Photoentrainment and pupillary light reflex are mediated by distinct populations of ipRGCs

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Supplementary text

Pupillary light reflex shows diurnal rhythm

We measured PLR in Opn4$^{Cre/+}$;Brn3b$^{Z\text{-}dta/+}$ mice at two light intensities in the middle of the day (ZT 8) and the middle of the night (ZT 20). The PLR in wild type mice shows a diurnal rhythm with higher constriction during the daytime (at ZT 8 pupil constriction is 95.61% under high light intensity [5.66 mW/cm^2]) and 79.47% under low light intensity [22 μW/cm^2]) compared to night time (at ZT 20 pupil constriction is 82.61% under high light intensity and 42.44% under the low light intensity) (Figure 3a and supplementary Figure 4a). In contrast, Opn4$^{Cre/+}$;Brn3b$^{Z\text{-}dta/+}$ mice showed highly attenuated PLR at ZT 8 under both high light and low light intensities, and no detectable PLR at ZT20 even under the high light intensity (Figure 3b and supplementary Figure 4b). This phenotype is remarkably similar to the PLR deficits observed in the Opn4$^{aDTA/aDTA}$ homozygous animals, although the Opn4$^{Cre/+}$;Brn3b$^{Z\text{-}dta/+}$ animals still have a single functional copy of the melanopsin gene. Heterozygous Opn4$^{aDTA/+}$ animals show mixed responses with PLR, especially at high light intensities[1].

Circadian and masking studies on Opn4$^{Cre/+}$;Brn3b$^{Z\text{-}dta/+}$ mice

To study circadian photoentrainment and masking in the Opn4$^{Cre/+}$;Brn3b$^{Z\text{-}dta/+}$ mice, we carried out the following procedures:

1- We first placed the animals under a 12h:12h light dark cycle. All Opn4$^{Cre/+}$;Brn3b$^{Z\text{-}dta/+}$ mice photoentrained to the light dark cycle by confining their activity to the dark, showing a stable phase relationship with the light dark cycle and producing an exact 24-hour period length.

2- In the second LD cycle, the dark was advanced by 6 hours to measure re-entrainment. Both the experimental and the control groups show the same ability for re-entrainment. The re-entrainment ability of mice to a 6-hr advance in the dark cycle depends on two factors: the phase of the clock (circadian) and direct dark activation of activity (masking). These experiments show that both experimental and control animals are capable of readjusting their activity to the new imposed light dark cycle.

3- We then delayed the dark onset in the LD cycle by 6 hours. Again, both the experimental and control animals appear to directly entrain. However, this is actually an artifact due to the fact that mice do not like to run under bright light conditions (masking). To see the speed of re-

entrainment, we have to observe the time at which mice cease their activity (indicated by the red line in the actograms showing a negative slope), which shows that all animals (control and experimental) require on average six days to re-entrain.

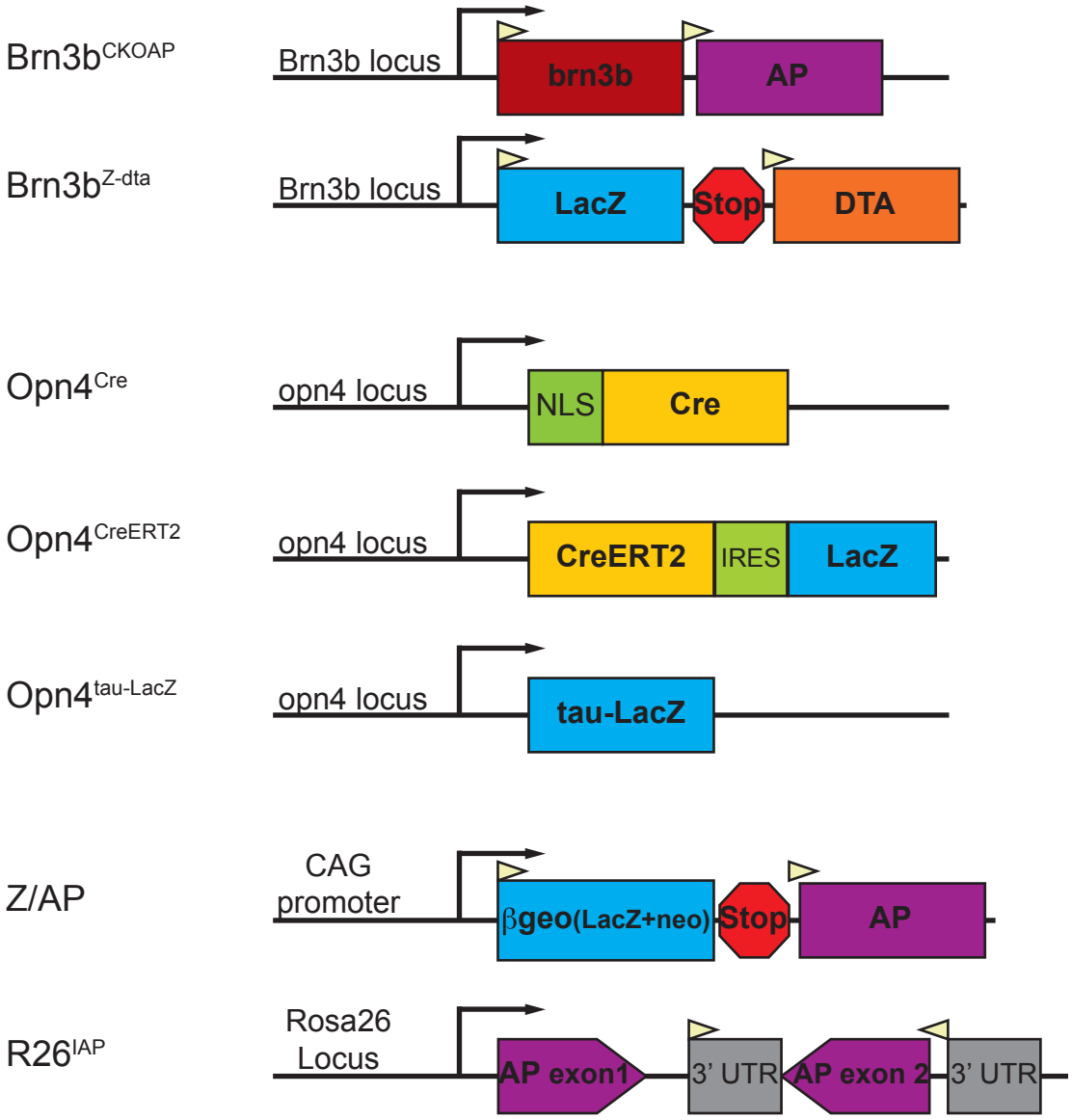
4- To measure if the circadian oscillator is functional, animals are placed in constant darkness. We observe little variation in the oscillator's period length between control and experimental group. Also, all animals (control and experimental) respond to a brief bright light pulse by shifting their onset in dark stably for several days after the administration of the light pulse.

