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Thermoregulation-Independent Regulation of Sleep by Serotonin Revealed in Mice Defective in Serotonin Synthesis

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Running Title Page

Running Title

5-HT and sleep

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Abbreviations:

5-hydroxytryptamine (5-HT)

LIM homeobox transcription factor 1 (*Lmx1b*)

Tryptophan hydroxylase 2 (*Tph2*)

Electroencephalographic (EEG)

p-chlorophenylalanine (pCPA)

5-hydroxytryptophan (5-HTP)

Rapid eye movement (REM)

Non-rapid eye movement (NREM)

Electromyography (EMG)

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ABSTRACTS

A role for 5-hydroxytryptamine (5-HT) or serotonin in sleep has been known for decades but was challenged by recent papers which concluded that the apparent sleep phenotype was secondary to defective thermoregulation. Those studies used mice lacking serotonergic neurons resulting from the loss of function mutations in the gene encoding the LIM homeobox transcription factor 1 (*Lmx1b*). Here we show that, while *Lmx1b* mutants failed to keep the physiological body temperature, they exhibited more activities at the room and elevated temperatures. More importantly, we used mice deficient in the gene encoding tryptophan hydroxylase 2 (*Tph2*) which could not synthesize 5-HT in the brain. *Tph2* mutants were capable of thermoregulation and keeping physiological body temperature when the environmental temperature was reduced and exhibited significantly more activities at both the room and elevated temperatures. Electroencephalographic (EEG) recording also showed decreased sleep in *Tph2* deficient mice. Our results indicate that 5-HT is important for sleep regulation but not thermoregulation.

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INTROUDCTION

The monoamine neurotransmitter 5-hydroxytryptamine (5-HT), also known as serotonin, is important in multiple physiological processes. It is surprising that one of the earliest suggested roles for 5-HT, i.e. that in sleep regulation, remains controversial after decades of physiological and pharmacological studies.

Early studies used p-chlorophenylalanine (pCPA), an inhibitor of tryptophan hydroxylase to deplete 5-HT from cats, rats, monkeys and humans (reviewed in Jouvet, 1972, 1969). Reduction of central 5-HT levels after pCPA administration in cats (Koella et al., 1968) and rats (Mouret et al., 1968) were found to correlate with reduction of sleep. Lesion of serotonergic neurons in the raphe nuclei of cats resulted in a decrease of sleep in a manner proportional to the size of the lesion (Jouvet, 1968; Jouvet et al., 1967). 5-hydroxytryptophan (5-HTP) is a precursor of 5-HT capable of crossing the blood-brain barrier and can bypass the effect of pCPA to restore the cerebral 5-HT level. Injection of 5-HTP proportionally increased sleep and 5-HT levels in cats whose sleep was disrupted by pCPA (Jouvet, 1968; Koella et al., 1968; Mouret et al., 1967; Pujol et al., 1971). These studies led to the hypothesis that central 5-HT promotes sleep.

However, the discovery that administration of either 5-HT or 5-HTP in animals with normal 5-HT level sometimes did not induce

physiological sleep put the hypothesis in question (Jouvet, 1972). Large doses of 5-HTP increased electroencephalography (EEG) synchronization, a hallmark of neural activity during non-rapid eye movement (NREM) sleep, while suppressing rapid eye movement (REM) sleep in cats (Pujol et al., 1971). In monkeys, 5-HTP caused hyperactivity followed by an inactive period alternating between dozing and staring blankly (Macchitelli et al., 1966). Gain-of-function experiments suggested that 5-HT was not simply promoting sleep or triggering sleep onset.

To further complicate the scenario, unit activities in raphe nuclei increased during waking while progressively decreased when animals transited from drowsiness to NREM sleep and was almost silent during REM sleep (Lydic et al., 1987; McGinty and Harper, 1976; Trulson and Jacobs, 1979). Microdialysis measurement of extracellular 5-HT concentration from the dorsal raphe nucleus in cats showed that the 5-HT level was the highest during waking and progressively decreased from NREM to REM sleep (Portas and McCarley, 1994). However, some atypical raphe neurons showed higher activity during REM or NREM sleep or both in freely moving cats (Sakai and Crochet, 2001).

Genetic depletion of serotonergic neurons was achieved by *ePet1*-cre driven knock-out of *Lmx1b* gene ($Lmx1b^{f/f/p}$) (Zhao et al., 2006). $Lmx1b^{f/f/p}$ mice exhibited increased wakefulness and decreased sleep (Buchanan and Richerson, 2010), but these results were attributed to the failure in

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maintaining body temperature, which caused the animals to exert hyperactivity in order to stay warm (Buchanan and Richerson, 2010; Hodges et al., 2008). This conclusion was not consistent with previous findings which suggested serotonergic neurons involved in thermoregulation were confined in he raphe magnus and the medullar nuclei (Berner et al., 1999; Nakamura et al., 2004; Tanaka et al., 2002), whereas the level of insomnia caused by lesion was proportional to the size of lesioned nuclei, which suggested that 5-HT concentration, rather than specific nuclei, was important for sleep-wake regulation (Jouvet, 1969). A large proportion of raphe serotonergic neurons also release glutamate (Hioki et al., 2010; Sos et al., 2016; Zhixiang Liu, 2014), raising the possibility that distinct neurotransmitters are involved in divergent functions of raphe neurons.

We have now used *Tph2* mutant mice, in which the raphe neurons are present but 5-HT could not be synthesized in the brain We found that the *Tph2*$^{-/-}$ mice were not defective in thermoregulation at cold or normal ambient temperature. Sleep was decreased in these mutant mice, as shown by locomotive activities and by electroencephalography/electromyography (EEG/EMG) recordings.

