Physiology & Behavior 105 (2012) 257–263

Contents lists available at SciVerse ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Developmental disruption of the serotonin system alters circadian rhythms

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ARTICLE INFO

Article history:
Received 29 September 2010
Received in revised form 3 August 2011
Accepted 24 August 2011

Keywords:
Pet-1
Serotonin
Suprachiasmatic nucleus
Circadian
Wheel-running
Locomotor

ABSTRACT

Serotonin (5-HT) plays an important role in circadian rhythms, acting to modulate photic input to the mammalian clock, the suprachiasmatic nucleus (SCN), as well as playing a role in non-photic input. The transcription factor Pet-1 is an early developmental indicator of neurons that are destined for a 5-HTergic fate. Mice lacking the Pet-1 gene show a 70% loss of 5-HT immunopositive cell bodies in adult animals. 5-HT neurotoxic lesion studies using 5,7-dihydroxytryptamine (5,7-DHT) have highlighted species-specific differences in response to 5-HT depletion and studies using knockout mice lacking various 5-HT receptors have helped to elucidate the role of individual 5-HT receptors in mediating 5-HT's effects on circadian rhythms. Here we investigate the effects of a developmental disruption of the 5-HT system on the SCN and circadian wheel-running behavior. Immunohistochemical analysis confirmed depletion of 5-HT fiber innervation to the SCN as well as greatly reduced numbers of cell bodies in the raphe nuclei in Pet-1 knockout mice. These mice also display significantly longer free-running periods than wildtype or heterozygote counterparts. In light–dark cycles, knockouts showed a shift in peak wheel running behavior towards the late night as compared to wildtype and heterozygote animals. When kept in constant darkness for 70 days, wildtype animals showed decreases in free-running period over time while the period of knockout animals remained constant. Immunohistochemical analysis for neuropeptides within the SCN indicates that the behavioral changes observed in Pet-1 knockout mice were not due to gross changes in SCN structure. These results suggest that developmental loss of serotonergic input to the clock has long-term consequences for both circadian clock parameters and the temporal organization of activity.

1. Introduction

In mammals, behavioral circadian rhythms are generated by an endogenous clock located in the suprachiasmatic nucleus (SCN) within the ventral hypothalamus. Rhythms are entrained to the 24 h light–dark cycle by external cues, with light functioning as the primary cue [1–3]. However, other non-photic stimuli such as temperature, food availability, or activity can also serve as cues for entrainment [4–6].

Serotonin (5-HT) is a neurotransmitter that plays an important role in circadian rhythms, modulating photic input to the SCN as well as playing a role in non-photic phase shifts [7]. One of the 5-HTergic pathways thought to convey non-photic information to the SCN, such as the activity level of the animal, as well as modulate photic effects is input from the median raphe (MR) [8]. There is also evidence suggesting direct 5-HTergic input to the SCN from the dorsal raphe (DR) [9]; the DR can also influence the SCN through an indirect connection via the intergeniculate leaflet (IGL) [8].

5-HT release in the brain is highest during periods of activity, and in nocturnal rodents peak 5-HT release occurs around the time of lights off. When induced to run on running wheels, animals show increases in 5-HT levels during mid-subjective day; suggesting that 5-HT release is correlated with arousal and locomotor activity [10]. In turn, locomotor activity attenuates light-induced phase shifts [11]. Interestingly, light-induced phase shifts can also be attenuated by pharmacological treatment with (±)-8-hydroxy-2-(dipropylamino) tetralin hydrobromide (8-OH-DPAT), a 5-$HT_{1A/7}$ receptor agonist [12], and 3-trifluoromethylphenylpiperazine (TFMPP), a 5-HT_{1B} agonist [13]. This differs from the circadian system's response to systemic injections of a 5-HT agonist during the mid-subjective day, which cause phase advances [14–20]. Conversely, inhibition of 5-HT release and/or administration of a 5-HT receptor antagonist potentiates light-induced phase shifts [21–25].

