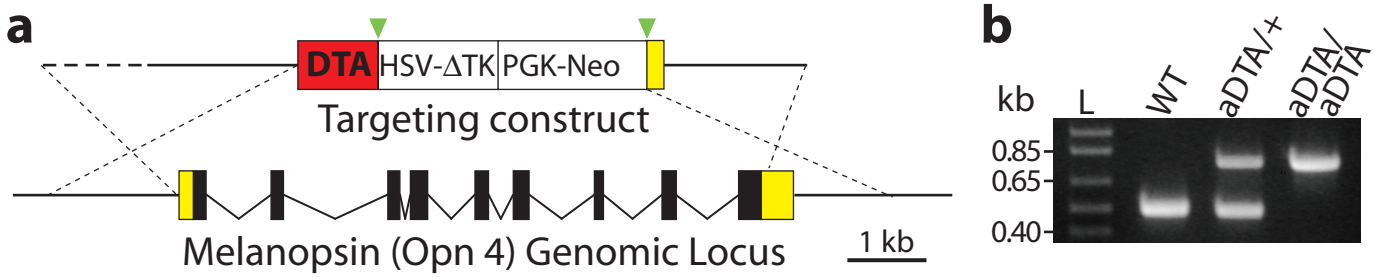
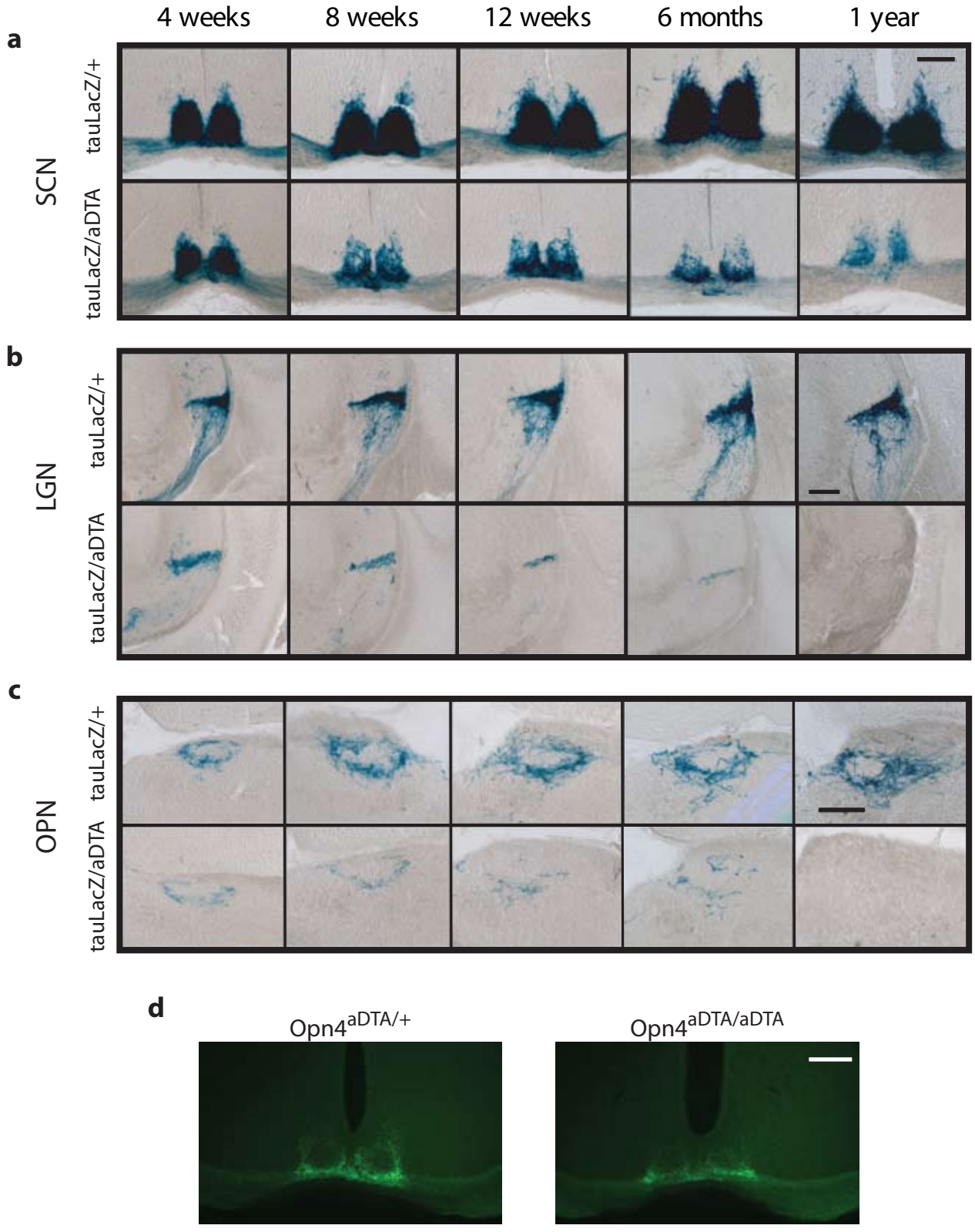