MATERIALS & METHODS

Experimental Animals

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All procedures and protocols were carried out under the instruction of Institutional Animal Care and Use Committee at Peking University. Briefly, mice were kept under a 12-12hr dark-light cycle at the ambient temperature of 25±1°C. Experiments were carried out between 12~18 weeks of age. *ePet1-Cre*, *Lmx1b*$^{f/f}$, *Tph2* mice were gifts from Dr. Zhoufeng Chen, respectively. Generation, breeding and genotyping of *Lmx1b*$^{f/f/p}$ (Zhao et al., 2006) and *Tph2*$^{-/-}$(Kim et al., 2014) mice have been described previously (Liu et al., 2011). Primers used for genotyping were: AGGCTCCATCCATTCTTCTC and CCACAATAAGCAAGAGGCAC for *Lmx1b*$^{f/f}$; ATTTGCCTGCATTACCGGTCG and CAGCATTGCTGTCACTTGGTC for *ePet1-Cre*; GGGCATCTCAGGACGTAGTAG, GGGCCTGCCGA TAGTAACAC and GCAGCCAGTAGACGTCTCTTAC for *Tph2*. All strains were backcrossed with C57B/L for over eight generations in our lab before these experiments. For *Tph2* mice, only males were used. While for *Lmx1b*$^{f/f/p}$, both male and female mice were used. Littermates with genotype *Lmx1b*$^{f/f}$ or *Tph2*$^{+/+}$ of the same age were used as controls for *Lmx1b*$^{f/f/p}$and *Tph2*$^{-/-}$, respectively. All mice were housed individually during experiments. Numbers of male and female mice: Figure 1 (n=7 for *Lmx1b*$^{f/f}$, 7 females, 0 males; n=4 for

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Lmx1b$^{f/f/p}$, 2 females, 2 males; n=6 for *Tph2*$^{+/+}$, 5 females, 1 males; n=7 for *Tph2*$^{-/-}$, 7 females, 0 males), Figure 2 (n=7 for *Lmx1b*$^{f/f}$, 7 females, 0 males; n=5 for *Lmx1b*$^{f/f/p}$, 3 females, 2 males; n=7 for *Tph2*$^{+/+}$, 6 females, 1 males; n=9 for *Tph2*$^{-/-}$, 8 females, 1 males), Figure 3 (RT: n=16 for *Lmx1b*$^{f/f}$, 12 females, 4 males; n=12 for *Lmx1b*$^{f/f/p}$, 6 females, 6 males; n=36 for *Tph2*$^{+/+}$, 29 females, 7 males; n=33 for *Tph2*$^{-/-}$, 27 females, 6 males. 33C: n=15 for *Lmx1b*$^{f/f}$, 9 females, 6 males; n=13 for *Lmx1b*$^{f/f/p}$, 7 females, 6 males; n=30 for *Tph2*$^{+/+}$, 21 females, 9 males; n=29 for *Tph2*$^{-/-}$, 20 females, 9 males), Figure 4 (all mice for EEG were male).

Core body temperature and gross motor activity measurement

The core body temperature and gross motor activity were measured by G2 E-Mitter implantable telemetry, acquired by ER4000 energizer/receiver, and analyzed by VitalView software (Starr Life Science, Oakmont, PA, USA). Mice were anesthetized with tribromoethanol (250 mg/kg, i.p.) and electrodes were implanted intraperitoneally at the abdomen. Mice were individually housed and recovered for 1 week before recording. Data were recorded every 6 minutes during experiments. Mice were provided with free access to food and water during recording. For experiments at 4°C, the *Lmx1b* mice were recorded in the cold room for at most 5

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hours (and *Tph2* mice for 9 hours) if they had not been removed because of drop in body temperature.

Video recording and processing

Mice were recorded in their home cages for three days and data acquired in the last 24 hours were used for analysis. For experiments at 33°C, mice were kept in climate chamber (MGC-450HP-2, Yiheng Instruments, Shanghai) with 12-12hr dark-light cycles and adequate food and water supply. Videos were captured at 1 frame per second (fps) and processed with customize-written software. Briefly, the scale of each video was normalized according to standard grid, the region of interest was specified by hand, the threshold to distinguish mouse from background was each selected from a binary representation of the original image and the centroid of each mouse was automatically calculated and traced for activity. Only mice not moving for consecutive 40 seconds were considered inactive according to previous studies (Pack et al., 2007), otherwise active or between activity bouts. Both the latter two states were counted as active.

EEG/EMG recording and processing

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Mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and chronically implanted with EEG and EMG electrodes for polysomnographic recordings according to previous studies (Qu et al., 2010). Two stainless steel screws (1 mm in diameter) were bilaterally inserted through the skull into the cortex (1.0 mm anteriorly from Bregma and 1.5 mm laterally to both sides from midline) according to the atlas (Paxinos and Franklin, 2008) to serve as EEG electrodes. Two Teflon-coated insulated stainless steel wires were placed bilaterally into trapezius muscles to serve as EMG electrodes. All electrodes were attached to a microconnector and fixed to the skull with dental cement.

The cable was connected through a slip ring to enable recording from free moving animals. Mice were individually housed for 10 days to recover, and allowed to habituate to the recording cable for 3~4 days before polygraphic recording started. Each animal was recorded for 24 hours from the onset of dark phase at 7:00 p.m.

EEG and EMG signals were amplified and filtered (EEG, 0.5-30 Hz; EMG, 20-200 Hz), digitized at a sampling rate of 128 Hz and recorded by using SLEEPSIGN (Kissei Comtec) as described earlier (Huang et al., 2005). The processed polygraphicsignals were then automatically scored off-line by 4s epochs as wakefulness, REM, and NREM sleep by SLEEPSIGN according to standard criteria (Huang et al., 2005; Kohtoh et

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al., 2008). The defined sleep-wake states were finally examined manually, and corrected, if necessary.

The Mann-Whitney test was carried out with GraphPad Prism.

RESULTS

Neither *Lmx1b*$^{f/f/p}$ nor *Tph2*$^{-/-}$ mice significantly differ in body temperature from wild type mice

When serotonergic neurons were depleted with *Lmx1b-floxp* crossed to *ePet1-cre* (*Lmx1b*$^{f/f/p}$), mice were found to have higher body temperature in the dark phase than *Lmx1b-floxp (Lmx1b*$^{f/f}$*)* controls during 12:12 hour dark:light cycles (Hodges et al., 2008). It was not possible to rule out whether this was attributable to heat generated from elevated activity.