In hamsters, destruction of 5-HT afferents by 5,7-dihydroxytryptamine (5,7-DHT), a neurotoxin that selectively kills serotonergic neurons, causes lengthening of the active phase and longer periods in constant light (LL), but no change in period length in constant darkness (DD) [26,27]. Mice treated with 5,7-DHT and blinded by enucleation also show lengthening of the active phase and exhibit longer circadian periods than controls. Additionally, these mice show greater total activity and altered distribution of activity within the active

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doi:10.1016/j.physbeh.2011.08.032

phase with delays in onset of peak running activity and increased occurrences of bimodal patterns of activity [28]. These data indicate that disruption of serotonergic neurotransmission can have profound consequences for the expression of circadian rhythms.

Pet-1, a transcription factor belonging to the ETS family, is an early developmental indicator of neurons destined for a 5-HTergic fate. Pet-1 expression is restricted to 5-HT neurons in the rat and mouse, appearing at embryonic day 12.5, about half a day before 5-HT is detectable. Pet-1 mRNA colocalizes with tryptophan hydroxylase (TPH) in neurons but not non-neuronal cells [29,30], and is necessary for the maintenance of a serotonergic phenotype in adults [31]. Mice lacking the Pet-1 gene appear normal and have normal feeding behavior, motor learning, balance, coordination but increased aggression and anxiety-like behaviors [32]. These animals also show a significant disruption of the 5-HT system, without effects on other monoamine systems, with a 70% loss of 5-HT immunopositive cell bodies in adult Pet-1 KOs. While the remaining 30% of 5-HT cell bodies retain their neuronal phenotype and appear to be normally positioned, they show little to no protein or mRNA expression for some of the substances that are common to the 5-HT neuron phenotype: TPH, 5-HT transporter, and vesicular monoamine transporter 2. Further, Pet-1 KO mice have a reduced density of 5-HT immunopositive fibers in target fields and the brain tissue content of 5-HT and 5-hydroxyindoleacetic acid in the Pet-1 KOs are only 10–15% of WT levels [32]. The effects of the loss of Pet-1 on peripheral serotonin levels are unknown.

It is clear from these studies that alteration of 5-HT input to the SCN changes the expression of behavioral circadian rhythms. What is unknown is how developmental disruption of the entire system can affect the SCN and behavioral clock output. The Pet-1 KO mouse provides an opportunity to examine this question, so in this study we examine the expression of behavioral rhythmicity in these mice, with the goal of further clarifying the role of 5-HT in circadian rhythm regulation.

2. Materials and methods

2.1. Animals

Pet-1 KO animals on a mixed 129Sv and C57BL/6 background were obtained from Dr. Evan Deneris (Case Western Reserve University, Cleveland, OH) [32]. Pet-1 heterozygote breeding pairs were used to produce all experimental animals, which were 2–5 months of age at the time of experimentation. Heterozygotes were used as Pet-1 homozygotes are deficient in maternal behaviors [33]. Experiments were conducted by matching siblings of different genotypes as much as possible to avoid bias from the mixed genetic background. At the time of weaning, animals were ear-tagged and a tail-snip was collected for DNA extraction and genotyping. Experimental animals were entrained to a 14:10 or 12:12 light–dark (LD) cycle prior to the start of the experiment as noted below. Food and water were available *ad libitum* and all experimental procedures were approved by the Kent State University Institutional Animal Care and Use Committee.