Supplementary Figure 1:

Targeting of the attenuated diphtheria toxin (aDTA) into the melanopsin locus. **a**, An attenuated form of the diphtheria toxin (aDTA) was inserted in the melanopsin gene locus using homologous recombination techniques. **b,** Specific targeting to the melanopsin locus was confirmed by PCR in both heterozygous (*Opn4*$^{aDTA/+}$) and homozygous (*Opn4*$^{aDTA/aDTA}$) mice. PCR primers were as follows: melanopsin forward primer: 5'- CCCCTGCTCATCATCATCTTCTG -3', melanopsin reverse primer: 5'-TGACAATCAGTGCGACCTTGGC -3', DTA forward primer: 5'-GAGCCACTGAGCATGTGTAGTC -3', DTA reverse primer: 5'-TAACGCTTTCGCCTGTTCCCAG -3'.

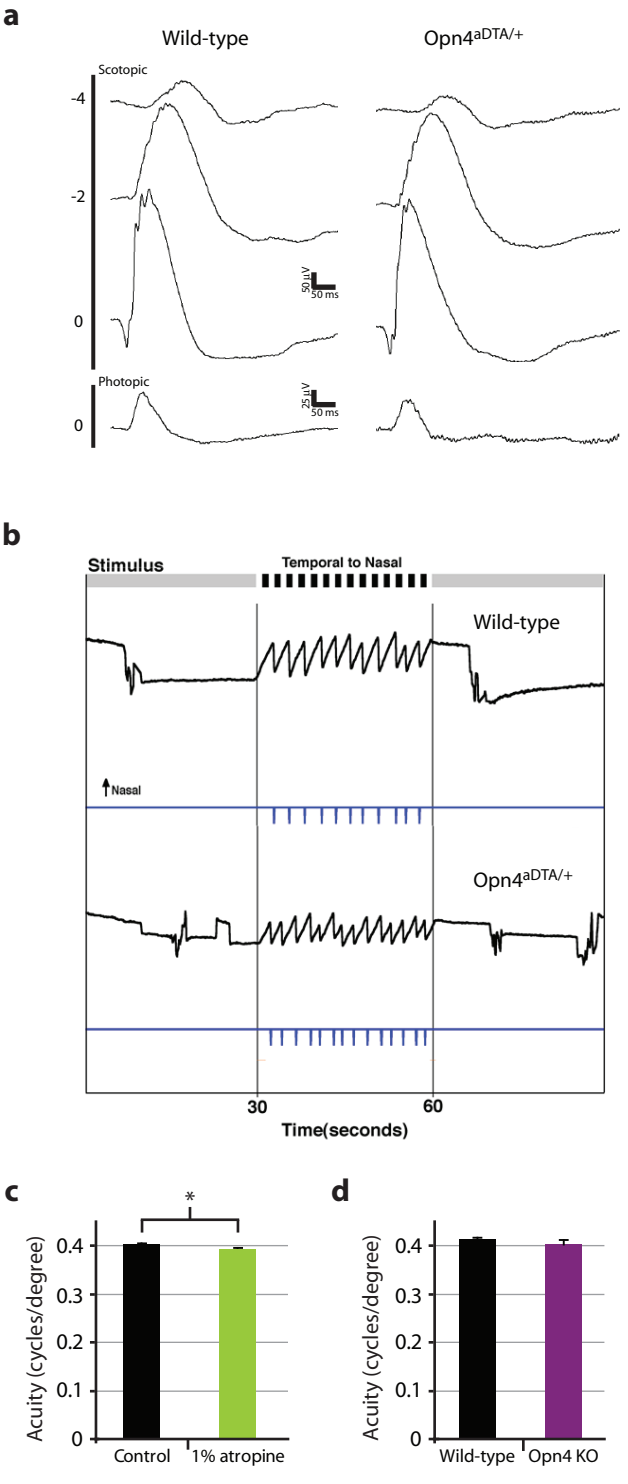


Supplementary Figure 2.