To examine whether *Tph2* deficient mice were abnormal in thermoregulation, we measured the body temperature and activities of both *Lmx1b*$^{f/f/p}$ and *Tph2* deficient mice by implantable emitter telemetry during a 24-hour light-dark cycle. All four groups of mice show fluctuations in body temperature, indicated as core temperature, within the 24-hour period: higher during the dark phase and lower during the light phase (Figure1). Different from the previous report (Hodges et al., 2008), we did not observe significant difference in body temperature

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between *Lmx1b*$^{f/f}$ versus *Lmx1b*$^{f/f/p}$ mice (Figure 1A and 1B), nor between *Tph2*$^{+/+}$and *Tph2*$^{-/-}$ mice (Figure 1C and 1D).

Movement of each mouse was measured as an indicator for activity simultaneously with the body temperature. All four groups of mice exhibited higher activity levels during the dark phase than those in the light phase (Figure 1). *Lmx1b*$^{f/f/p}$ mice exhibited higher level of cumulative activity than *Lmx1b*$^{f/f}$ controls during both the dark and the light phases (Figure 1A and 1B), whereas the cumulative activity of *Tph2*$^{-/-}$mice was only higher than the *Tph*$^{+/+}$ during the light phase (Figure 1Cand 1D,), when nocturnal animals spend most of the time sleeping. Elevated activities were also observed in previous studies (Buchanan and Richerson, 2010; Hodges et al., 2008) and were suggested to increase the body temperature. This explanation is not consistent with our results, because neither *Lmx1b*$^{f/f/p}$ nor *Tph2*$^{-/-}$mice had elevated body temperature when compared with their controls.

Neither *Lmx1b*$^{f/f/p}$ nor *Tph2*$^{-/-}$ mice exhibited excessive activity during cold challenge despite the failure of *Lmx1b*$^{f/f/p}$ to maintain body temperature

To test the hypothesis that cold stress is the cause of elevated activity in the*Lmx1b*$^{f/f/p}$ deficient as suggested in the previous paper (Buchanan and Richerson, 2010), we measured the body temperature and locomotion

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of all four groups of mice at 4°C for 5 hours. *Lmx1b*$^{f/f/p}$ mice indeed failed to maintain the body temperature which dropped from 36.7±0.3°C to around 25°C (Figure 2A and 2C). By contrast, *Tph2*$^{-/-}$ mice were able to maintain their body temperature within the same range as the *Tph2*$^{+/+}$ mice (Figure 2D and 2F), suggesting that molecules other than 5-HT is involved in thermoregulation. Furthermore, we did not observe significantly elevated activity in either *Lmx1b*$^{f/f/p}$ or *Tph2*$^{-/-}$ mice compared to their controls (Figure2B and 2C, 2E and 2F), which is against the hypothesis that excessive activities are responsible for counteracting body temperature loss.

Increased activity in *Lmx1b*$^{f/f/p}$mice was not diminished at elevated ambient temperature

Since telemetry only offered a gross indicator of activity by recording the number of times that animals changed their locations, we also measured locomotion by video recording. Each group of mice were recorded in their home cages at either the room temperature or 33°C for a 12:12 hour dark:light cycle. All mice retained normal 24-hour circadian cycles with more activities during the dark phase than the light phase. At the room temperature, both *Lmx1b*$^{f/f/p}$ and *Tph2*$^{-/-}$ mice continuously displayed more activities than their controls. *Lmx1b*$^{f/f/p}$ mice moved a distance of 134.1±13.1 m whereas *Lmx1b*$^{f/f}$ mice moved 30.9±5.0 m

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during the dark phase, and 27.9±3.5 versus 7.8±1.3 m during the light phase (Figure 3A and 3B), and also spent more time moving than *Lmx1b*$^{f/f}$ (Figure 3C and 3D). Similarly, the distances travelled by *Tph2*$^{-/-}$ versus *Tph2*$^{+/+}$ mice (Figure 3E and 3F) and duration of locomotion differed significantly (Figure 3G and 3H). Similar to a recent publication using adult-specific conditional knockout of *Tph2* gene (Whitney et. al., 2016), the active state of *Tph2*$^{-/-}$ lasted for more than an hour after the light was on, whereas it was not observed in *Lmx1b*$^{f/f/p}$mice at 25°C (Figure 3A, 3C, 3E and 3G).

In contrast to the previous report of thermo-sensitive sleep recovery of *Lmx1b*$^{f/f/p}$ mice at 33°C vs the room temperature (Buchanan and Richerson, 2010), we observed that *Lmx1b*$^{f/f/p}$ mice displayed higher activity levels during both the dark and light phases at 33°C (Figure 3I-3J). Similar to our finding with telemetry (Figure 1), *Tph2*$^{-/-}$ mice only exhibited elevated activity during the light phase at 33°C (Figure 3M-3P). There was no significant differences in distance or duration during the dark phase at 33°C (Figure3M-3P). The diminished difference in locomotion between *Tph2*$^{-/-}$ and *Tph2*$^{+/+}$ mice during the dark phase was not due to decreased activity with *Tph2*$^{-/-}$, because the locomotion distances travelled at higher temperature increased with both *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ groups but the increase with *Tph2*$^{+/+}$ mice was significantly larger (Figure S1). While *Lmx1b*$^{f/f/p}$ mice exhibited prolonged activity at 33°C,

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Tph2$^{-/-}$ mice displayed even higher level of activity after light on (Figure 3I, 3K, 3M and 3O).

REM sleep was decreased in *Tph2*$^{-/-}$ mice

To directly verify whether 5-HT was involved in regulating sleep, we recorded the EEG/EMG to distinguish wakefulness, NREM and REM sleep in *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ mice for 24-hr cycle at either the room temperature or 33°C. The EEG signal is high in frequency with low in voltage during wakefulness and REM sleep and these features are reversed during NREM sleep with low frequency and high voltage. The EMG signal is high during wakefulness and low during both REM and NREM sleep, which, when combined with the EEG signal, unambiguously distinguishes the three states of sleep-wake cycle.