5- To further measure the influence of light on these animals, we carried out constant light exposure experiments. These studies revealed deficits in light responsiveness of the experimental group under LL conditions. Note that in the control group, some animals barely run on the wheel in LL and are nearly arrhythmic (animals 1 and 3). This is an indication of a strong effect of light on their behavior. In the experimental group, several parameters highlight their attenuated light responses in LL: a- lack of complete arrhythmicity, b- higher activity levels, c- weaker lengthening of the period and d- an appearance of two period lengths (animal 1 of the experimental group). Thus although animals with Brn3b-ipRGCs deleted are completely capable of photoentrainment, phase-shifting, and masking responses under the ultradian cycle (see point 7), they do show deficits in light-responsiveness under LL conditions. This indicates that brain regions distinct from the SCN, such as the IGL, which receives weaker innervation patterns in the Brn3b-ipRGCs deleted animals, may mediate LL responsiveness. Further experiments are needed to completely prove this possibility.

6- Even after prolonged LL, all mice are able to re-entrain to LD including animals that show little activity in LL (animals 1 and 3 of the control group).

7- All animals respond similarly to an ultradian cycle composed of 3.5 hours of light and 3.5 hours of dark that measures masking responses to light.

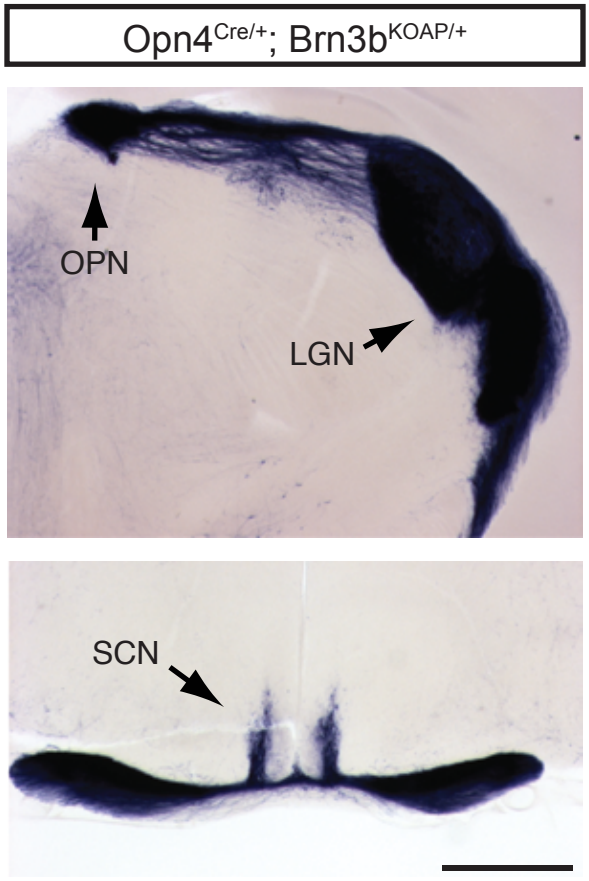
8- We also measured the ability of $Opn4^{Cre/+}$;$Brn3b^{Z\text{-}dta/+}$ mice to entrain to a skeleton photoperiod and show that both control and experimental group entrain to the skeleton photoperiod.