The ipRGC fibres in non-image forming centers in the brain decrease in aDTA expressing mice with age. a, b, and c, X-gal staining in the *Opn4*$^{tau\text{-}LacZ/aDTA}$ mice (tauLacZ/aDTA; n = 2-4 for each time point) show that ipRGC innervation of the SCN, IGL and OPN is decreased to a great extent with time as compared to the *Opn4*$^{tau\text{-}LacZ/+}$ control littermates (tauLacZ/+; n = 2-4 for each time point). At 4 weeks, there are extensive fibres from ipRGCs that innervate the different brain regions. Note that the fibre density decreases with age and by one year, the innervations in the IGL and OPN are absent. **d,** Since PACAP is co-expressed in ipRGCs, this peptide could be used to assess the number of remaining ipRGCs in homozygous compared to heterozygous/wild-type animals. Unfortunately, PACAP is also expressed in other cell types in the retina, and hence is not exclusive to melanopsin cells, making the analysis hard to interpret[27, 28]. Therefore, we compared coronal sections from the SCN of cholera toxin injected eyes of 4.5 months old *Opn4*$^{aDTA/+}$ and *Opn4*$^{aDTA/aDTA}$ mice. The 4.5 months old time point allows for optimal discrimination of the remaining RGC fibers between genotypes. Indeed, the intensity of RGC fiber innervations in the SCN is less in the *Opn4*$^{aDTA/aDTA}$ compared to *Opn4*$^{aDTA/+}$ mice (n=2). Scale bars; 200 μm.

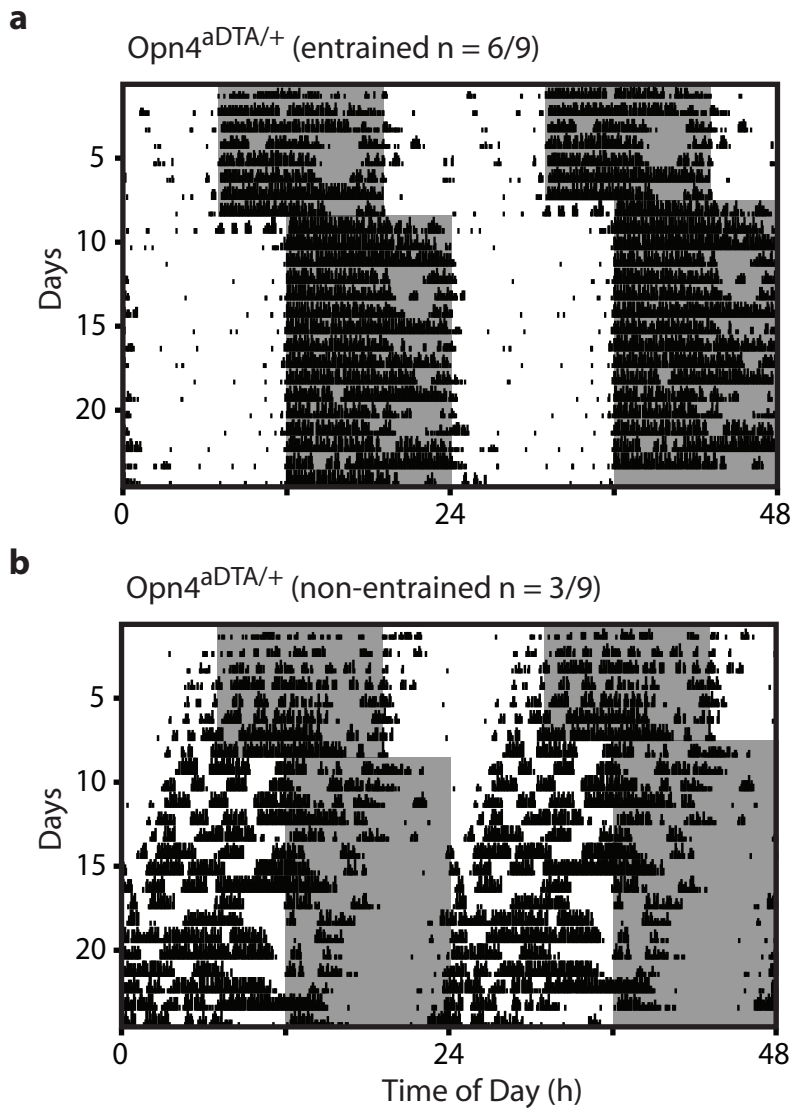
27. Seki, T., Shioda, S., Izumi, S., Arimura, A. & Koide, R. Electron microscopic observation of pituitary adenylate cyclase-activating polypeptide (PACAP)-containing neurons in the rat retina. *Peptides* 21, 109-13 (2000).