To our surprise, at the room temperature, *Tph2*$^{-/-}$ mice did not exhibit significantly more wakefulness than *Tph2*$^{+/+}$ mice (Figure 4A-4C). Neither did they differ in NREM sleep (Figure 4A-4D). At the room temperature, *Tph2*$^{-/-}$ mice showed significantly less REM than *Tph2*$^{+/+}$ during the light phase but not the dark phase (Figure 4A, 4B and 4E).

At 33°C, interestingly, the differences of wakefulness, NREM and REM become larger between *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ mice. During the dark phase, *Tph2*$^{-/-}$ mice stayed awake for longer period (Figure 4F-H) and spent less time in both NREM (Figure 4F, 4G and 4I) and REM sleep

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(Figure 4F, 4G and 4J). During the light phase, *Tph2*$^{-/-}$ mice also had less REM sleep than the *Tph2*$^{+/+}$ (Figure 4F, 4G and 4J). We did not observe significant difference in the amount of wakefulness (Figure 4F-4H) or NREM sleep (Figure 4F, 4G and 4I) during the light phase at 33°C. These results indicate that the sleep phenotype of *Tph2*$^{-/-}$ mice is present at 33°C.

DISCUSSION

5-HT and central serotonergic neurons in the raphe nuclei have been implicated in multiple physiological processes, which complicated the endeavor to distinguish one role from another, and also making it confusing whether a role was played by 5-HT or by serotonergic neurons but with other transmitters. The function of 5-HT in sleep-wake regulation has remained under debate for many years, with recent papers concluding that the apparent sleep phenotype in mice lacking serotonergic neurons was explained by the primary role of serotonergic neurons in thermoregulation (Buchanan and Richerson, 2010). Here we provide multiple pieces of evidence indicating that the role of 5-HT in sleep is not attributable to its role in thermoregulation.

Mice depleted of serotonergic neurons were found to be defective in body temperature control as they were not able to keep normal temperature during coldness challenge (Hodges et al., 2008). However, a

paper with a *Tph2*$^{-/-}$ strain, which deleted the coding part of *Tph2* gene exon 1 and 2, did not report body temperature changes in *Tph2* deficient mice (Alenina et al., 2009). We utilized two types of mouse mutants to study the roles of serotonergic neurons and 5-HT in sleep regulation. One of them, *Lmx1b*$^{f/f/p}$ crossed with *ePet1-cre*, is the same as has been studied previously (Buchanan and Richerson, 2010). Mice lacking serotonergic neurons showed higher body temperature during the dark phase and were unable to maintain normal body temperature when challenged by coldness at 4°C. We also found that the temperature of *Lmx1b*$^{f/f/p}$ mice dropped dramatically at 4°C within 5 hours (Figure 2). Since hindbrain *ePet1*-positive neurons also contain other neurotransmitters such as glutamate and neuropeptides such as substance P, we utilized *Tph2*$^{-/-}$ mice to study the role of 5-HT (Zhao et al., 2006). We found that the body temperature of *Tph2*$^{-/-}$ mice did not differ from that of wild type (WT) mice and remained at the normal range at 4°C. Another study found that, while the body temperature of the *Tph2*$^{-/-}$ mutants dropped lower than the WT at the beginning of cold challenge, it recovered slowly to the same level as the WT at the end of the 4th hour (Alenina, 2009), supporting that mice lacking 5-HT were not defective in thermoregulation.

Previous work with *Lmx1b*$^{f/f/p}$ mice demonstrated that increased wakefulness and decreased sleep with the *Lmx1b*$^{f/f/p}$ disappeared as the ambient temperature increased from 24°C to 33°C, leading to the

conclusion that elevated activity and reduced sleep was due to deregulation of body temperature and perception of 24°C as cold stress. However, our results do not support this conclusion. First of all, another study from the same group demonstrated that *Lmx1b*$^{f/f/p}$ mice preferred ambient temperature of 30.6°C which was similar to the *Lmx1b*$^{f/f}$ (Hodges et al., 2008), suggesting that temperature perception in *Lmx1b*$^{f/f/p}$ mice was not different from the WT. Secondly, despite of decreased body temperature at 4°C, no elevated activity of *Lmx1b*$^{f/f/p}$ mice was observed by us during cold challenge (Figure 2), raising the possibility that activity was not increased in these mice to generate heat. Moreover, *Lmx1b*$^{f/f/p}$ mice exhibited higher level of locomotion activity at both room temperature and 33°C. Similar to a recent publication with the *Tph2* gene conditionally deleted in the raphe nuclei of adult mice (Whitney *et al*., 2016), the *Tph2*$^{-/-}$ mice exhibited higher activity level in locomotion and activity time during both the dark and light phases at the room temperature, while elevation was only observed during the light phase at 33°C (Figure 3). Activity was increased in *Tph2*$^{-/-}$ mice as well as *Lmx1b*$^{f/f/p}$ at both the normal and warm ambient temperatures, suggesting a direct role of 5-HT in sleep-wake regulation. These results demonstrated that absence of 5-HT did not dramatically disrupt body temperature control. Therefore, the elevated activity in *Tph2*$^{-/-}$ mice could not be attributed to hypothermia. Thirdly, the EEG/EMG measurements

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further support our point. Compared to *Tph2*$^{+/+}$ mice, REM duration was reduced in *Tph2*$^{-/-}$ mice both at the room temperature and 33°C (Figure 4). The percentage of wakefulness and NREM sleep duration is unclear. We only observed marginal difference between *Tph2*$^{-/-}$ and *Tph2*$^{+/+}$ mice at the room temperature. It was not significant until the ambient temperature was 33°C and only during the dark phase. The different tendency of changes from the room temperature to 33°C between activity and sleep/wake duration may indicate an increase in passive wakefulness with *Tph2*$^{-/-}$ while increased activity with *Tph2*$^{+/+}$at warm temperatures.