Chen et al., Supplementary Figure 1

Supplementary Figure 1. Schematic representation of the mouse genetic lines

Schematic representation of all the genetic mouse lines that we utilized in this paper, yellow triangles indicate the LoxP site. Brn3b^{CKOAP}, which was previously validated[2] has 2 LoxP sites flanking the Brn3b open reading and upon Cre excision, an alkaline phosphatase gene becomes in frame with the Brn3b promoter. Therefore, the expression of AP will be restricted to Brn3b positive cells only upon Cre expression. Brn3b$^{Z\text{-}dta}$ was published previously[3] and has 2 LoxP sites flanking the βgeo cassette (LacZ-neo), which prevents the downstream DTA from being expressed in the absence of Cre. Therefore, DTA will only be expressed upon the Cre dependent excision of the intervening cassette. Opn4^{Cre} was used in our previous study to reveal the diversity of ipRGCs and their targets in the brain[4], whereas Opn4$^{tau\text{-}LacZ}$ mice were published several times and show labeling of only M1 ipRGCs[5-7]. Opn4$^{CreERT2/+}$ is a recently generated animal that was validated in this study upon mating with previously published animal R26^{IAP} mice[8] which in the presence of Cre causes an inversion that restores the functional open reading frame of AP. Finally, Z/AP mice[9] is possibly one of the most widely used Cre-dependent reporter line. We used R26^{IAP} instead of Z/AP in our conditional Cre analysis, since we observed a higher rate of recombination with this animal upon tamoxifen injection.


Opn4Cre/+; Brn3bKOAP/+
OPN
LGN
SCN

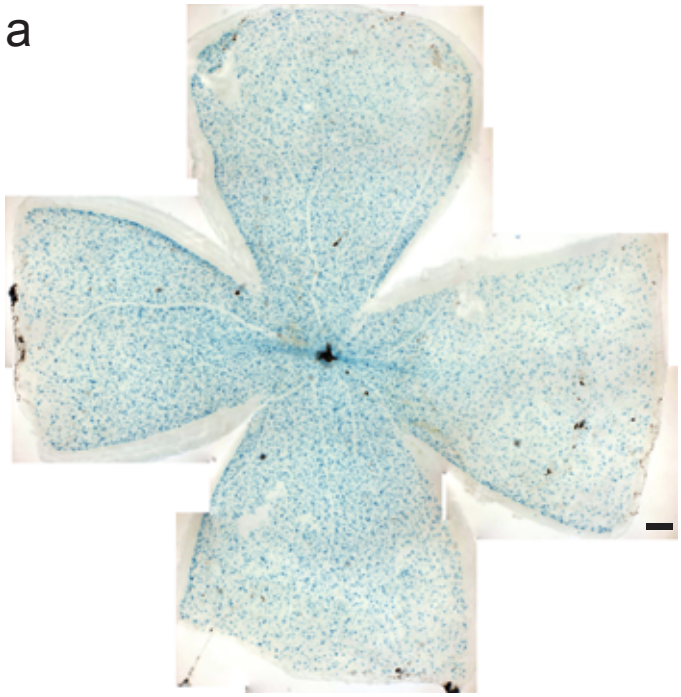
Supplementary Figure 2. Brain innervation pattern in Opn4$^{Cre/+}$; Brn3b$^{CKOAP/+}$ line

Representative images using histochemical staining with AP in coronal sections from the brain of an Opn4$^{Cre/+}$; Brn3b$^{CKOAP/+}$ mouse. Note the intense labeling of the LGN and the OPN in agreement with the conditional staining (Figure 1h and i). Higher labeling intensity is expected since we used the conventional Cre line, which labels all Brn3b-positive ipRGCs. Interestingly, despite the intense labeling in the LGN and the OPN, the staining of SCN is still restricted to the lateral edges of the nucleus. Scale bar is 400 μm.

