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Distinct ipRGC subpopulations mediate light's acute and circadian effects on body temperature and sleep

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1 **Abstract**

2 The light environment greatly impacts human alertness, mood, and cognition by acute
3 regulation of physiology and indirect alignment of circadian rhythms. Both processes
4 require the melanopsin-expressing intrinsically photosensitive retinal ganglion cells
5 (ipRGCs), but the relevant downstream brain areas remain elusive. ipRGCs project
6 widely in the brain, including to the central circadian pacemaker, the suprachiasmatic
7 nucleus (SCN). Here we show that body temperature and sleep responses to light are
8 absent after genetic ablation of all ipRGCs except a subpopulation that projects to the
9 SCN. Furthermore, by chemogenetic activation of the ipRGCs that avoid the SCN, we
10 show that these cells are sufficient for acute changes in body temperature. Our results
11 challenge the idea that the SCN is a major relay for the acute effects of light on non-
12 image forming behaviors and identify the sensory cells that initiate light's profound
13 effects on body temperature and sleep.

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1 **Main body**

2 **Introduction**

3 Many essential functions are influenced by light both indirectly through alignment
4 of circadian rhythms (photoentrainment) and acutely by a direct mechanism (sometimes
5 referred to as 'masking') (Mrosovsky et al., 1999; Altimus et al., 2008; Lupi et al., 2008;
6 Tsai et al., 2009; Legates et al., 2012). Dysregulation of the circadian system by
7 abnormal lighting conditions has many negative consequences, which has motivated
8 decades of work to identify the mechanisms of circadian photoentrainment (Golombek
9 and Rosenstein, 2010). In contrast, it has only recently become apparent that light
10 exposure can also acutely influence human alertness, cognition, and physiology
11 (Chellappa et al., 2011). As a result, there is a developing awareness of light quality in
12 everyday life (Lucas et al., 2014). It is therefore essential to human health and society to
13 elucidate the circuitry and coding mechanisms underlying light's acute effects.

14 Intriguingly, a single population of retinal projections neurons—intrinsically
15 photosensitive retinal ganglion cells (ipRGCs)—have been implicated in the circadian
16 and acute effects of light on many functions, including activity, sleep, and mood (Göz et
17 al., 2008; Güler et al., 2008; Hatori et al., 2008; Legates et al., 2012; Fernandez et al.,
18 2018). ipRGCs integrate light information from rods, cones, and their endogenous
19 melanopsin phototransduction cascade (Schmidt et al., 2011), and relay that light
20 information to over a dozen central targets (Hattar et al., 2006; Ecker et al., 2010).
21 However, the circuit mechanisms mediating ipRGC-dependent functions are largely
22 unknown.

23 One notable exception is the control of circadian phototentrainment. It is
24 accepted that ipRGCs mediate photoentrainment by direct innervation of the master

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1 circadian pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus (Göz et
2 al., 2008; Güler et al., 2008; Hatori et al., 2008; Jones et al., 2015). This is supported by
3 studies demonstrating that genetic ablation of ipRGCs results in mice with normal
4 circadian rhythms that 'free-run' with their endogenous rhythm, independent of the
5 light/dark cycle (Göz et al., 2008; Güler et al., 2008; Hatori et al., 2008). Further, mice
6 with genetic ablation of all ipRGCs except those that project to the SCN and
7 intergeniculate leaflet (IGL) display normal circadian photoentrainment (Chen et al.,
8 2011), suggesting that ipRGC projections to the SCN/IGL are sufficient for
9 photoentrainment.

10 In comparison, the mechanisms by which ipRGCs mediate acute light responses
11 remains largely a mystery. Genetic ablation of ipRGCs or their melanopsin
12 phototransduction cascade blocks or attenuates the acute effects of light on sleep
13 (Altimus et al., 2008; Lupi et al., 2008; Tsai et al., 2009), wheel-running activity
14 (Mrosovsky and Hattar, 2003; Güler et al., 2008), and mood (Legates et al., 2012;
15 Fernandez et al., 2018). This dual role of ipRGCs in circadian and acute light responses
16 suggests they may share a common circuit mechanism. However, whether the circuit
17 basis for ipRGCs in the acute effects of light and circadian functions is through common
18 or divergent pathways has yet to be determined. ipRGCs project broadly in the brain
19 beyond the SCN (Hattar et al., 2002, 2006; Gooley et al., 2003; Baver et al., 2008).
20 Additionally, ipRGCs are comprised of multiple subpopulations with distinct genetic,
21 morphological, and electrophysiological signatures (Baver et al., 2008; Schmidt and
22 Kofuji, 2009; Ecker et al., 2010; Schmidt et al., 2011) and distinct functions (Chen et al.,
23 2011; Schmidt et al., 2014). Though there are rare exceptions (Chen et al., 2011;

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1 Schmidt et al., 2014), the unique roles played by each ipRGC subsystem remain largely
2 unknown.
3 It is currently unknown whether distinct ipRGC subpopulations mediate both the
4 acute and circadian effects of light, and two major possibilities exist for how this occurs:
5 (1) ipRGCs mediate both acute and circadian light responses through their innervation of
6 the SCN or (2) ipRGCs mediate circadian photoentrainment through the SCN, but send
7 collateral projections elsewhere in the brain to mediate acute light responses. To date,
8 the predominant understanding has centered on a role for the SCN in both acute and
9 circadian responses to light (Muindi et al., 2014; Morin, 2015). However, this model has
10 been controversial due to complications associated with SCN lesions (Redlin and
11 Mrosovsky, 1999) and alternative models proposing a role for direct ipRGC input to other
12 central targets (Redlin and Mrosovsky, 1999; Lupi et al., 2008; Tsai et al., 2009;
13 Hubbard et al., 2013; Muindi et al., 2014). Here, we sought to address the question of
14 how environmental light information—through ipRGCs—mediates both the circadian and
15 acute regulation of physiology. To do so, we investigated the ipRGC subpopulations and
16 coding mechanisms that mediate body temperature and sleep regulation by light. We
17 find that a molecularly distinct subset of ipRGCs is required for the acute, but not
18 circadian, effects of light on thermoregulation and sleep. These findings suggest that,
19 contrary to expectations, functional input to the SCN is not sufficient to drive the acute
20 effects of light on these behaviors. These findings provide new insight into the circuits
21 through which light regulates behavior and physiology.

22 **Results**

23 **Brn3b-positive ipRGCs are required for light's acute effects on thermoregulation**

24

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1 To identify mechanisms of acute thermoregulation, we maintained mice on a 12-
2 hr/12-hr light/dark cycle and then presented a 3-hr light pulse two hours into the night
3 (Zeitgeber time 14, ZT14) while measuring core body temperature (Fig. 1A). The
4 nocturnal light pulse paradigm is well-established for studying acute regulation of sleep
5 and wheel-running activity (Mrosovsky et al., 1999; Mrosovsky and Hattar, 2003; Altimus
6 et al., 2008; Lupi et al., 2008). We focused first on body temperature because of its
7 critical role in cognition and alertness (Wright et al., 2002; Darwent et al., 2010), sleep
8 induction and quality (Kräuchi et al., 1999), metabolic control (Kooijman et al., 2015) ,
9 and circadian resetting (Buhr et al., 2010).