Serotonergic receptors have been studied for their roles in sleep regulation (reviewed in Monti e*t al*., 2010). For example, 5-HT_{1A} receptor is thought to act as a pre-synaptic self-inhibitory receptor, and REM sleep was increased in mouse mutants lacking 5-HT_{2A}, which was opposite to the effect of 5-HT depletion (Boutrel *et al*., 2002). NREM sleep was decreased, wakefulnees was increased, but REM sleep was not affected in 5-HT_{2A} mutant mice (Popa *et al*., 2005). Inhibition of 5-HT_{1A} receptors by antagonist WAY100635 increased REM sleep whereas the agonist 8-OH-DPAT decreased REM sleep, both in mice and cats (Portas *et al*., 1996; Boutrel *et al*., 2002). REM was increased in mice lacking 5-HT_{1B} (Boutrel *et al*., 1999), which is thought to be able to act as an inhibitory receptor. Wakefulness was increased, NREM decreased, but REM unaffected in mutants lacking the 5-HT_{2C} receptor (Frank *et al*., 2002),

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which is similar to the sleep phenotype of mice lacking 5-HT_{2A}. REM was decreased in mice lacking the 5-HT_7 receptor (Hedlund *et al*., 2005). In the future, it will be ideal to study mutants of 5-HT receptors of identical genetic background and with identical methods under the same conditions.

In summary, we found that dysfunction in thermoregulation could not explain the elevated wakefulness and decreased sleep level in animals lacking 5-HT, in that a) animals deficient in 5-HT synthesis were able to maintain body temperature in cold environment; b) animals depleted of serotonergic neurons preferred the same ambient temperature as the wt mice; c) animals lacking serotonergic neurons exhibited more activity at an elevated ambient temperature; d) animals deficient in 5-HT synthesis woke more and slept less even at an elevated ambient temperature. Thus, our study supports a role of 5-HT in sleep-wake regulation independent of thermoregulation.

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Authorship Contribution

Participated in research design: YR, ZLH, XZ, HMY

Conducted experiments: HMY, XZ, YJL

Performed data analysis: HMY, XZ, YJL

Wrote or contributed to the writing of the manuscript: XZ, HMY, YR

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Footnotes

The first two authors are co-first authors.

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Figure Legends

Figure 1. Both *Lmx1b*$^{f/f/p}$ and *Tph2*$^{-/-}$ mice exhibited elevated locomotion but maintained body temperature

(A) Mean body temperature (T_{core}; top) and activity [arbitrary units (A.U.); bottom] of *Lmx1b*$^{f/f/p}$ (red) versus *Lmx1b*$^{f/f}$ (blue) mice over 24h at the room temperature (RT).

(B) Mean T_{core} and activity by phase (light and dark) of *Lmx1b* mice at RT (p=0.0061 for both dark and light, Mann-Whitney tests; n=7 for *Lmx1b*$^{f/f}$, n=4 for *Lmx1b*$^{f/f/p}$; Data are mean±sem).

(**C**) T_{core}(top) and activity (arbitrary units bottom) of *Tph2*$^{-/-}$ (red) versus *Tph2*$^{+/+}$ (blue) mice over 24h atRT.

(**D**) Mean T_{core} and activity by phase (light and dark) of *Tph2* mice at RT (p=0.0082, Mann-Whitney test for the light phase; n=6 for *Tph2*$^{+/+}$; n=7 for *Tph2*$^{-/-}$; Data are mean±sem).

Figure 2. Body temperature was maintained by *Tph2*$^{-/-}$ mice but not by *Lmx1b*$^{f/f/p}$ mice when the ambient temperature was 4°C

(**A**) T_{core} of *Lmx1b*$^{f/f/p}$ (red) versus *Lmx1b*$^{f/f}$ (blue) mice recorded for 5hrs at 4°C (mean±sem, sem indicated in gray). (**B**) Activity of *Lmx1b*$^{f/f/p}$ (red) versus *Lmx1b*$^{f/f}$ (blue) mice recorded for 5hrs at 4°C (mean±sem, sem indicated in gray). (**C**) Mean T_{core} and activity of *Lmx1b*mice at 4°C (for

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mean T_{core}, p<0.01, Mann-Whitney test; n=7 for *Lmx1b*$^{f/f}$, n=5 for *Lmx1b*$^{f/f/p}$; data are mean±sem). (**D**) T_{core} of *Tph2*$^{-/-}$ (red) versus *Tph2*$^{+/+}$ (blue) mice recorded for 5hrs at 4°C (mean±sem, sem indicated in gray). (**E**) Activity of*Tph2*$^{-/-}$ (red) versus *Tph2*$^{+/+}$(blue) micerecorded for 5hrs at 4°C (mean±sem, sem indicated in gray). (**F**) Mean T_{core} and activity of *Lmx1b*mice at 4°C(n=7 for *Tph2*$^{+/+}$, n=9 for *Tph2*$^{-/-}$; data are mean±sem).

Figure 3. **Both *Lmx1b*$^{f/f/p}$ and *Tph2* mice exhibited elevated activity at room temperature (RT) and 33°C**

(**A** and **B**) Mean locomotion distance of *Lmx1b*$^{f/f/p}$ (red) versus *Lmx1b*$^{f/f}$ (blue) mice at RT (p<0.0001 for both dark and light phase in B, Mann-Whitney tests; data are mean±sem). (**C** and **D**) Mean active time of *Lmx1b*$^{f/f/p}$ (red) versus *Lmx1b*$^{f/f}$ (blue) mice at RT (p<0.0001 for dark phase and p=0.0112 for light phase in D, Mann-Whitney tests; data are mean±sem; n=16 for *Lmx1b*$^{f/f}$, n=12 for *Lmx1b*$^{f/f/p}$). (**E** and **F**) Mean locomotion distance of*Tph2*$^{-/-}$(red) versus *Tph2*$^{+/+}$ (blue) mice at RT (p=0.0002 for dark and p0.0006 for light phase in F, Mann-Whitney tests; data are mean±sem). (**G** and **H**) Mean active time of *Tph2*$^{-/-}$(red) versus *Tph2*$^{+/+}$ (blue) mice at RT (p=0.0005 for dark phase and p<0.0001 for light phase in H, Mann-Whitney tests; data are mean±sem; n=36 for *Tph2*$^{+/+}$, n=33 for *Tph2*$^{-/-}$). (**I** and **J**) Mean locomotion distance of *Lmx1b*$^{f/f/p}$ (red)