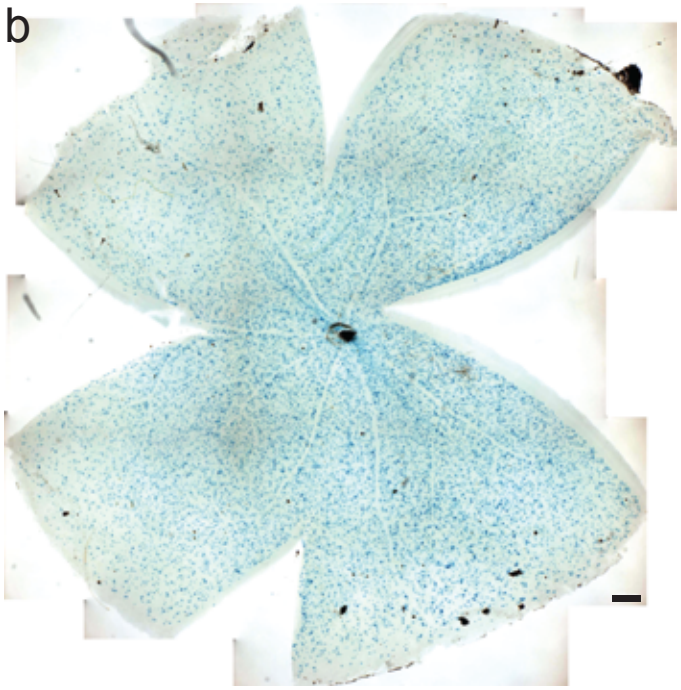
opn4$^{+/+}$; Brn3b$^{Z\text{-}dta/+}$

opn4$^{Cre/+}$; Brn3b$^{Z\text{-}dta/+}$

a

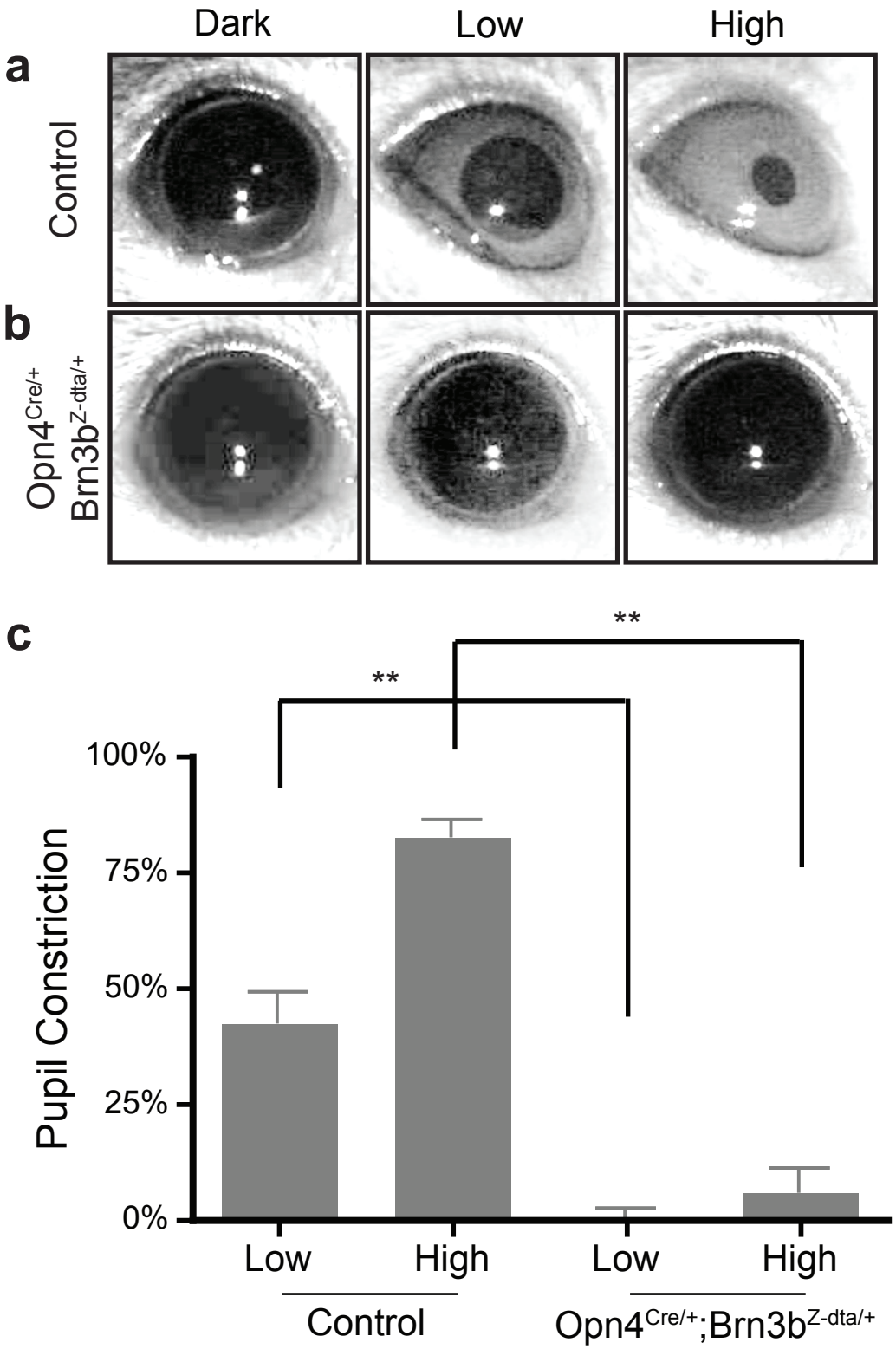


b



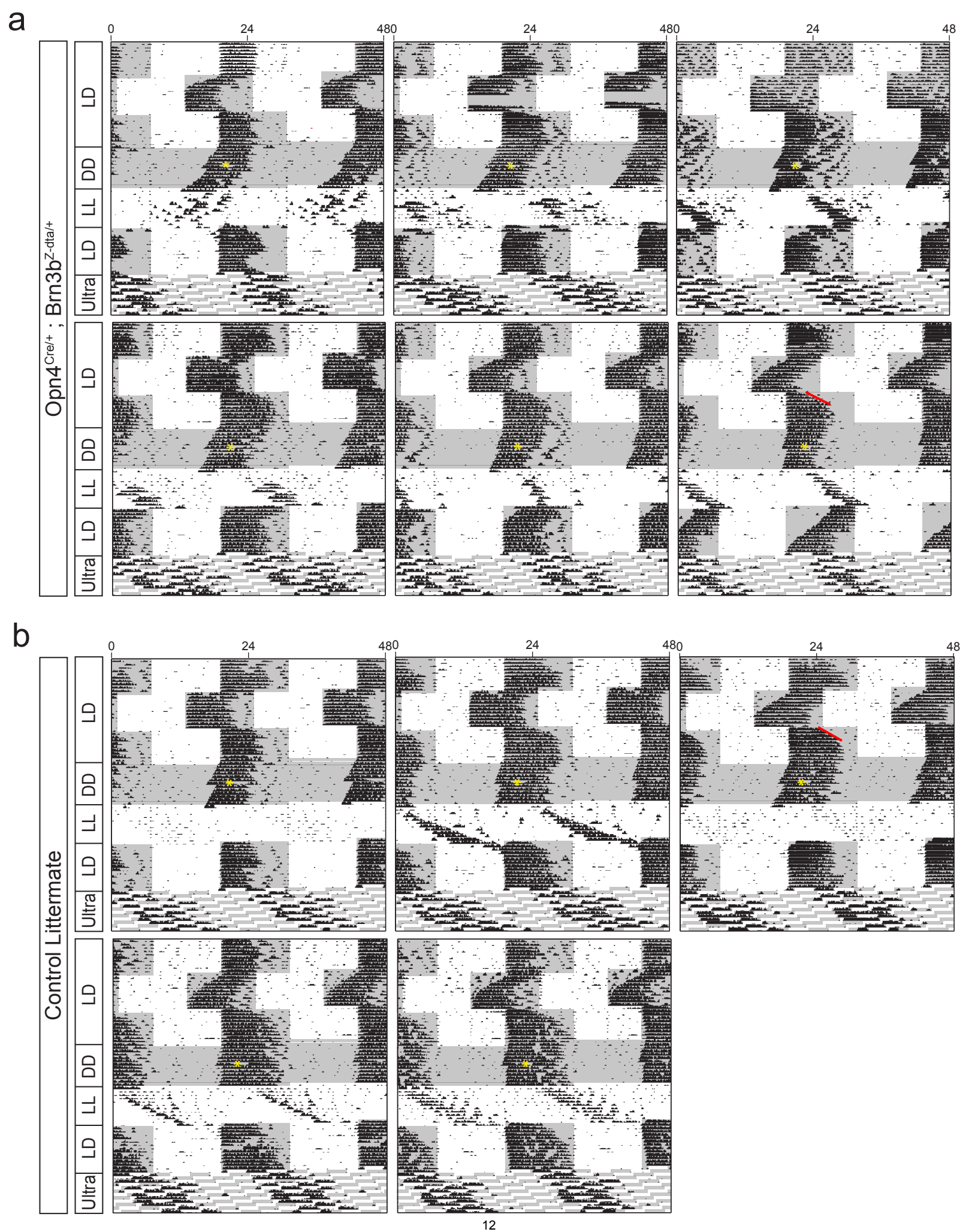
Supplementary Figure 3. Total number of RGCs is similar in control and experimental group

X-gal histochemical staining of β-galactosidase from: a. $Opn4^{+/+}$; $Brn3b^{Z\text{-}dta/+}$ and b. $Opn4^{Cre/+}$; $Brn3b^{Z\text{-}dta/+}$ mouse retinas. The deletion of Brn3b-positive ipRGCs does not impact the total number of RGCs in the retina in agreement with the visual acuity test (Figure 2g). Scale bar is 200 μm.


Dark
Low
High
a
Control
b
Opn4Cre/+
Brn3bZ-dta/+
c
**
**
100%
75%
50%
25%
0%
Pupil Constriction
Low
High
Low
High
Control
Opn4Cre/+;Brn3bZ-dta/+

Supplementary Figure 4. PLR shows highly attenuated responses in Opn4$^{Cre/+}$; Brn3b$^{Z\text{-}dta/+}$ mice