10 Body temperature photoentrains to the light/dark cycle with peaks during the
11 night and troughs during the day (Fig. 1B). Both rodents and humans utilize ocular light
12 detection to acutely adjust body temperature in response to a nocturnal light pulse (Dijk
13 et al., 1991; Cajochen et al., 2005), though how this body temperature change is initiated
14 by the retina and relayed to the brain is unknown. When we presented wildtype mice
15 with a nocturnal light pulse, we observed a decrease in both body temperature and
16 general activity compared to the previous night (Fig. 1C). The decrease in body
17 temperature and activity was sustained for the entire 3-hr stimulus, with moderate
18 rundown (Fig. 1C).

19 We observed that acute body temperature regulation only occurred at relatively
20 bright light intensities (>100 lux) (Fig. S1). This, in combination with previous reports that
21 body temperature regulation is most sensitive to short-wavelength light (Cajochen et al.,
22 2005), suggested that it might be mediated by the insensitive and blue-shifted
23 melanopsin phototransduction (Lucas et al., 2001; Do et al., 2009). To test this, we
24 measured body temperature in mice lacking either functional rods and cones

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1 (melanopsin-only: *Gnat1*$^{-/-}$; *Gnat2*$^{-/-}$) or lacking melanopsin (melanopsin KO: *Opn4*$^{-/-}$).
2 Both genotypes photoentrained their body temperature (Fig. 1D,E), with an amplitude
3 indistinguishable from wildtype (Fig. 1F). However, we found that acute body
4 temperature decrease to a nocturnal light pulse was present in melanopsin-only mice
5 (*Gnat1*$^{-/-}$; *Gnat2*$^{-/-}$) (Fig. 1G,H), but absent from melanopsin knockout mice (*Opn4*$^{-/-}$) (Fig.
6 1I,J). This indicates that melanopsin is critical for light's ability to drive acute body
7 temperature decreases, as it is for acute sleep induction (Altimus et al., 2008; Lupi et al.,
8 2008; Tsai et al., 2009). These results suggest that ipRGCs are the only retinal cells that
9 are necessary and sufficient for acute thermoregulation by light.
10 ipRGCs comprise multiple subtypes with distinct gene expression profiles, light
11 responses, and central projections (Schmidt et al., 2011), prompting us to ask which
12 subtypes mediate acute thermoregulation. Brn3b(+) ipRGCs project to many structures
13 including the olivary pretectal nucleus (OPN) and dorsal lateral geniculate nucleus
14 (dLGN), but largely avoid the SCN (Chen et al., 2011). In contrast, Brn3b(–) ipRGCs
15 project extensively to the SCN and intergeniculate leaflet (IGL), while avoiding the OPN
16 and dLGN (Chen et al., 2011). Ablation of Brn3b(+) ipRGCs using melanopsin-Cre and a
17 Cre-dependent diphtheria toxin knocked into the *Brn3b* locus (Brn3b-DTA:
18 *Opn4*$^{Cre/+}$;*Brn3b*$^{zDTA/+}$) removes virtually all ipRGC input to brain areas aside from the
19 SCN and IGL, and these mice retain circadian photoentrainment of wheel-running
20 activity (Chen et al., 2011).
21 When we measured body temperature in Brn3b-DTA mice, we found that their
22 body temperature was photoentrained with a similar amplitude to controls (Fig. 2A–C).
23 However, despite the presence of melanopsin in Brn3b-DTA mice
24 (*Opn4*$^{Cre/+}$;*Brn3b*$^{zDTA/+}$), they did not acutely decrease body temperature in response to a
25 nocturnal light pulse (Fig. 2F,G). Importantly, melanopsin heterozygous littermate

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1 controls (*Opn4*$^{Cre/+}$) displayed normal acute thermoregulation by light (Fig. 2D,E),
2 indicating that halving melanopsin gene dosage is not the cause of the impaired body
3 temperature decrease in Brn3b-DTA mice. These results demonstrate that Brn3b(+)
4 ipRGCs are required for acute thermoregulation regulation by light but not
5 photoentrainment of body temperature and reveal that light information to the SCN is
6 sufficient for circadian photoentrainment of body temperature, but not its acute
7 regulation.
8
9 **Brn3b-positive ipRGCs are sufficient for acute thermoregulation**
10 Our data thus far suggest that there are two functionally distinct populations of
11 ipRGCs that regulate the same physiological function: (1) Brn3b(–) ipRGCs that project
12 to the SCN to mediate circadian photoentrainment of body temperature and (2) Brn3b(+)
13 ipRGCs that project elsewhere in the brain to mediate acute thermoregulation. To test if
14 Brn3b(+) ipRGCs are sufficient for acute thermoregulation, we expressed a
15 chemogenetic activator in Brn3b(+) RGCs (Fig. 3A, *Brn3b*$^{Cre/+}$ with intravitreal AAV2-
16 hSyn-DIO-hM3Dq-mCherry, we refer to these mice as Brn3b-hM3Dq. This technique
17 allows us to acutely activate the Brn3b(+) RGCs with the DREADD agonist clozapine N-
18 oxide (CNO) (Armbruster et al., 2007). We found that after intravitreal viral delivery,
19 many RGCs were infected, including melanopsin-expressing ipRGCs (Fig. 3A).
20 Importantly, Brn3b-hM3Dq mice photoentrained to a normal light/dark cycle (Fig.
21 3B). Following CNO administration at ZT14 to depolarize the Brn3b(+) RGCs, we
22 observed a robust decrease in body temperature that lasted at least 6 hours (Fig. 3D).
23 Importantly, PBS administration in Brn3b-hM3Dq mice (Fig. 3C) and nocturnal CNO
24 administration in wildtype control mice (Fig. S2) had no measurable effect on body
25 temperature. Together, these results demonstrate that Brn3b(+) ipRGCs mediate the

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1 acute effects of light on body temperature though extra-SCN projection(s), while Brn3b(–
2) ipRGCs mediate circadian photoentrainment by projections to the SCN and/or IGL.
3
4 **Brn3b-positive ipRGCs are required for light's acute effects on sleep**
5 We next examined the contribution of Brn3b(+) and Brn3b(-) ipRGCs to sleep. To
6 do this, we used EEG and EMG recordings to compare the sleep behavior of Control
7 (*Opn4*$^{Cre/+}$) and Brn3b-DTA mice. We first analyzed the daily sleep patterns and
8 proportion of rapid eye movement (REM) and non-REM (NREM) sleep in Control and
9 littermate Brn3b-DTA animals. We found that Brn3b-DTA mice show normal
10 photoentrainment of sleep and similar percent time of sleep across the 24 hour day, with
11 only one 30 minute bin at ZT12 (light offset) showing a significant difference between
12 Control and Brn3b-DTA animals (Fig. 4A,B). This is consistent with previous reports of
13 normal circadian photoentrainment of daily activity rhythms in Brn3b-DTA mice (Chen et
14 al., 2011). Control and Brn3b-DTA mice also showed similar total percent time awake or
15 asleep across an entire day (Fig. 4C), though Brn3b-DTA mice showed a small, but
16 significant, increase in the proportion of total sleep that was classified as NREM and
17 decrease in the proportion of total sleep that was classified as REM (Fig. S3A).
18 We hypothesized that this small difference in sleep at lights-off in Brn3b-DTA
19 mice could be due to a defect in their acute response to light for sleep modulation. To
20 test this, we subjected mice to a 3-hr light pulse from ZT14–17 (Altimus et al. 2008),
21 when the homeostatic drive for sleep is low and Control and Brn3b-DTA animals display
22 similar amounts of sleep (Fig. 4A,B). We found that in Control mice, a light pulse
23 decreased time awake and increased time asleep relative to baseline (previous day)
24 (Fig. 4C,D), while in Brn3b-DTA mice a light pulse caused no change in total percent
25 time asleep or awake (Figure 4F,G), but moderately increased sleep in the first 30-min
26 bin (Fig. 4F). Neither Control nor Brn3b-DTA animals showed any change in proportion