versus *Lmx1b*$^{f/f}$ (blue) mice at 33°C ($p<0.01$ for both dark and light phase in J, Mann-Whitney tests; data are mean±sem). (**K** and **L**) Mean active time of *Lmx1b*$^{f/f/p}$ (red) versus *Lmx1b*$^{f/f}$ (blue) miceat 33°C ($p<0.01$ for both dark and light phase in L,Mann-Whitney tests; data are mean±sem;n=15 for *Lmx1b*$^{f/f}$, n=13 for *Lmx1b*$^{f/f/p}$). (**M** and **N**) Mean locomotion distance of *Tph2*$^{-/-}$(red) versus *Tph2*$^{+/+}$ (blue) mice at 33°C ($p<0.01$ for the light phase in N, Mann-Whitney tests; data are mean±sem). (**O** and **P**) Mean active time of *Tph2*$^{-/-}$(red) versus *Tph2*$^{+/+}$ (blue) mice at 33°C ($p<0.01$ for the light phase in P, Mann-Whitney tests; data are mean±sem;n=30 for*Tph2*$^{+/+}$, n=29 for *Tph2*$^{-/-}$). For A, C, E, G, I, K, M, and O: *, $p<0.05$; ‡, $p<0.01$; †, $p<0.001$; #, $p<0.0001$; Mann-Whitney tests.

Figure 4. **Analysis of EEG/EMG patterns reveals that *Tph2*$^{-/-}$ mice exhibited less sleep both at RT and 33°C**

(**A** and **B**) Examples of time spent on wake, NREM and REM by *Tph2*$^{+/+}$ (A) and *Tph2*$^{-/-}$ (B) over 24 hrs at RT. (**C**-**E**) Mean percentage of wake (C), NREM (D) and REM (E) by phase (light and dark) at RT($p<0.01$ for light phase in E, Mann-Whitney test; data are mean±sem; n=20 for *Tph2*$^{+/+}$, n=22 for *Tph2*$^{-/-}$). (**F** and **G**) Examples of time spent on wake, NREM and REM by *Tph2*$^{+/+}$ (F) and *Tph2*$^{-/-}$ (G) over 24 hrs at 33°C. (**H**-**J**) Mean percentage of wake

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(H), NREM (I) and REM (J) by phase (light and dark) at 33°C ($p<0.01$ for dark phase in H, $p<0.01$ for dark phase in I, $p<0.01$ for both dark and light phase in J; Mann-Whitney test; data are mean±sem; n=20 for *Tph2*$^{+/+}$, n=21 for *Tph2*$^{-/-}$).

