Supplemental Figures

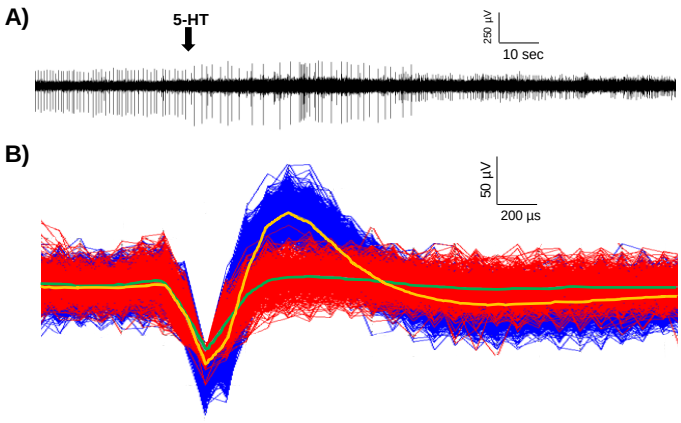
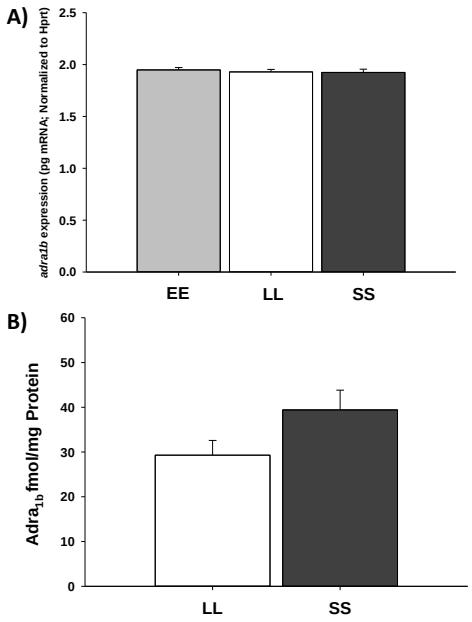
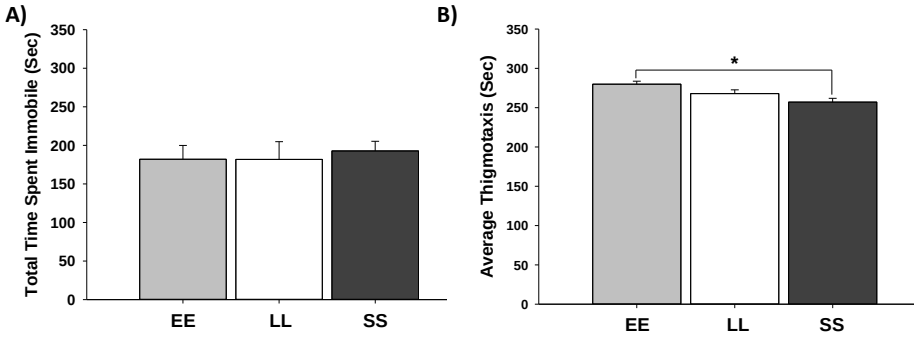


Figure S1, Related to Figure 1. Representative traces of multielectrode array recordings in DRN slices. (A) Representative spike train from a multielectrode array recording in the DRN. Large amplitude spikes represent 5-HT neuronal firing. 5-HT was added after at least 4 minutes of recording to verify serotonergic neuronal firing by 5-HT1a mediated autoinhibition, indicated by arrow. (B) Spikes were sorted in offline sorter using a combination of a K-means scan automated sorting software and manual verification. Blue traces indicate individual sorted serotonergic spikes, the bold yellow line indicates the average waveform of all serotonergic spikes. Red traces indicate individual sorted spikes not classified as serotonergic, the bold green line indicates the average waveform of all non-serotonergic spikes.



<u>Figure S2, Related to Figure 1</u>. **No change across photoperiods in the mRNA expression of the adra1b receptor or ADRA1b receptor binding in the midbrain**. (A) No change across photoperiods in adra1b receptor expression. n= 6 mice for each photoperiod (p= 0.583). (B) No change between short and long in ADRA1b receptor binding. n=7 mice for LL and 5 mice for SS (p=0.089).