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1 of non-REM or REM sleep in response to the light pulse (Fig. S3B,C). These data show
2 that Brn3b(+) ipRGCs are necessary for the acute light induction of sleep. Consistent
3 with our body temperature data, although Brn3b-DTA mice have apparently normal input
4 to the SCN and show normal circadian photoentrainment of wheel-running activity (Chen
5 et al., 2011), body temperature (Fig. 2), and sleep (Fig. 4), this ipRGC innervation of the
6 SCN is not sufficient to drive the normal light induction of sleep. These disruptions in
7 light's acute effects on thermoregulation and sleep are circuit specific effects because
8 Brn3b-DTA mice showed robust inhibition of wheel running behavior to a 3-hr light pulse
9 delivered from ZT14-17 (Fig. S4).
10

11 **Discussion**

12 We show here that for the same physiological outcome, the acute effects of light
13 are relayed through distinct circuitry from that of circadian photoentrainment, despite
14 both processes requiring ipRGCs. Our results suggest that for thermoregulation and
15 sleep, ipRGCs can be genetically and functionally segregated into Brn3b(+) 'acute' cells,
16 and Brn3b(–) 'circadian' cells. Because Brn3b(+) cells largely avoid the SCN, and
17 Brn3b(–) cells preferentially target the SCN, our results discount a critical role for the
18 SCN in acute light responses in these two behaviors, and instead implicate direct ipRGC
19 projections to other brain areas (Gooley et al., 2003; Hattar et al., 2006). Surprisingly,
20 Brn3b(-) cells are sufficient to drive the acute and circadian effects of light on wheel
21 running activity, demonstrating further divergence in the circuits mediating the acute
22 effects of light on behavior, and suggesting that, unlike for thermoregulation and sleep,
23 acute and circadian regulation of activity is driven via the SCN.

24 The specific Brnb(+) ipRGC subtypes that mediate the light's acute effects on
25 body temperature and sleep remain a mystery. A majority of all known ipRGC subtypes

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1 (M1–M5) are lost in Brn3b-DTA mice (Chen et al., 2011), with the exception of a subset
2 of ~200 M1 ipRGCs. In agreement with this, ipRGC projections to all minor hypothalamic
3 targets are lost in Brn3b-DTA mice, while innervation of the SCN and part of the IGL
4 remains intact (Chen et al. 2011, Li and Schmidt, 2018). This suggests that all non-M1
5 subtypes and a portion of M1 ipRGCs are Brn3b(+). Each subtype has a distinct reliance
6 on melanopsin versus rod/cone phototrasnsduction for light detection (Schmidt and
7 Kofuji, 2009). The necessity and sufficiency of melanopsin in mediating acute effects of
8 light on body temperature (Fig. 1) and sleep (Altimus et al., 2008; Lupi et al., 2008; Tsai
9 et al., 2009) suggests that a subtype with strong melanopsin, but weak rod/cone
10 photodetection is responsible – possibly either M1 or M2 cells. However, experiments to
11 tease this apart will require novel methods to specifically manipulate ipRGC subtypes
12 that are currently unavailable.

13 The brain areas that mediate the acute effects of light on physiology are
14 essentially unknown. There are many candidate areas that both receive direct ipRGC
15 innervation and have been documented to be involved in light's acute effects on
16 physiology, including the preoptic areas (Muindi et al. 2014), the ventral
17 subparaventricular zone (Kramer et al., 2001), and the pretectum/superior colliculus
18 (Miller et al., 1998). Aside from the SCN, ipRGC projections to the median (MPO) and
19 ventrolateral preoptic (VLPO) areas have been the most widely supported. The preoptic
20 areas are involved in sleep and body temperature regulation (Szymusiak and McGinty,
21 2008; Nakamura, 2011) and are activated by an acute light pulse (Lupi et al., 2008; Tsai
22 et al., 2009). In support of our behavioral findings, ipRGC projections to each of these
23 areas is lost in Brn3b-DTA animals (Li and Schmidt, 2018). However, ipRGC projections
24 to these areas are sparse (Gooley et al., 2003; Hattar et al., 2006), suggesting their
25 activation by light may be indirect.

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1 In contrast, the superior colliculus (SC) and pretectum receive robust innervation
2 from ipRGCs (Hattar et al., 2002, 2006; Gooley et al., 2003; Ecker et al., 2010), their
3 lesioning blocks light's acute effects on sleep (Miller et al., 1998), and melanopsin
4 knockout mice lose light-induced cFOS expression in the SC (Lupi et al., 2008).
5 However, the SC and pretectum receive robust innervation from many conventional
6 RGCs, making the requirement for melanopsin and ipRGCs in acute sleep and body
7 temperature regulation difficult to reconcile. It is also possible (and perhaps probable),
8 that multiple ipRGC target regions are involved, with distinct areas mediating distinct
9 physiological responses. Future studies silencing each retinorecipient target while
10 activating Brn3b(+) ipRGCs will be necessary to tease apart the downstream circuits
11 mediating light's acute effects on physiology.
12 Alternatively, it remains possible that direct ipRGC control of body temperature is
13 the primary and critical step for many acute responses to light that are mediated by
14 ipRGCs. In support of this possibility, changes in body temperature and heat loss can
15 directly influence sleep induction (Kräuchi et al., 1999). This change in sleep is in turn
16 presumably causative of at least some of light's effects on wheel-running and general
17 activity (Mrosovsky et al., 1999). Further, core body temperature can acutely regulate
18 cognition and alertness (Wright et al., 2002; Darwent et al., 2010). It is therefore possible
19 that ipRGCs can have widespread influence on an animal's basic physiology and
20 cognitive function simply by regulating body temperature.
21 Together, our identification of the photopigment and the retinal circuits mediating
22 acute body temperature and sleep induction will facilitate better methods to promote or
23 avoid human alertness and cognition at appropriate times of day (Chellappa et al.,
24 2011). Our results support many recent efforts to capitalize on the specific light-detection
25 properties of melanopsin (Lucas et al., 2014), such as its insensitivity and short-
26 wavelength preference, to promote or avoid its activation at different times of day.

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1 However, this approach is problematic because acute activation of melanopsin to
2 promote alertness has the unintended effect of shifting the circadian clock (Provencio et
3 al., 1994), thereby making subsequent sleep difficult. Our identification that the Brn3b(+)
4 ipRGCs specifically mediate light's acute effects on body temperature provides a cellular
5 basis for developing targeted methods for promoting acute alertness, while minimizing
6 circadian misalignment.