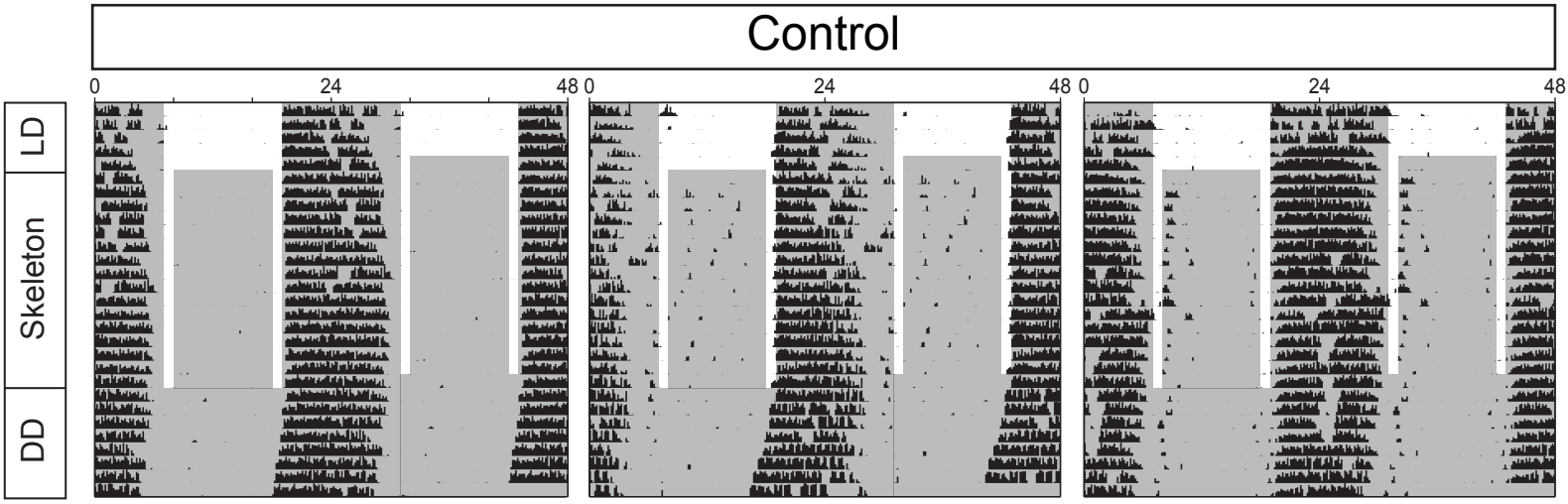
a and b. Representative images of pupillary light reflex from control (a) and Opn4$^{Cre/+}$; Brn3b$^{Z\text{-}dta/+}$ mice (b) at ZT 20. The dash circles mark the edge of pupil. The left panels show pupils under dark, the middle panels show pupils under low light intensity (22 μW/cm^2) and the right panels show pupils under high light intensity (5.66 mW/cm^2). Each row represents images from the same animal. There is almost no detectable PLR in the Opn4$^{Cre/+}$; Brn3b$^{Z\text{-}dta/+}$ mice even at high light intensity. Note that both control and Opn4$^{Cre/+}$; Brn3b$^{Z\text{-}dta/+}$ groups show a less pupil constriction during the night compared to the day (Figure 3). c. Quantification of PLR data from control (n=5) and Opn4$^{Cre/+}$; Brn3b$^{Z\text{-}dta/+}$ (n=6) animals. ** indicates $p<0.01$ with 1-way ANOVA. Error bars represent SEMs.


a
Opn4Cre/+ ; Brn3bZ-dta/+
LD
DD
LL
LD
Ultra
0
24
480
24
480
24
48
b
Control Littermate
LD
DD
LL
LD
Ultra
0
24
480
24
480
24
48

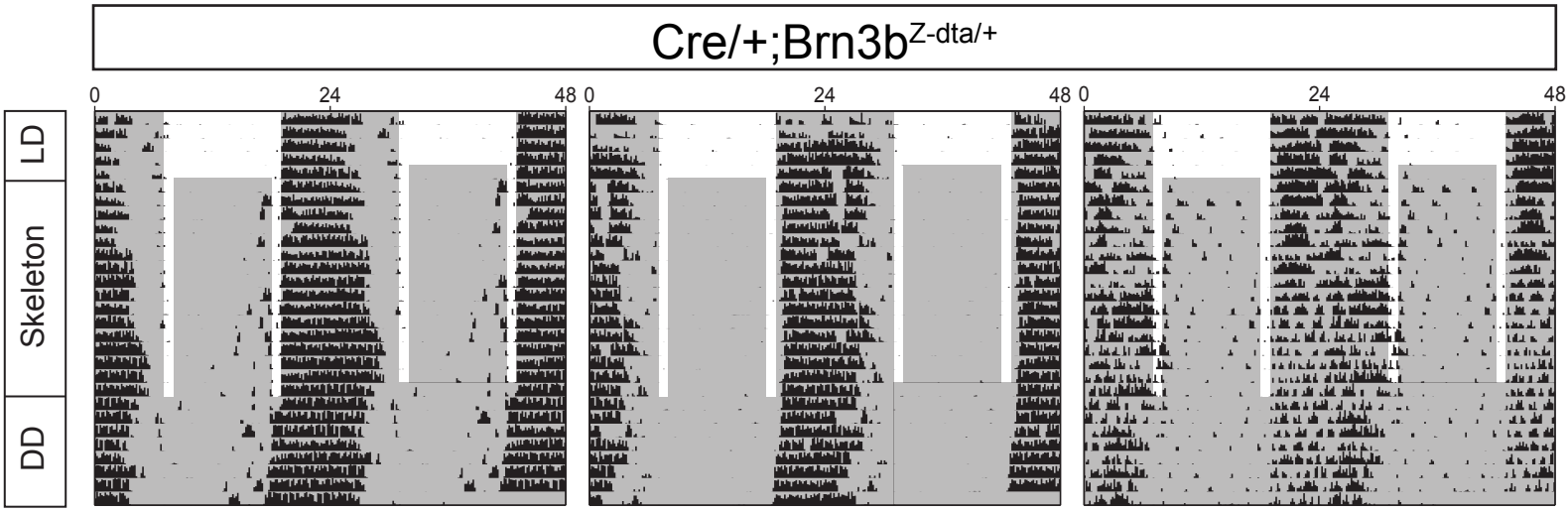
Supplementary Figure 5. All actograms of experimental and control groups