<u>Figure S3, Related to Figure 2.</u> Open Field Test and Tail Suspension Test. (A) No significant differences across photoperiod in the TST. EE; n= 12, LL; n=15, SS; n=20 (p=0.244). (B) The EE group displays an increase in thigmotaxis over the SS group but not the LL group (p=0.011). There is no difference between the SS and LL groups. OFT: EE; n=10, LL; n=15, SS; n=20.

	EE	LL	SS	F	p
Max (Spikes/Sec)	1.18 ± 0.19	1.60 ± 0.04	0.86 ± 0.10	F (2, 7) = 10.91	0.0071*
Min (Spikes/ Sec)	0.18 ± 0.17	0.29 ± 0.12	0.16 ± 0.05	F (2, 7) = 0.3005	0.7496
EC50 (µM)	2.93 ± 0.97	1.34 ± 0.53	7.13 ± 5.69	F (2, 7) = 0.9872	0.4191

Table S1, Related to Figure 1. Fitted curve values for the dose response curve to Phenylephrine. The maximum is higher in the LL photoperiod compared to EE and SS photoperiods (p=0.0071). However, there is not a significant difference between the minimum (p= 0.7496) and EC50 values (p=0.4191), One-Way ANOVA.

	EE	LL	SS	F	p
Max (Spikes/Sec)	0.897 ± 0.09	1.205 ± 0.11	0.718 ± 0.14	F (2, 8) = 4.486	0.0494*
Min (Spikes/Sec)	0	0.04 ± 0.03	0	F (2, 8) = 0.8485	0.4633
IC50 (nM)	208.3 ± 56.5	140.4 ± 31.3	95.4 ± 10.9	F (2, 8) = 2.354	0.1571

Table S2, Related to Figure 1. Fitted curve values for the dose response curve to 8-OH-DPAT. The maximum spike rate before adding 8-OH-DPAT is higher in the LL photoperiod compared to EE and SS photoperiods (p= 0.494), reflecting the LL photoperiod-induced increase in spike rate (Figure 1). However, there is not a significant difference between the minimum (p= 0.4633) or IC50 (p=0.1571), One-Way ANOVA.

Supplemental Experimental Methods

Animals and Housing

Melatonin receptor 1 knock out mice (MT1KO), on a C3Hf$^{+/+}$ background [S1], were also developed on the light cycles stated above. Light cycles were aligned so that the tissue isolation and testing times on all cycles were at the same time local time corresponding to the middle of the light period for each photoperiod, between 1100 and 1300 (except the forced swim test which ranged from 1100-1500 due to equipment scheduling constraints). Experiments were performed in accordance with the Vanderbilt University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Ex vivo culture

Mice were euthanized by cervical dislocation, brains were extracted and mounted in cold, oxygenated (95%O_2-5%CO_2) dissecting media (in mM: 114.5 NaCl, 3.5 KCL, 1 NaH2PO4, 1.3 MgSO4, 2.5 CaCl2, 10 D(+)-glucose, and 35.7 NaCHO3), and 235 µm thick coronal slices were taken using a Vibroslicer (Campden Instruments). Dissecting media was frozen and chunks of dissecting media ice were added to the bath to maintain the low temperature of the bath while slicing. The dorsal raphe nuclei were isolated by removing the extraneous cortical tissue and placed sample in a slice chamber full of room temperature, oxygenated, extracellular recording media (in mM: 124 NaCl, 3.5 KCl, 1 NaH2PO4, 1.3MgSO4, 2.5 CaCl2,10 D(+)-glucose, and 20 NaHCO3).

Electrophysiological Recording and 5-HT Neuron Identification

As there are differences in the electrophysiological properties of neurons in the medial and lateral wing subfields of the DR, placement on the array and the dimensions of the electrode grid were used to ensure that only the ventromedial DR neurons (vmDR) were recorded from. Mid-DRN slices of 280 micron thickness were taken between -4.5 mm and -4.75 mm back

from bregma. We used a 6X10 perforated array with electrodes that have a diameter of 30 microns and 100 microns spacing between electrodes. The slice was placed so the electrodes cover an area spreading 1200 microns down from the cerebral aqueduct in the ventral direction and 340 microns laterally on either side of the midline (for a 680 micron total recording width). Putative 5HT neurons were identified by feedback inhibition evoked by application of 5-HT (40μM) (for general firing rate experiments) or 8-OH-DPAT (for dose response experiments) for 5 min, with those cells demonstrating a minimum of 50% spike rate suppression being included in the analysis, as we have done in previously published studies [S2, S3].