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Fig 1

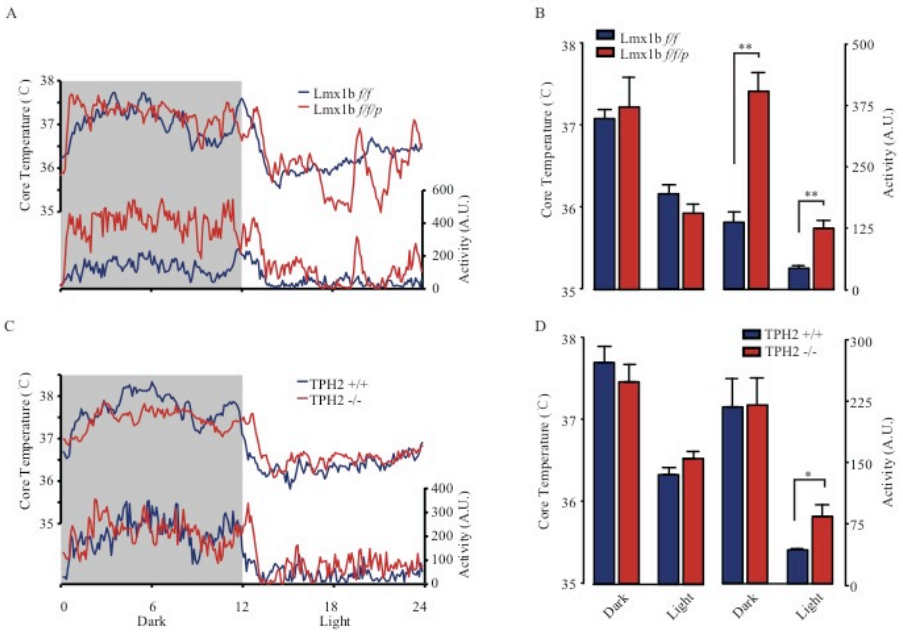

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Fig 2

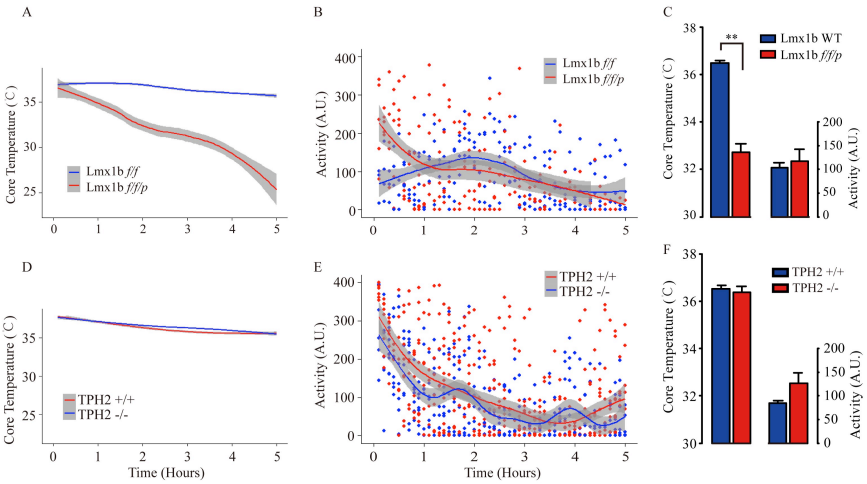

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Fig 3

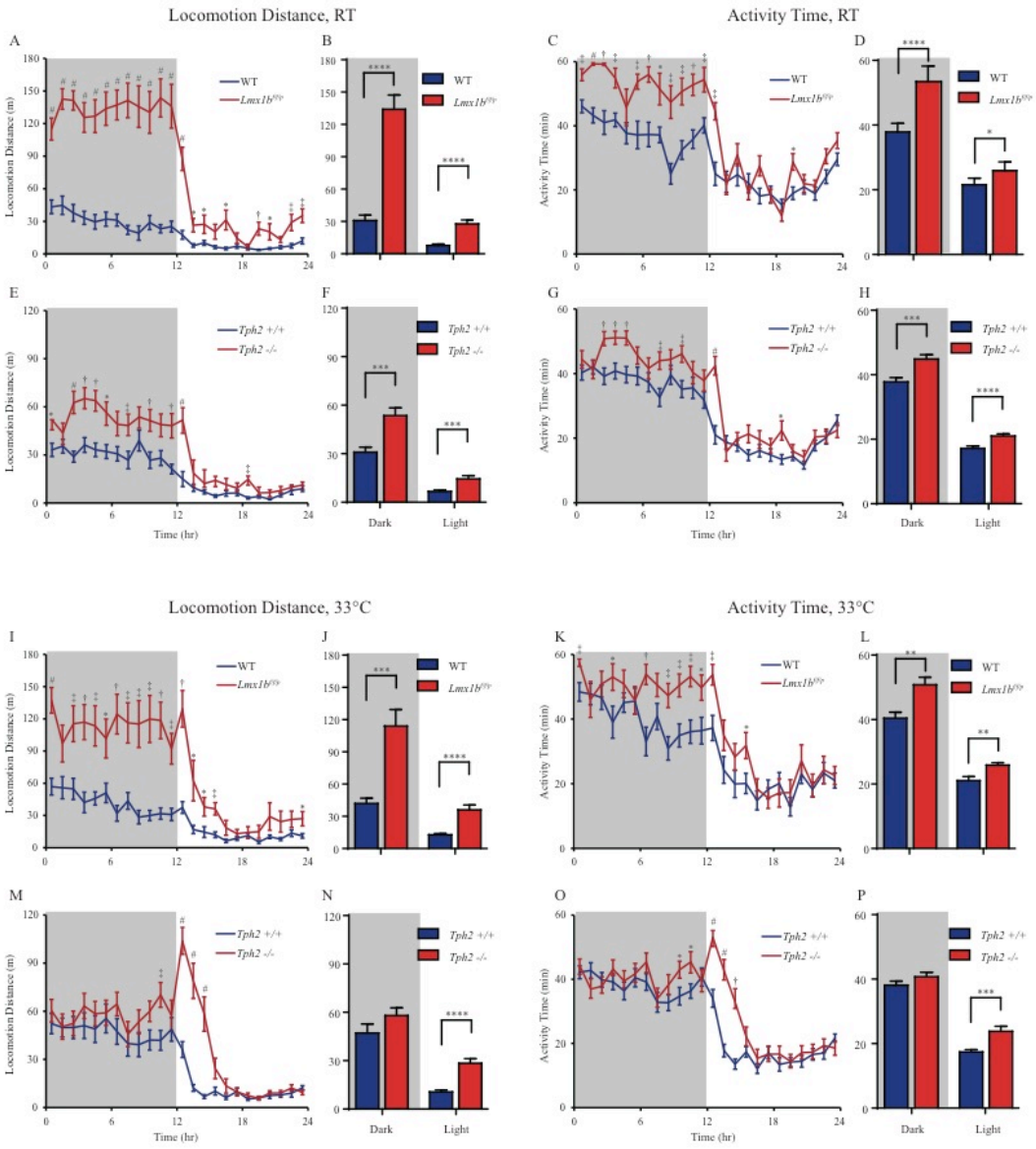

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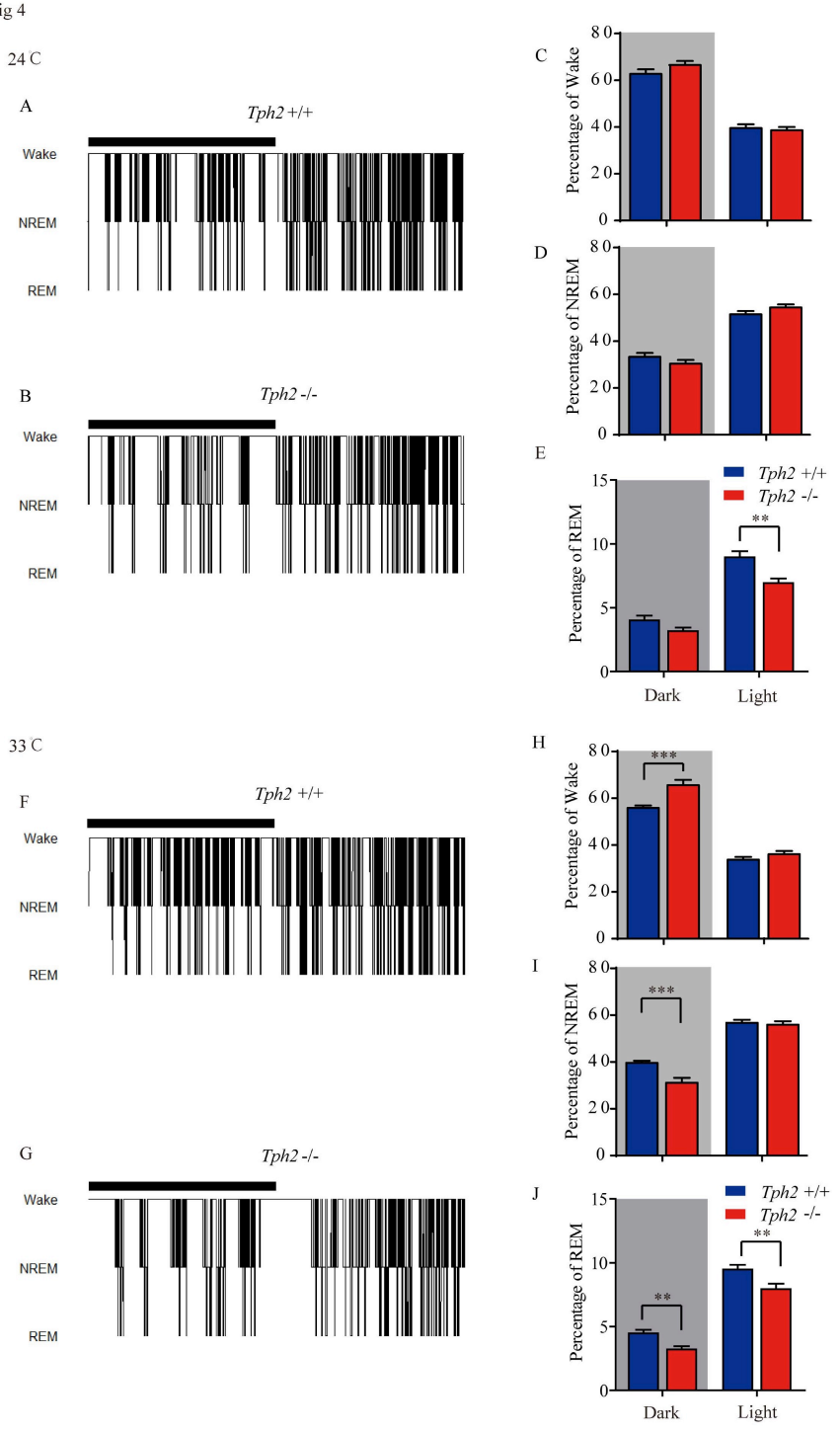
Fig 4