a and b, Actograms from all $Opn4^{Cre/+}$; $Brn3b^{Z\text{-}dta/+}$ mice (a) and control mice (b). The yellow * indicates 15 min light pulse for shifting the circadian oscillator. Red line shows re-entrainment after the shift in the LD cycle. Note that all the treatments are exactly the same as those explained in Figure 4.

a


Control
0
24
48
LD
Skeleton
DD

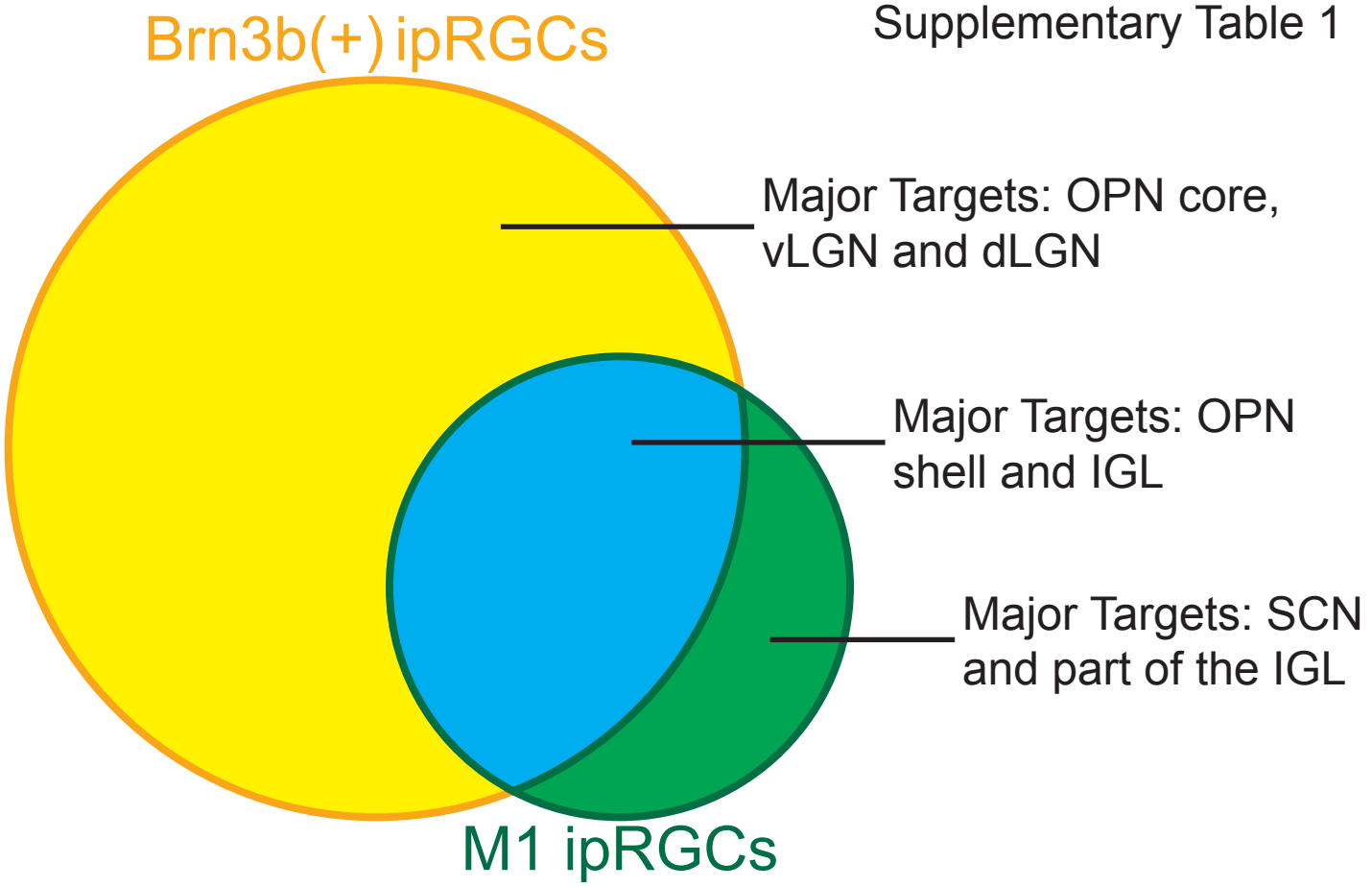
b


Cre/+;Brn3b^Z-dta/+
0
24
48
LD
Skeleton
DD

Supplementary Figure 6. All actograms of experimental and control groups under skeleton photoperiod