Although there is overlap in the electrophysiological characteristics of dorsal raphe serotonergic and non-serotonergic neurons [S4, S5], these selection characteristics, as well as the placement of the array to record from the ventromedial DR region, assured that a high proportion of cells in our recordings were serotonergic. The vmDR is highly enriched in serotonergic neurons. In the rat this has been carefully quantified with 75-95% of the neurons containing immunoreactivity to 5-HT, and 70-95% of neurons expressing the 5-HT1A receptor also expressing 5-HT [S5]. In addition, spike duration is significantly broader in 5-HT vs non-5-HT neurons in the dorsal raphe and the 5-HT1A mediated response is significantly greater in 5-HT neurons [S4]. The mouse DRN is thought to be similarly highly enriched for serotonergic neurons in the vmDR region, and 84% of genetically identified 5-HT neurons respond with 5-HT1a inhibition [S6]. In our dose-response experiments 156 out of 198 total cells decreased their firing rate by at least 50% upon application of 8-OH-DPAT, indicating that ~80% of the neurons recorded from express functional 5-HT_{1a} receptors and therefore are likely serotonergic. Thus, while we cannot be sure that each and every neuron we putatively identified as serotonergic indeed expressed serotonin, the combination of our anatomical recording site

and physiological criteria assures that the overwhelming majority of neurons in our study were serotonergic.

Data files were saved as .mcd files and analyzed in offline sorter. For analysis a Besel filter with a 150 Hz frequency cut off was applied to the raw data traces. The threshold for detection was set manually to a level that will include all legitimate spikes with the least amount of unipolar noise spikes included (between 13 µV and 35 µV). Once spikes were detected they were sorted by a combination of a K means scan method and manual verification. The manual verification was conducted after the K means scan was run and divided spikes into groups based on criterion such as amplitude, power under the curve and spike duration (for full list of criterion see offline sorter V3 manual under the K means scan, Plexon Inc.). Once waveforms were sorted into groups and judged to be biologically relevant each spike was validated by eye and spikes that did not fit the average waveform shape were invalidated. Also all unsorted spikes were visualized manually and any spikes that matched the average waveform shape in the relevant group were added to that group. Once the total number of spikes was determined for the period of the recording before the application of 5-HT or 8-OH-DPAT that number was divided by the total time in which those spikes occurred to produce a measure of spikes per second.

Gene Expression Detection and Analysis

Mouse mid-brains were removed between 11am-1pm, and then frozen in a 1.5 ml tube in liquid nitrogen. Samples were stored at -80°C until RNA extraction. Total RNA was extracted using a Qiagen RNeasy mini kit (Qiagen Inc., Valencia, CA, USA, Cat. No. 74104), measured by a Nanodrop system (ThermoScientific), and reverse-transcribed (~200ng) into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen Inc., Valencia, CA, USA, Cat. No. 205311). qRT-PCR reactions were performed in 20 µL total volume with 2 µL cDNA, 10µL of SsoAdvanced

SYBR Green Supermix (Bio-Rad, Hercules, CA), 6μL sterile water and 1μL of 300 nM intron-spanning gene specific forward and reverse primers in a Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Quantification of transcript levels were performed by comparing the threshold cycle for amplification of the unknown to those of six concentrations of standard cDNAs for each respective transcript, then normalizing the standard-calculated amount to hypoxanthine guanine phosphoribosyl transferase (*Hprt*) in each sample. Each sample was assayed in duplicate. Statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant.