7 **Methods**

8 **Animals (body temperature)**

9 All procedures were conducted in accordance with NIH guidelines and approved
10 by the Institutional Animal Care and Use Committee of Johns Hopkins University. All
11 mice were maintained on a mixed C57Bl/6J; 129Sv/J background and kept on ad libitum
12 food and water under a 12-hr/12-hr light/dark cycle in group housing until
13 experimentation, with temperature and humidity control. Male and female mice between
14 the ages of 2 and 6 months were used for analysis.
15

16 **Body temperature recordings**

17 Each mouse was single-housed at the time of experiment. Surgery was
18 conducted under tribromoethanol (Avertin) anestheshia and a telemetric probe (Starr G2
19 E-Mitter) was implanted in the peritoneal cavity to monitor core body temperature and
20 general activity. Data was collected in continuous 1- or 2-min bins using VitalVIEW
21 software and analyzed in Microsoft Excel. All experiments were conducted at least 10
22 days after surgery. Lights were controlled by a programmable timer and all lights were
23 6500K CFL bulbs illuminated each cage at ~500 lux. Light intensity (Figure S1) was
24 modulated using

25 *Brn3b*$^{Cre/+}$ mice were anesthetized with tribromoethanol (Avertin) and 0.5–1 μl
26 AAV2-hSyn-DIO-hM3Dq-mCherry (UNC Vector Core) was injected intravitreally in one

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1 eye using a picospritzer and pulled pipet. At least one week later, animals underwent
2 surgery for implantation of telemetric probes (as above). All experiments were conducted
3 at least 10 days after telemetric probe implantation and at least three weeks after viral
4 injection. After behavior, the eyes of each animal were inspected to ensure that >50%
5 infection had been achieved. We routinely saw >80% of the retinas were infected as we
6 have described previously (Keenan et al. 2016).
7 Diurnal amplitude was measured by subtracting the mean body temperature for
8 the light cycle (ZT0-12) from the mean body temperature for the dark cycle (ZT12-24).
9 Mean body temperature during testing used all data from ZT14-17. For CNO
10 experiments, injections were carried out near ZT14, but specific times were recorded for
11 each mouse to align the data to the time of injection. Comparisons of mean body
12 temperature after PBS or CNO utilized the 6 hours following injection.
13 Clozapine-N-oxide (Sigma) was prepared as a 0.1 mg/ml solution in PBS and
14 injected at 1 mg/kg intraperitoneally at ZT14. After behavior, the eyes of each animal
15 were inspected to ensure that >50% infection had been achieved. We routinely saw
16 >80% of the retinas were infected as we have described previously (Keenan et al. 2016).
17
18 **Animals (Sleep)**
19 All procedures were conducted in accordance with NIH guidelines and approved
20 by the Institutional Animal Care and Use Committee of Northwestern University.
21 Opn4Cre and Brn3bz-dta were maintained on a mixed C57Bl/6J; 129Sv/J background
22 (Hattar et al., 2002, 2006; Mu et al., 2005). Male and female littermate *Opn4*$^{Cre/+}$ and
23 *Opn4*$^{Cre/+}$; *Brn3b*$^{z\text{-}dta/+}$ animals between the ages of 2 and 3 months were used for sleep
24 analysis.
25
26 **Sleep recording**

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1 Male and female littermate *Opn4*$^{Cre/+}$ and *Opn4*$^{Cre/+}$; *Brn3b*$^{z\text{-}dta/+}$ mice were used
2 for sleep recordings. Electroencephalogram (EEG) and electromyogram (EMG)
3 electrode implantation was performed simultaneously at 8 weeks of age. Mice were
4 anesthetized with a ketamine/xylazine (98 and10 mg/kg respectively) and a 2-channel
5 EEG and 1-channel EMG implant (Pinnacle Technology) was affixed to the skull. Mice
6 were transferred to the sleep-recording cage 6 days after surgery, tethered with a
7 preamplifier, and allowed 3 days to acclimate to the new cage and tether. Mice were
8 housed in 12:12 light/dark conditions before and after EEG implantation. EEG and EMG
9 recording began simultaneously at the end of the habituation period, which were
10 displayed on a monitor and stored in a computer for analysis of sleep states. The high
11 pass filter setting for both EEG channels was set at 0.5 Hz and low pass filtering was set
12 at 100 Hz. EMG signals were high pass filtered at 10 Hz and subjected to a 100 Hz low
13 pass cutoff. EEG and EMG recordings were collected in PAL 8200 sleep recording
14 software (Pinnacle Technology) and scored, using a previously described, multiple
15 classifier, automatic sleep scoring system, into 10-sec epochs as wakefulness, NREM
16 sleep, or REM sleep on the basis of rodent sleep criteria (Gao et al., 2016). Light source
17 for all sleep experiments was a 3000 Kelvin light source at 500 lux.

18 **Wheel-running activity and Masking experiment**

19 Mice were placed in cages with a 4.5-inch running wheel, and their activity was
20 monitored with VitalView software (MiniMitter). Analyses of wheel running activity were
21 calculated with ClockLab (Actimetrics). We used 500 lux light intensity. Mice were
22 initially placed under 12:12 LD masking experiments. Mice were exposed, in their home
23 cage, to a timer-controlled 3-hour light pulse at ZT14-ZT17. Percent activity for each
24 mouse was normalized to its own activity at ZT13 (1 hour before light pulse), and
25 analyzed in 30-minute bins.

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1

2 **Statistics**

3 All statistical tests were performed in Graphpad Prism or R 3.4.4. Specific tests
4 are listed in the text and figure legends.

5

6

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1 **Figure legends**

2 **Figure 1: Melanopsin mediates the acute effects of light on body temperature. (A)**
3 Paradigm to measure body temperature continuously in a 12:12 light dark cycle with a 3-
4 hour light pulse at ZT14. (**B**) 48 hours of continuous body temperature monitoring in
5 wildtype male mice (n = 13) (**C**) Body temperature in WT during light pulse, compared to
6 baseline (ZT14). $P < 0.001$, paired t-test of mean temperature compared to previous
7 night. (**E**) Melanopsin-only mice (*Gnat1*$^{-/-}$; *Gnat2*$^{-/-}$, n = 11) and (**E**) melanopsin knockout
8 (*Opn4*$^{-/-}$, n = 6) 48-hour diurnal body temperature. (**F**) Diurnal body temperature
9 amplitude in the three groups. $P > 0.347$ for effect of group by one-way ANOVA. (**G**)
10 Body temperature in melanopsin-only during light pulse, relative to baseline (ZT14). (**H**)
11 Paired comparison of body temperature during light pulse compared to previous night. P
12 < 0.001 by paired t-test. (**I**) Body temperature in melanopsin knockout during light pulse,
13 relative to baseline (ZT14). (**J**) Paired comparison of body temperature during light pulse
14 compared to previous night.
15

16 **Figure 2: Brn3b-negative ipRGCs are insufficient for acute body temperature**
17 **regulation via the SCN.** (**A**) Diurnal body temperature in control (*Opn4*$^{Cre/+}$, n = 9) and
18 (**B**) Brn3b-DTA (*Opn4*$^{Cre/+}$;*Brn3b*$^{DTA/+}$, n = 7). (**C**) Diurnal body temperature amplitude in
19 the two groups. $P = 0.223$ by t-test. (**D**) Body temperature in control during light pulse,
20 relative to baseline (ZT14). (**E**) Paired comparison of body temperature during light pulse
21 compared to previous night. $P = 0.001$ by paired t-test. (**F**) Body temperature in Brn3b-
22 DTA during light pulse, relative to baseline (ZT14). (**G**) Paired comparison of body
23 temperature during light pulse compared to previous night. $P = 0.287$ by paired t-test.
24

25 **Figure 3: Activation of Brn3b-positive RGCs is sufficient to drive sustained body**
26 **temperature decreases.** (**A**) Diagram of intravitreal delivery of AAV2-hSyn-DIO-hM3Dq-