a and b, Actograms from all $Opn4^{Cre/+}$; $Brn3b^{Z\text{-}dta/+}$ mice (a) and control mice (b) under a skeleton photoperiod. Both groups show normal circadian photoentrainment as revealed by the onset of their free running activity in constant dark conditions.

Supplementary Table 1



Opn4$^{CreERT2/+}$; Brn3b$^{CKOAP/+}$:	Labels all Brn3b+ ipRGCs subtypes, including all non-M1 and a subset of M1 ipRGCs.	labels all known ipRGC brain targets with minimal innervation of the SCN
Opn4$^{CreERT2/+}$; R26$^{IAP/+}$:	Labels all ipRGCs subtypes	labels all known ipRGC brain targets
Opn4$^{Cre/tau\text{-}LacZ}$;Brn3b$^{Z\text{-}dta/+}$:	Eliminates all Brn3b+ ipRGCs, which includes all non-M1 and a subset of M1 ipRGCs	Labeling with X-gal staining is only observed in SCN and partially in the IGL
Opn4$^{Cre/+}$; Brn3b$^{Z\text{-}dta/+}$; Z/AP :	Eliminates all Brn3b+ ipRGCs, which includes all non-M1 and a subset of M1 ipRGCs	Labeling with AP staining is only observed in SCN and partially in the IGL (non-M1 not labeled)
Opn4$^{Cre/+}$; Brn3b$^{Z\text{-}dta/+}$:	Eliminates all Brn3b+ ipRGCs, which includes all non-M1 and a subset of M1 ipRGCs	This line was used for behavior: pupillary light reflex deficit and normal photoentrainment
Opn4$^{aDTA/aDTA}$ (from Guler 2008 *Nature*)	SCN and shell of OPN are not labeled	Pupillary light reflex and circadian photoentrainment deficits only in homozygotes

Supplementary Table 1

Summary table with a Venn diagram explaining the rationale for the use of the genetic mouse lines. A subpopulation of ipRGCs are M1 ipRGCs (Green Circle). Yellow circle represents all Brn3b-positive ipRGCs that are M1 or non-M1. Yellow color (not merged with green) represents non-M1 ipRGCs that are Brn3b positive (nearly 100% are Brn3b positive). Blue (yellow and green circles merge) represents the M1 ipRGCs that are Brn3b-positive and project to the IGL and the shell of the OPN, whereas crescent green represents M1 ipRGCs that predominantly target the SCN and are Brn3b negative. It is noteworthy to mention that the area of the circles correspond to the percentage of the different subtypes of ipRGCs. Note that no data was included in this study from the $Opn4^{aDTA/aDTA}$ animals, which were previously published.

Reference:

1 Guler, A.D. *et al.*, Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. *Nature* 453 (7191), 102-105 (2008).

2 Badea, T.C., Cahill, H., Ecker, J., Hattar, S., & Nathans, J., Distinct roles of transcription factors brn3a and brn3b in controlling the development, morphology, and function of retinal ganglion cells. *Neuron* 61 (6), 852-864 (2009).

3 Mu, X. *et al.*, Ganglion cells are required for normal progenitor- cell proliferation but not cell-fate determination or patterning in the developing mouse retina. *Curr Biol* 15 (6), 525-530 (2005).

4 Ecker, J.L. *et al.*, Melanopsin-expressing retinal ganglion-cell photoreceptors: cellular diversity and role in pattern vision. *Neuron* 67 (1), 49-60 (2010).

5 Baver, S.B., Pickard, G.E., & Sollars, P.J., Two types of melanopsin retinal ganglion cell differentially innervate the hypothalamic suprachiasmatic nucleus and the olivary pretectal nucleus. *Eur J Neurosci* 27 (7), 1763-1770 (2008).

6 Hattar, S. *et al.*, Central projections of melanopsin-expressing retinal ganglion cells in the mouse. *J Comp Neurol* 497 (3), 326-349 (2006).

7 Hattar, S., Liao, H.W., Takao, M., Berson, D.M., & Yau, K.W., Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. *Science* 295 (5557), 1065-1070 (2002).

8 Badea, T.C. *et al.*, New mouse lines for the analysis of neuronal morphology using CreER(T)/loxP-directed sparse labeling. *PLoS ONE* 4 (11), e7859 (2009).

9 Lobe, C.G. *et al.*, Z/AP, a double reporter for cre-mediated recombination. *Dev Biol* 208 (2), 281-292 (1999).