Adra1b receptor binding

Mice were killed by brief isoflurane anesthesia exposure and decapitation. Brains were quickly dissected and placed into a cold, clean brain matrice, and mibrain was cut free coronally using clean razor blades. The midbrain section was then dissected by making two cuts 2mm each side of the dorsal midline to the aqueduct of sylvius to further isolate dorsal and median raphe regions. The tissue was homogenized in ice-cold 50-mM Tris assay buffer (50 mM Tris–HCl, 10 mM MgCl2, 0.1 mM EDTA (pH 7.3)). The homogenate was centrifuged at 20,000 g for 20 min at 4°C. The supernatant was discarded, and the pellet was resuspended in assay buffer, briefly homogenized, and protein concentration was measured by Bradford assay (Bio-Rad). A single point saturating concentration was chosen based on pilot experiments of complete saturation binding curves for [^{3}H] prazosin. Homogenized midbrain membranes (60 μg/tube) were incubated with [^{3}H] prazosin [6 nM] in the presence or absence of prazosin [10 μM] to measure non-specific and total binding, respectively. Reaction buffer included the alpha 1A receptor antagonist WB 4101 [10 μM]. Specific binding was calculated by subtracting non-specific binding from total binding and expressed as bound ligand (fmol) per mg protein. All experiments were performed in duplicate. Following 30 min incubation at 37°C, free radioligand was

separated from bound by vacuum filtration through Whatman GF/B glass filters (Brandel, Gaithersburg, MD, USA). Filters were placed in vials with scintillation cocktail and counted 12 h later in a liquid scintillation counter.

Monoamine analysis

Mouse mid-brains were removed between 1100-1300. brains were quickly dissected and placed into a cold, clean brain matrice, and mibrain was cut free coronally using clean razor blades. The midbrain section was then dissected by making two cuts 2mm each side of the dorsal midline to the aqueduct of sylvius to further isolate dorsal and median raphe regions. These sections were then frozen in a 1.5 ml tube in liquid nitrogen. Samples were stored at -80°C until the tissue was homogenized, using a tissue dismembrator, in 100-750 µl of 0.1M TCA, which contained 10^{-2} M sodium acetate, 10^{-4} M EDTA, 5ng/ml isoproterenol (as internal standard) and 10.5 % methanol (pH 3.8). Samples were spun in a microcentrifuge at 10000 x g for 20 minutes. The supernatant was removed and stored at –80°C . The pellet was saved for protein analysis. Supernatant was then thawed and spun for 20 minutes. Samples of the supernatant were then analyzed for biogenic monoamines. Biogenic amines were determined by a specific HPLC assay utilizing an Antec Decade II (oxidation: 0.4) (3mm GC WE, HYREF) electrochemical detector operated at 33°C . Twenty µl samples of the supernatant were injected using a Waters 2707 autosampler onto a Phenomenex Kintex (2.6u, 100A) C18 HPLC column (100 x 4.60 mm). Biogenic amines were eluted with a mobile phase consisting of 89.5% 0.1M TCA, 10^{-2} M sodium acetate, 10^{-4} M EDTA and 10.5 % methanol (pH 3.8). Solvent was delivered at 0.6 ml/min using a Waters 515 HPLC pump. Using this HPLC solvent the following biogenic amines eluted in the following order: noradrenaline, Adrenaline, DOPAC, Dopamine, 5-HIAA, HVA, 5-HT, and 3-MT (2). HPLC control and data acquisition were managed by Empower software. For C3Hf$^{+/+}$ mice 5-HT and NE measurements, statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant. For MT1

KO mice 5-HT and NE measurements, statistical significance was determined by a one-way Kruskal-Wallis ANOVA on ranks because the data was determined not to be normal with a p value less than 0.05 considered significant.

Behavioral Testing

Mice - No mouse underwent more than one test daily and no mouse underwent the same test more than once. The experimenter was the same for all tests conducted. There was no pseudo-randomization in the order of the mice tested, they were tested on a cage by cage basis. That is to say all the mice in one cage (and therefore one genotype and photoperiod) were tested in succession and then the next cage was tested. Mice were allowed to acclimate to the testing room for 30 minutes prior to all tests. Sex differences in behavioral results within photoperiods were not observed (t-test with Holm-Sidak correction for multiple comparisons).

