are mitral cells in the mitral cell layer (MCL) and tufted cells in the external plexiform layer of the OB. Finally, granule and Blanes cells reside in the granule cell layer (GCL). A subset of the PG cells and the majority of cells in the GCL release the inhibitory neurotransmitter GABA and inhibit mitral and tufted cell activity.

Melatonin itself is released from the pineal gland into the bloodstream (though the retina and other tissues have been reported to synthesize melatonin; see Gómez-Corvera et al., 2009 and Itoh et al., 2007), and is synthesized from serotonin by two enzymes: arylalkylamine N-acetyltransferase (AANAT) and hydroxyindole-O-methyltransferase (HIOMT; also called acetylserotonin methyltransferase, or ASMT). AANAT mRNA has been shown in the OB (Uz et al., 2002). HIOMT mRNA has been shown in multiple brain areas, but not in the OB (Ribelayga et al., 1998).

We pursued three hypotheses for this study, using a combination of PCR, qPCR, immunoblotting, cell culture, immunohistochemistry, and electrophysiology: first, that melatonin receptors and HIOMT are present in the OB; second, that melatonin receptors and melatonin biosynthesis enzymes fluctuate over 24 h; and third, that melatonin receptor activation mediates transcriptional, translational, and electrical changes in OB cells.

EXPERIMENTAL PROCEDURES

Animals

Male and female Sprague Dawley rat pups, aged postnatal days 21–23, (Charles River, Raleigh, NC, USA) were kept in standard rat cages under a 12-h light, 12-h dark cycle (lights on at 0700 h EST; ZT0). Water and rat chow were available *ad libitum.* Male and female wild-type, melatonin receptor 1 knock-out (MT1R-KO; Liu et al., 1997), melatonin receptor 2 knock-out (MT2R-KO; Jin et al., 2003), and MT1R-KO/MT2R-KO mice (C3H strain) were provided by the Olcese laboratory at the Florida State University. MT1R-KO and MT2R-KO mice were genotyped and assayed for MT1R and MT2R transcripts by sequencing using a HiSeq sequencer (Illumina, San Diego, CA, USA) to ensure that KO animals did not express melatonin receptors. For tissue collection, animals were anesthetized using isoflurane and killed by decapitation. During the night, tissue collection was performed under dim red light. For immunohistochemical samples, animals were anesthetized with a mixture of ketamine and xylazine and killed by transcardial perfusion. The animals for these experiments were used according to the guidelines of our protocol approved by the Florida State University's Animal Care and Use Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

RNA extraction

Rat pups of ages P21, P22, and P23 were killed at 3-h intervals over 48-h and their OBs were excised and placed immediately in RNAlater reagent (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. For processing, samples were removed from RNAlater, placed in ice-cold TRIzol reagent (Life Technologies, Carlsbad, CA, USA), and homogenized with a rotor–stator homogenizer (PRO Scientific, Oxford, CT, USA) at 50% amplitude with autoclaved probes washed in diethylpyrocarbonate-treated water. Samples homogenized in TRIzol were processed according to manufacturer's instructions with the following changes: the chloroform-addition step was repeated, samples in isopropanol were left at –20 °C overnight, and the samples were not heated for resuspension. The resulting pellet was resuspended in 100 μl of RNase-, DNase-free water (EMD Millipore, Billerica, MA, USA) and processed through the RNEasy Mini Kit (Qiagen) according to the manufacturer's RNA Cleanup protocol with DNase I treatment (Qiagen). Purified RNA samples were spun for 40 min in a vacuum concentrator and stored at –20 °C until use.

Reverse transcription, PCR, and quantitative PCR

Purified RNA samples were kept on ice and their concentrations assayed with a Nanodrop 1000 spectrophotometer (Thermo Scientific, Rockford, IL, USA). Sample integrity was assayed by gel electrophoresis with SYBR Safe DNA Dye (Life Technologies). Five micrograms of total RNA from tissue or 2 μg of total RNA from MOB brain slices were used for the reverse-transcription reaction with a Superscript III Reverse Transcription Supermix Kit (Life Technologies), according to the manufacturer's instructions except for inclusion of both random oligonucleotides and $oligo_{dT(20)}$ as primers for the reverse transcription, from the protocol of Resuehr and Spiess (2003). The resulting cDNA was diluted 1:10 in RNase-, DNase-free water.

Polymerase chain reaction (PCR) solutions were composed of HotStarTaq Master Mix (Qiagen), 2 μl of the diluted cDNA, and 500 nM primers, according to manufacturer instructions. PCR was run on a Veriti thermal cycler (Life Technologies). Reactions were run as follows: 95 °C for 15 min; 40 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; 72 °C for 10 min, 4 °C until samples were prepared for gel electrophoresis. PCR products were analyzed using

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