28. Hannibal, J., Hindersson, P., Knudsen, S. M., Georg, B. & Fahrenkrug, J. The photopigment melanopsin is exclusively present in pituitary adenylate cyclase-activating polypeptide-containing retinal ganglion cells of the retinohypothalamic tract. *J Neurosci* 22, RC191 (2002).



Supplementary Figure 3.

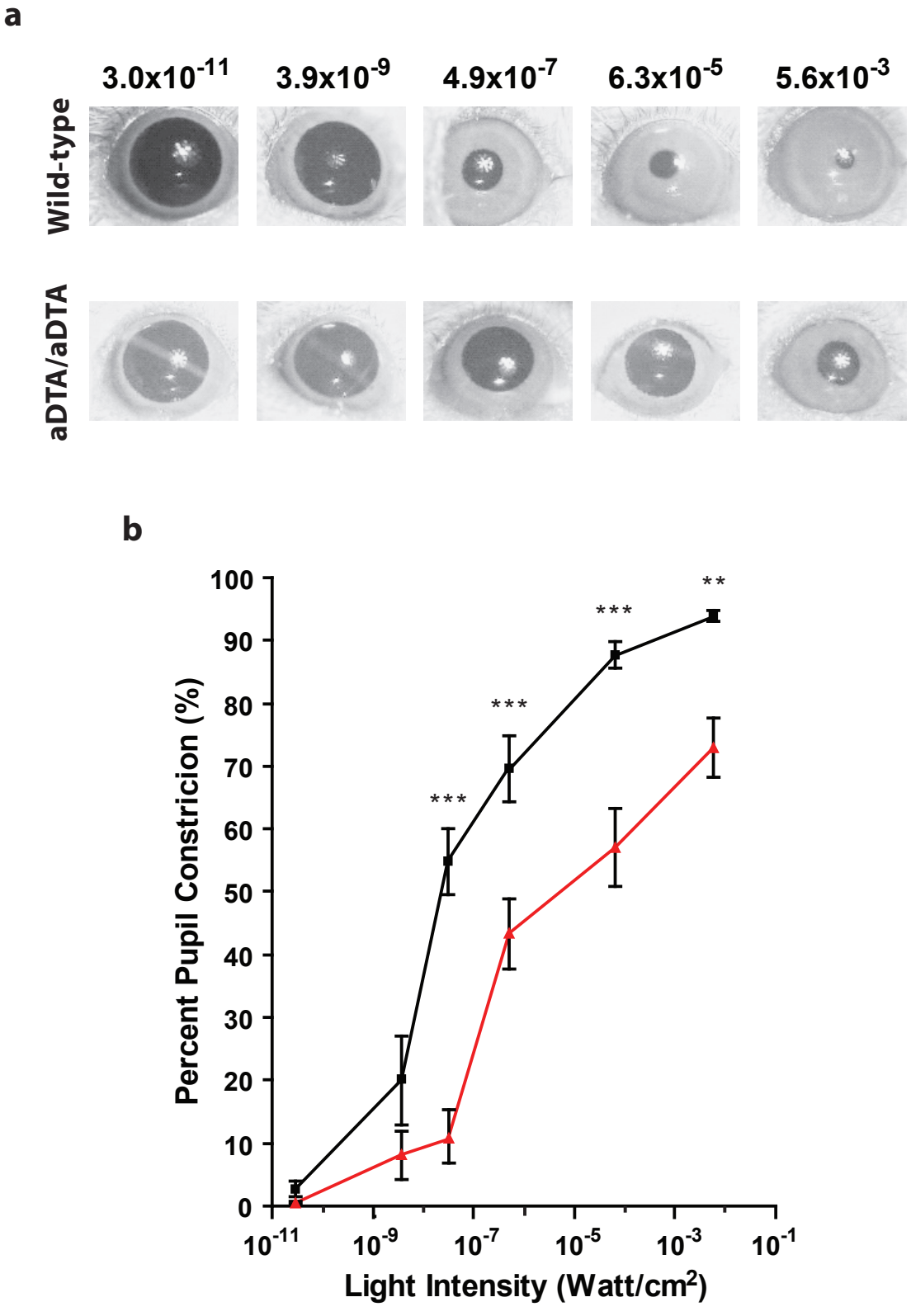
Pattern vision is intact in aDTA expressing animals. a, Electroretinogram (ERG) responses are similar in aDTA expressing and wild-type mice. Representative electroretinograms from an *Opn4*$^{aDTA/+}$ animal and a littermate wild-type control are indistinguishable when recorded under both scotopic and photopic conditions. All 6 *Opn4*$^{aDTA/+}$ mice tested (including the 3 animals with defective pupils at high light intensity) showed robust rod (scotopic ERG) and cone (photopic ERG) pathway responses, with ERG waveforms similar to those of wild types recorded under the same conditions (n=3). Quantitative analysis of the major ERG components failed to reveal differences in ERG sensitivity to account for the deficit in pupil response to sub-saturating stimuli observed in these animals (data not shown). **b**, OKN responses in *Opn4*$^{aDTA/+}$ mice were similar to wild-type controls. Representative eye tracking movements in *Opn4*$^{aDTA/+}$ (n=5) and wild-type (n=6) mice to a 30-second temporal to nasal grating stimulus. The downward spikes below the trace represent the number of eye tracking movements made in the 30 seconds interval. Null images of equal brightness were presented pre and post stimulus. The intensity of the light was ~100 lux, which was not sufficient to induce pupil constriction in two of the *Opn4*$^{aDTA/+}$ mice despite normal tracking in these animals. **c,** The differences in acuity between *Opn4*$^{aDTA/aDTA}$ and wild-type animals might be attributed to the pupil size. The acuity of the wild-type animals 18 hours after 1% atropine treatment (n = 6; green bar) is significantly lower than animals that are untreated (n = 6; black bar). **d,** Comparison between wild-type (n = 4; black bar) and *Opn4*$^{tau\text{-}LacZ/tau\text{-}LacZ}$ (n = 4; purple bar; Opn4 KO) animals showed no difference in the acuity.



Supplementary Figure 4:

***Opn4*$^{aDTA/+}$ mice that have normal pupil constriction at high light intensity photoentrain while mice that have defective pupil constriction do not photoentrain.**