2.2. Genotyping

Tail snips from all animals were placed into an extraction buffer containing 20 mM Tris pH 8.0, 1 mM EDTA, 400 mM NaCl, and 0.5% SDS with 20 mg/mL Proteinase K added in order to digest the snip. Samples were maintained at 55 °C with slow rotation overnight. Supernatant from each sample was spun down in order to pellet DNA, rinsed with ethanol and resuspended in TE buffer. DNA of interest was amplified using PCR with primers from IDT (3′ PET: 5′ GCC TGA TGT TCA AGG AAG ACC TCG G 3′ 5′ PET: 3′ CGC ACT TGG GGG GTC ATT ATC AC 3′ 5′ LOX: 5′ CGG TGG ATG TGG AAT GTG TGC G 3′) and thermocycler conditions beginning with a 3 min hold at 94 °C followed by 42 cycles of 94 °C for 50 s, 62 °C for 30 s and 72 °C for 40 s. PCR product was analyzed using the Agilent 2100 Bioanalyzer system. WT animals were identified by DNA fragments of 209 bp while Pet-1 KO animals were defined by DNA fragments of 361 bp. The number of animals of each sex and genotype were counted to assess whether breeding results corresponded to the expected Mendelian ratios. Differences from expected ratios were tested with a G-test of goodness-of-fit while differences between sexes were assessed by a G-test for independence.

2.3. Behavioral monitoring

WT (n = 8), heterozygote (HET) (n = 8) and KO (n = 8) animals were placed into individual cages equipped with running wheels so that activity patterns could be monitored. Food and water was available *ad libitum*. Wheel-running activity was monitored for at least 21 days under each of three light schedules: LD 14:10, DD, and LL using Clocklab (Actimetrics) software. An additional group of animals was profiled in LD 12:12. Actograms for each animal were examined for differences in activity patterns between genotypes. Hourly counts of the number of wheel revolutions per hour were averaged across animals and normalized to the baseline 24-hour activity total for each animal to eliminate the effects of running wheels with different frictional resistances on the results. Periods in LD, DD, and LL were calculated by chi-squared periodogram for each animal using Clocklab software then grouped and averaged according to genotype. For the extended DD experiment, WT (n = 8) and KO animals (n = 6) were housed in LD 12:12 then transferred to and maintained in DD for 70 days. Periods were calculated for 10 day increments, starting at day 11 and ending at day 70, and averaged across genotypes. Statistical analysis for all behavioral experiments was performed using repeated measures ANOVAs, with posthoc comparisons to control groups performed using Dunnett's test.

2.4. Histology

For the examination of SCN neuropeptide expression, animals were given a lethal overdose of sodium pentobarbital intraperitoneally (i.p.) and perfused transcardially with 4% ice-cold paraformaldehyde. Brains were removed and post-fixed overnight before being transferred into PBS. Anterior and posterior portions of brains were sectioned at 40 μm using either a vibratome (for arginine vasopressin (AVP) and 5-HT) or cryostat (for vasoactive intestinal polypeptide (VIP)); if a cryostat was used, brains were first cryoprotected in a 30% sucrose solution. Sections from each animal were then processed for immunohistochemistry for 5-HT (Immunostar rabbit anti-5-HT 1:2000 and Jackson Research Cy2 secondary 1:500) and images of the SCN, median raphe, and dorsal raphe were taken for analysis. 5-HT fluorescent staining was examined to confirm the level of 5-HT depletion in each mouse brain. Levels of AVP and VIP were also examined using immunohistochemistry (Chemicon rabbit anti-AVP 1:50,000 and Immunostar rabbit anti-VIP 1:10,000, Jackson Research biotinylated donkey anti-rabbit secondary 1:500 and processed for peroxidase reaction using a Vector Laboratories DAB kit) and images taken for comparison. ImageJ software was used to perform counts of AVP immunopositive cells in the SCN of WT (n = 5), HET (n = 5) and KO (n = 5) animals. Counts of AVP immunoreactive cells were also performed in the paraventricular nucleus (PVN) for each genotype. Statistical analysis of AVP cell counts was done using a one-way ANOVA. VIP immunopositive cells in the SCN could not be counted due to the density of the labeling which obscured individual cells, so only a qualitative inspection was performed.

3. Results

3.1. 5-HT immunohistochemistry in the SCN, MR, and DR

Immunohistochemical staining was performed for 5-HT in order to visualize the extent of 5-HT fiber innervation to the SCN (Fig. 1A–C).