Forced swim test - Mice were exposed to the FST for 6 min during the light phase 1100-1500 hours. The FST container was a Plexiglas cylinder 45 cm high and 20 cm in diameter filled with 30-35 cm of water (21 ± 2˚C). Testing was conducted in normal room light. During the entire duration of the task, an experimenter was present and watching the mice. The videos were then scored by the same experimenter as performed the tests. Before scoring the scorer was blinded to the genotypes and photoperiods of the mice in each video. Time immobile was defined as any period of time the animal was not making any active escape movements or floating without struggling. Statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant. For all post hoc analysis Holm-Sidak pair-wise comparisons were performed.

Tail suspension test - The tail of a mouse was taped to a force meter attached to the top of an open chamber so that it cannot escape. The mouse was suspended for 6 min. Latency to stop

struggling, and amount of time spent struggling were measured through the force meter with a threshold of 7 (arbitrary units) as the limit for struggling. Testing was conducted in normal room light. Mice used in this experiment were similar ages (50 to 90 days old) and there were no large weight discrepancies between mice tested. Data was gathered and processed by Med Associates Inc. tail suspension software. Statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant. For all post hoc analysis Holm-Sidak pair-wise comparisons were performed.

Open field test - Exploratory locomotor activity was measured in an open field measuring 27 x 27 cm, with a light intensity between 90-110 lux across all chambers, over a 60 minute period. Infrared beams and detectors automatically record movement in the open field. Thigmotaxis was defined as time spent on the outer rim of the open field, which was defined as the area 4.25 cm from the wall of the open field. This area constitutes 50% of the total area of the open field. Thigmotaxis was measured throughout the 60 minute period in 5-minute blocks. The average thigmotaxis within a 5-minute block was calculated from all 12 blocks within 60 minutes of testing which is what is represented in the data. Total distance traveled was also measured, there was no difference between photoperiods in $C3Hf^{+/+}$ mice. MT1 KO mice developed on an EE photoperiod showed an increase in total distance traveled over both SS and LL mice. Statistical significance was determined by a one-way ANOVA with a p value less than 0.05 considered significant. For all post hoc analysis Holm-Sidak pair-wise comparisons were performed.

Elevated zero maze - The zero maze consisted of one circular platform approximately 50 cm. Two discontinuous portions of the maze has walls, approximately 20 cm high with a light intensity within these arms of 71-91 lux and 344-355 lux outside these arms. The remaining portions have no walls. Mice were placed in one of the on-walled arms at the beginning of the

session. Number of entries into the closed arms, and amount of time spent in closed arms was used as a measure of anxiety. The zero maze task was conducted once per mouse for 6 min. Statistical significance was determined by a one-way Kruskal-Wallis ANOVA on ranks because the data was determined not to be normal with a p value less than 0.05 considered significant. For all post hoc analysis Dunn's method pair-wise comparisons were performed.

Data Analysis

Spike traces from multielectrode arrays were analyzed using offline sorting (Plexon) and spikes were sorted using a combination of manual identification and automatic K means based sorting software. For spontaneous firing rate data of wt C3Hf+/+ mice, where cell/mouse counts were unequal between mice, two-level nested one-way ANOVA was used to rule out significant within-photoperiod variability (mice within photoperiods, $p = 0.3609$, $F(13,394) = 1.0813$, among photoperiods, $p < 0.0001$, $F(13,4) = 23.5042$). All cells within each mouse were then averaged, and those values were used for ordinary One-Way ANOVA. Holm-Sidak's Multiple Comparisons test was used post-hoc for One-Way ANOVA, and Tukey's Multiple Comparisons test for Two-Way ANOVA. All statistical analyses were performed with $\alpha = 0.05$. Monoamine measurements values exceeding $\pm$ 1.5 SD from the mean were excluded. DRC data were analyzed with Two-Way ANOVA (factors: concentration, photoperiod, interaction) and with non-linear regression modeling to identify unshared parameters and plot curves. Parameter values for individual mice were determined using least-squares non-linear regression constraining "Bottom" values greater than zero. Parameter values for each photoperiod were then analyzed using One-Way ANOVA (Supplemental Tables 1 and 2). For intrinsic excitability curves regression analysis and slope significance testing was calculated as per [S7].

Supplemental References

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