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1 mCherry to *Brn3b*$^{Cre/+}$ mice, and confirmation of infection of ipRGCs. (**B**) 54-hr
2 continuous diurnal body temperature recordings in Brn3b-hM3Dq mice, with injections of
3 PBS then CNO on consecutive nights at ZT14. (**C**) Change in body temperature after
4 PBS injection, relative to baseline (time of injection). (**D**) Change in body temperature
5 after CNO injection, relative to baseline (time of injection). (**E**) Paired comparison of the
6 change in body temperature with either PBS or CNO injection. *P* = 0.002 by paired t-test.
7
8 **Figure 4. Brn3b-positive M1 ipRGCs are not required for circadian**
9 **photoentrainment of sleep, but are required for its acute induction by light.** (A–C)
10 Percent time spent asleep in 1-hr bins across the 24-hr day for (A) Control (black) mice
11 (n = 14) and (B) Brn3b-DTA (blue) mice (n = 13) lacking Brn3b-positive ipRGCs. Both
12 lines showed normal photoentrainment of sleep, with no main effect of genotype
13 compared to Control by repeated-measures two-way ANOVA (F (1, 25) = 1.108, *P* =
14 0.303). Brn3b-DTA mice showed a significant reduction in sleep only at lights off (ZT 12)
15 by Sidak's multiple comparisons test (*P* = 0.029). (C) Percent time spent awake and
16 asleep in Control (black) and Brn3b-DTA mice (blue). No differences were observed
17 between genotypes by t-test (*P* = 0.316). (D–G) Percent time spent asleep for (D)
18 Control mice (black) and (F) Brn3b-DTA mice (blue) at baseline (dark line) and during
19 the three hour light pulse (light line). Significant difference from baseline determined by
20 repeated measures two-way ANOVA. Significant effect of treatment for Controls (F (1,
21 13) = 38.09, P < 0.001), but not for Brn3b-DTA (F (1, 12) = 0.8496, *P* = 0.375). (E)
22 Control mice show significantly more sleep and less wake during a light pulse (paired t-
23 test) while (G) Brn3b-DTA mice showed no change in percent sleep or wake during the
24 same period. Data is mean for ZT14–17.
25