Supplementary Figure 1 for Zhang et al.

Thermoregulation-Independent Regulation of Sleep by Serotonin Revealed in Mice Defective in Serotonin Synthesis.

Molecular Pharmacology

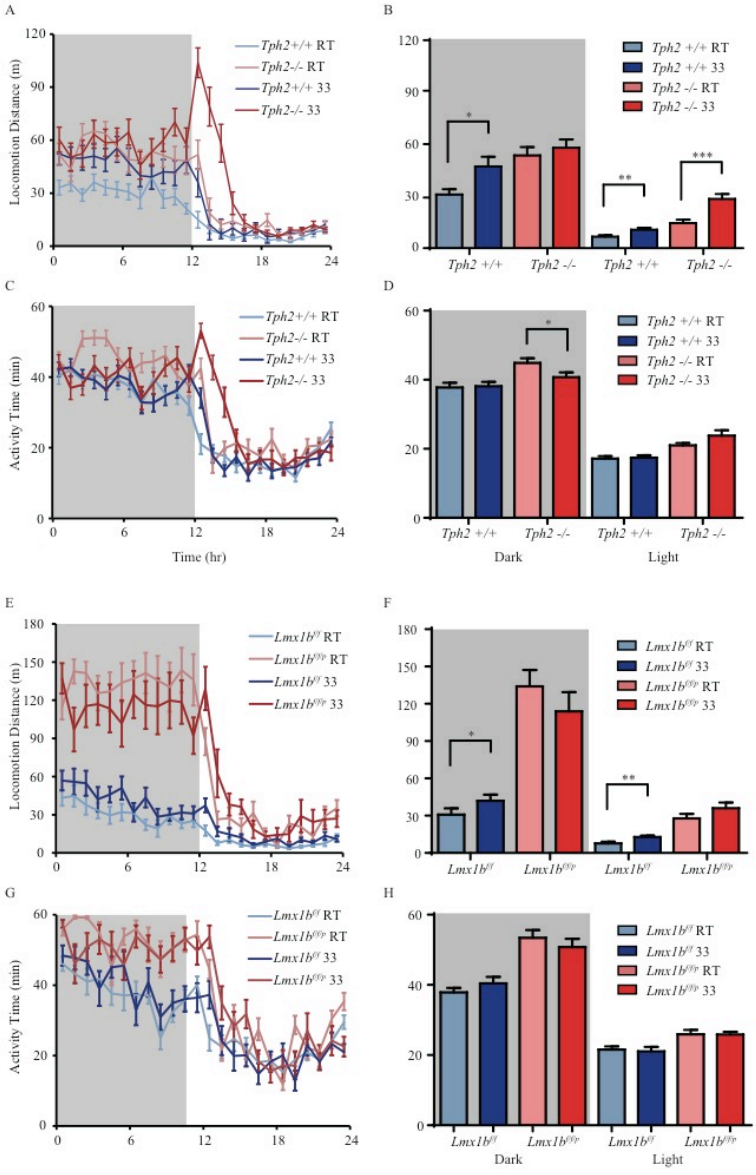


Figure S1**.** Comparison of locomotion activity of mice with same genotype between room temperature and 33°C. A-D, Locomotion distance (A) and activity time (C) of *Tph2* mice over 24 hrs. Mean locomotion distance was significantly increased at 33°C during both dark (p=0.0145) and light phase (p=0.0043) with wt mice and only during light phase (p=0.0002) with $Tph2^{-/-}$ mice (B). Mean activity time was significantly decreased at 33°C during dark phase (p=0.0384) with $Tph2^{-/-}$ mice (D). *E-H*, Locomotion distance (E) and activity time (G) of *Lmx1b* mice over 24 hours. Mean locomotion distance was increased at 33°C during both dark (p=0.0488) and light phase (p=0.0026) with $Lmx1b^{f/f}$ mice (F). Mean activity time did not differ significantly between RT and 33°C with both $Lmx1b^{f/f}$and $Lmx1b^{f/f/p}$ mice (H). Data are mean±sem. Unpaired t-test with Welch's correction for *Tph2* mice and Mann-Whitney test for *Lmx1b* mice (n=30 for $Tph2^{+/+}$; n=29 for $Tph2^{-/-}$;n=15for $Lmx1b^{f/f}$;, n=13 for $Lmx1b^{f/f/p}$).