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Figure 1

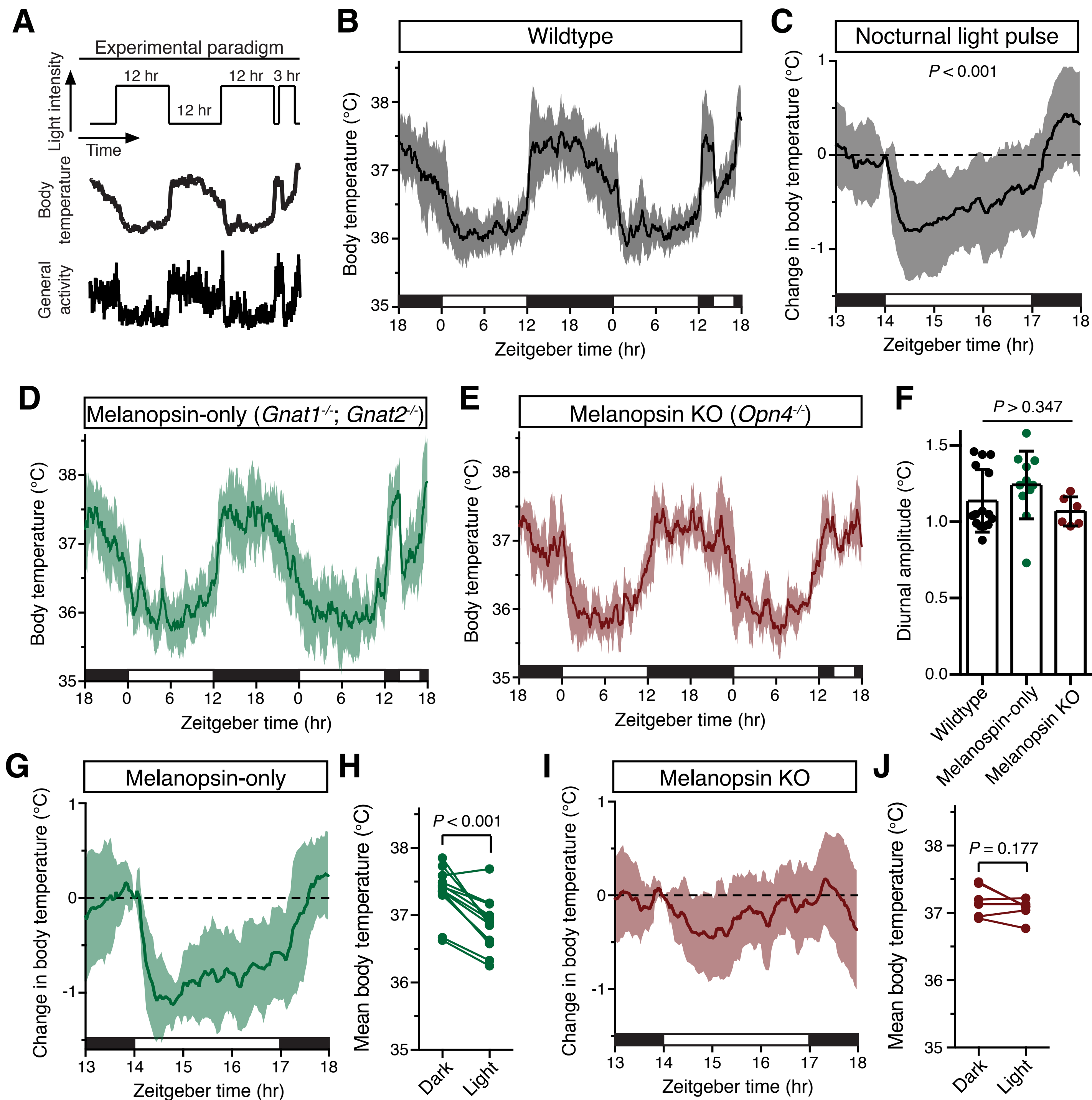

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Figure 2

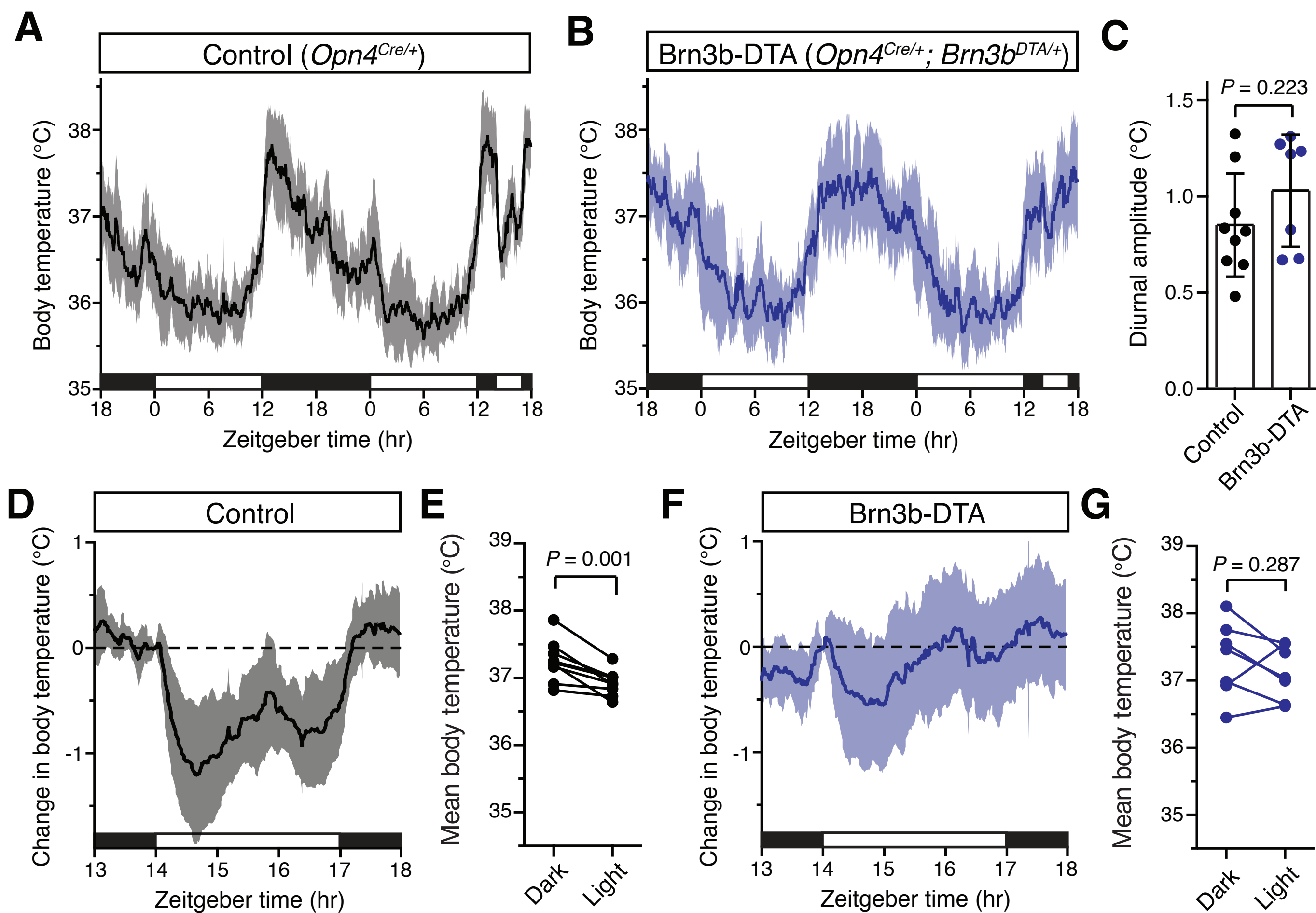

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Figure 3

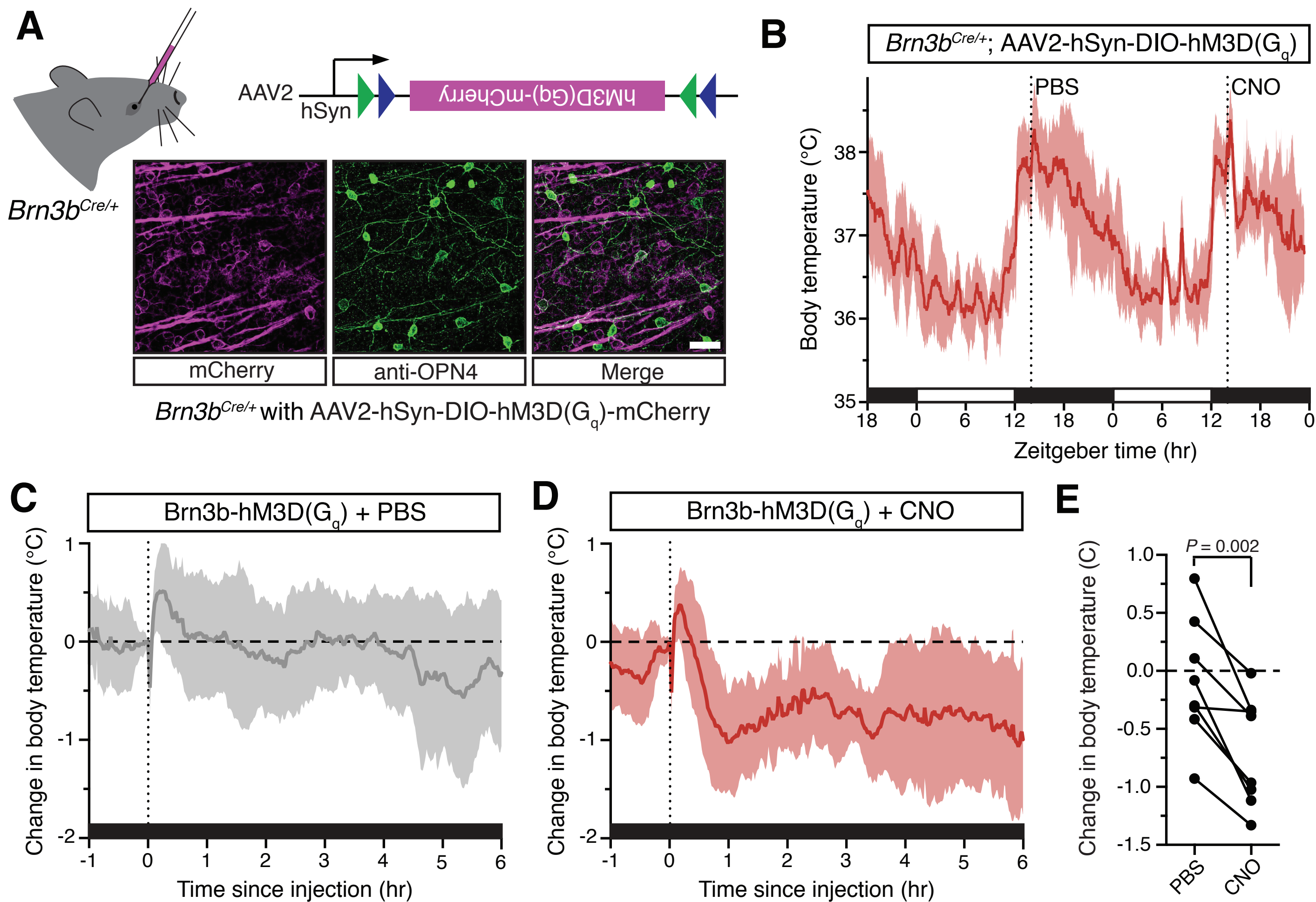

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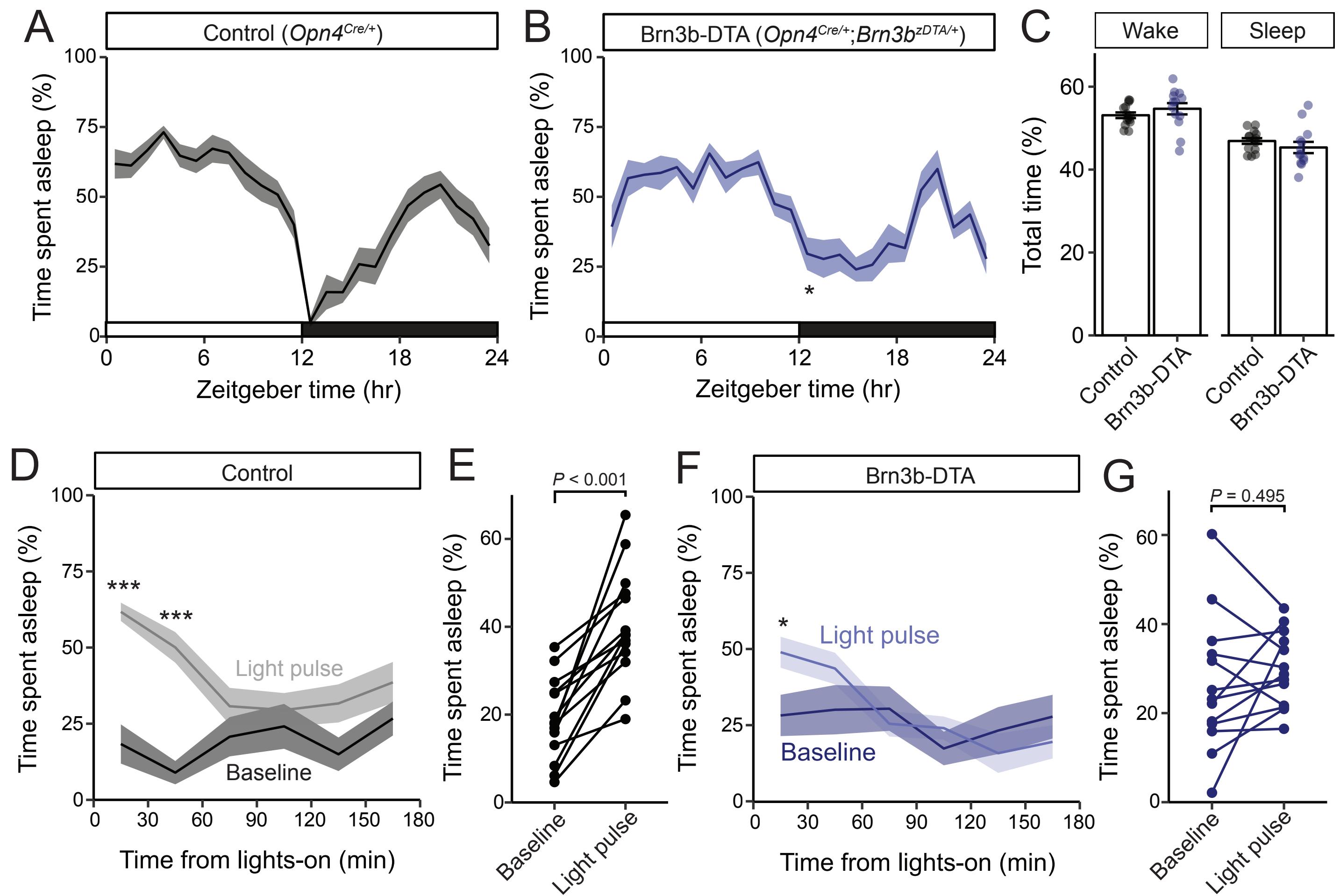
Figure 4



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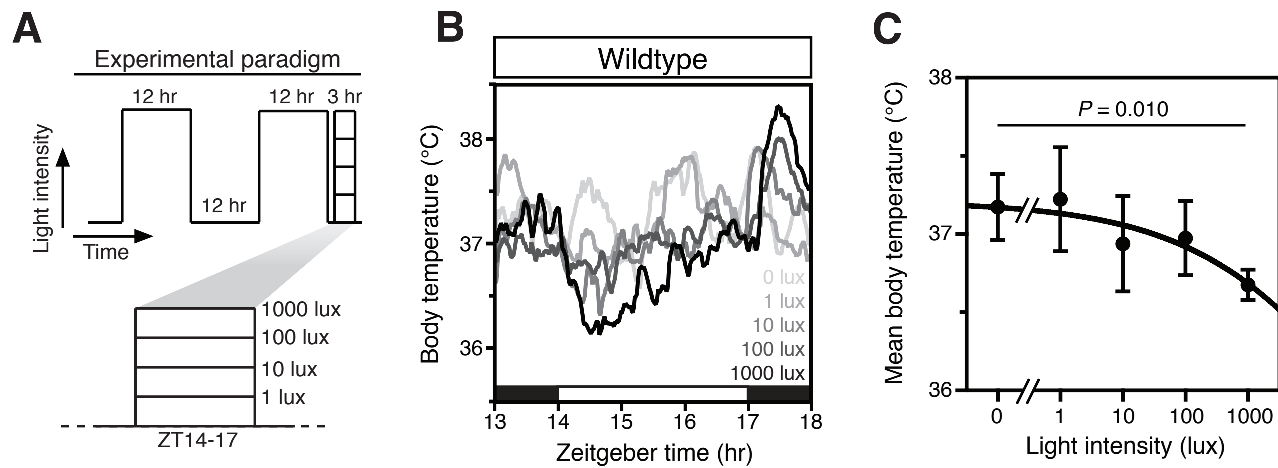

Figure S1: Intensity-dependent decrease in core body temperature during a nocturnal light pulse. (**a**) Experimental paradigm consisting of a 12-hr/12-hr light/dark cycle with a single 3-hr light pulse starting at Zeitgeber time (ZT) 14 (i.e. 2 hours after lights-off). Each experimental night, a light pulse was given at a specific envirnomental light intensity ranging from 1 to 1000 lux in $\log_{10}$ increments. (**b**) Mean body temperature for wildtype mice ($n = 4$) that were administered a light pulse at ZT14 of varying intensity (shown as shades of gray). Robust thermoregulation by light only occurs at bright intensities. Black and white bars on the *x* axis refer to time of lights-off and lights-on. (**c**) Quantification of the mean body temperature during the 3-hr light pulse for the wildtype mice in **b** ($n = 4$, mean ± SD) fit with a sigmoidal dose-response curve. There is a statistically significant effect of light intensity on body temperature ($P = 0.010$), as determined by the main effect of a repeated measures one-way ANOVA.

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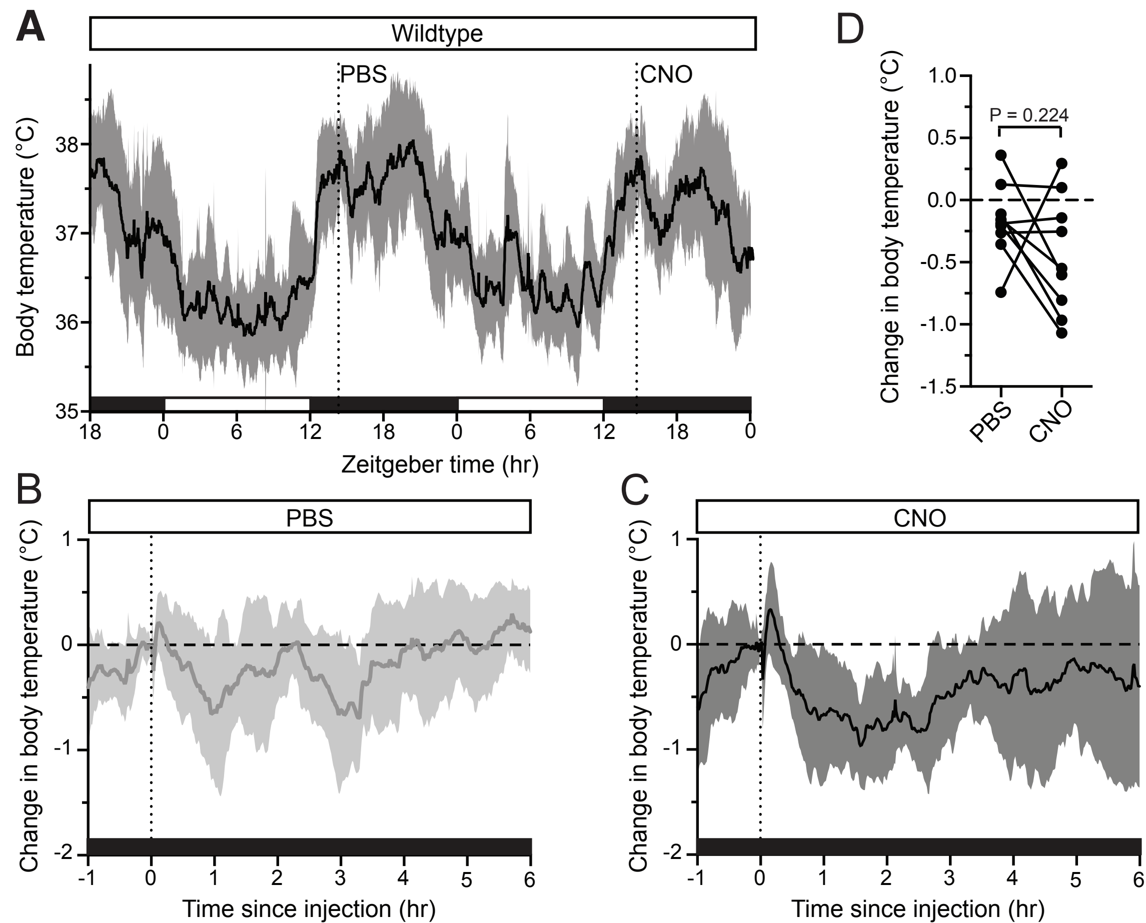

Figure S2: No effect of CNO on body temperature in wildtype mice. (**A**) Wildtype mice body temperature (n = 9) was monitored continuously and PBS was injected on night 1 at ZT14, followed by CNO injection (1 mg/kg) on night 2 at ZT14. (**B**,**C**) Normalized body temperature of either (**B**) PBS or (**C**) CNO injection. Both injections generate a rapid body temperature increase, followed by a dip below the reference value, before returning to normal. (**D**) Paired comparisons of body temperature changes in response to either PBS or CNO administration.

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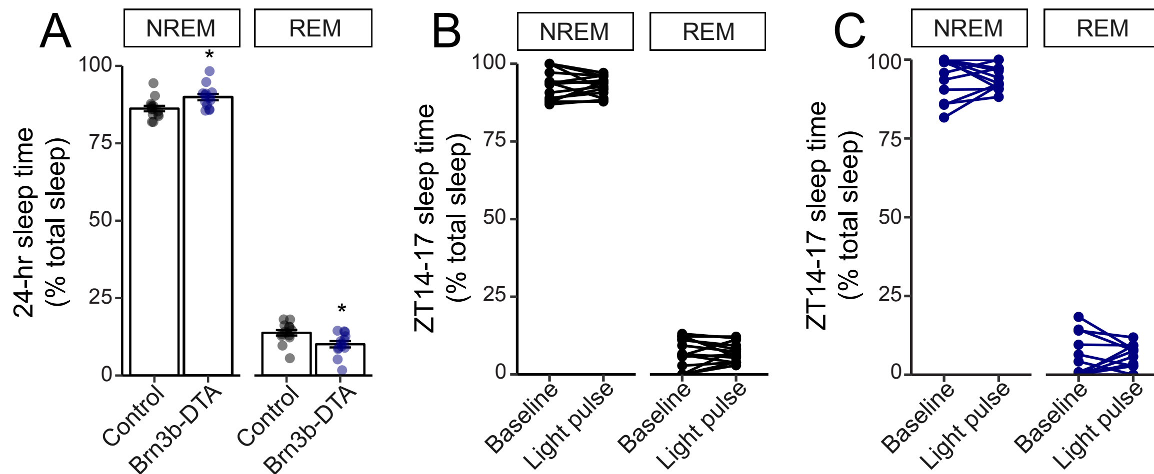

Figure S3: NREM and REM measurements in Control and Brn3b-DTA mice. (A) Percent sleep recorded across the 24 hour day as NREM vs. REM in Control (black) and Brn3b-DTA mice (blue). Brn3b-DTA mice showed a small but significant increase in NREM and decrease in REM sleep compared to Control mice. REM: rapid eye movement, NREM: non-REM. Control: n = 14. Brn3b-DTA: n = 13. *P = 0.011 by t-test. (B,C) Sleep stage quantification during ZT14-17, either baseline night or light pulse. No significant differences were seen in either (B) Control or (C) Brn3b-DTA mice by paired t-test.

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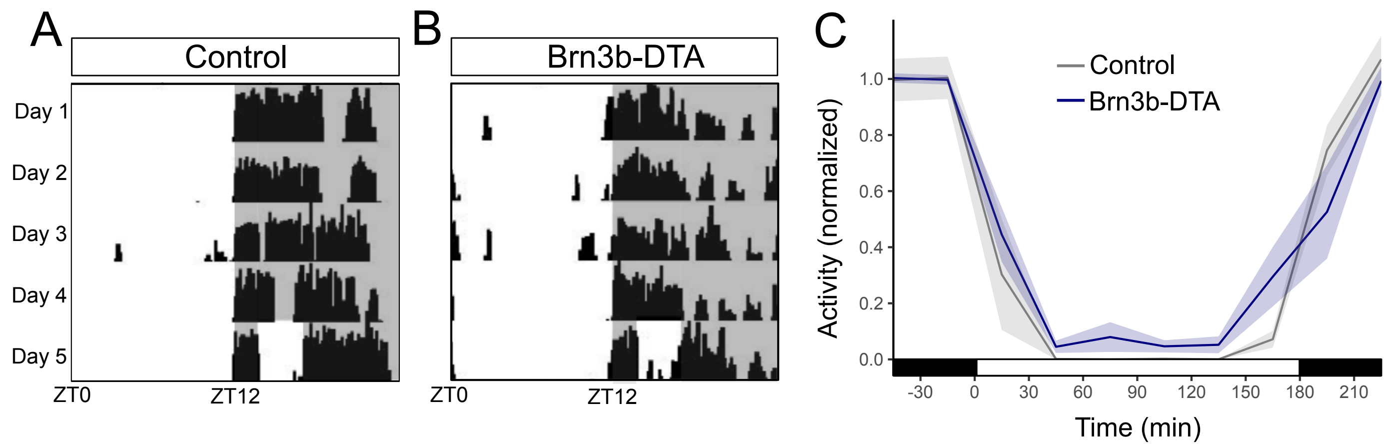

Supplemental Figure 4: Wheel-running activity in Brn3b-DTA mice. (**A**) Control ($Opn4^{Cre/+}$, n = 5) and (**B**) Brn3b-DTA ($Opn4^{Cre/+}$; $Brn3b^{DTA/+}$, n = 6) were housed in a 12:12 LD cycle and subjected to a 3-hr light pulse starting at ZT14. (**C**) Activity counts in 30 minute bins for both groups. Wheel revolutions were normalized to the average activity for the 1 hour preceding the light pulse. Shading represents SEM. While both groups display robust wheel-running inhibition in response to the light pulse, Brn3b-DTA mice had a mild deficit compared to Controls ($P = 0.039$ by linear mixed model).