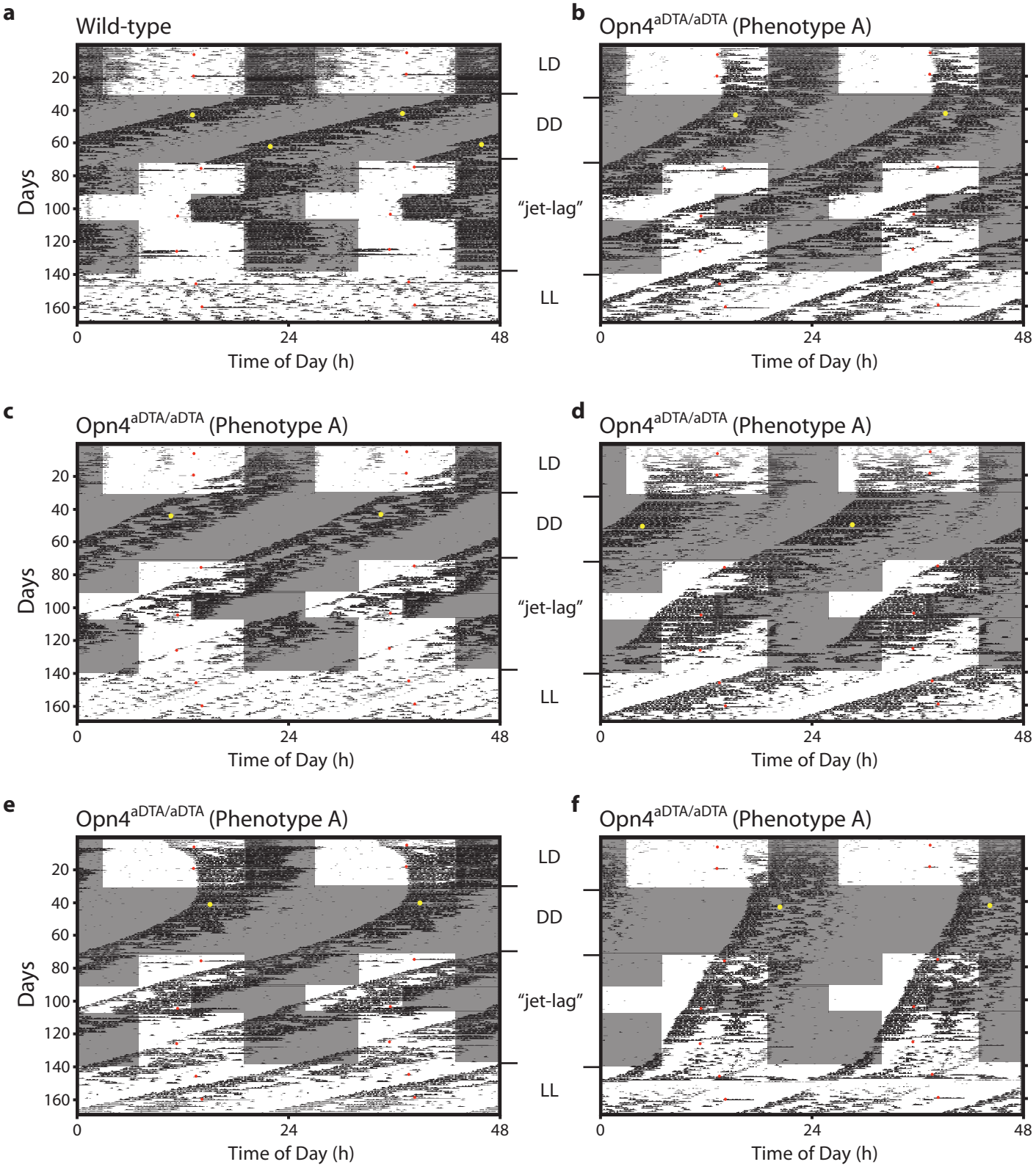
a, Representative actogram of a mouse that had normal pupil constriction at high light intensity, showing normal photoentrainment to a 5 hr delay in light/dark cycle. **b**, Representative actogram of a mouse that had defective pupil constriction showing lack of photoentrainment to a 5 hr delay in light/dark cycle. Grey and white backgrounds indicate dark and light (181 lux in animal cage), respectively.



Supplementary Figure 5.

Irradiance response curve shows that *Opn4*$^{aDTA/aDTA}$ mice are significantly less sensitive in PLR than wild-type controls at all light intensities. a, Representative images of 8 months old wild-type and *Opn4*$^{aDTA/aDTA}$ (aDTA/aDTA) pupils in response to a 30 second 470 nm monochromatic light stimulation of varying light intensities as indicated (Watt/cm^2). Representative images for each genotype are obtained from a single animal. At the ~ 50% pupil constriction in the wild-type animals (3.1X10^{-8} Watts/cm^2), the light intensity is similar to that emitted from a full moon on a clear night. At the highest intensity used, the light is equivalent to that emitted from the sun on an average clear day. These results demonstrate substantial impairment in pupillary responses, including at irradiances at which the constriction is driven solely by input from rods and/or cones. This impairment in PLR indicates that the shell of the OPN (highly innervated by ipRGCs[15], and eliminated in Opn4^{aDTA} animals {Figure 2c and 2f}) receives illuminance changes for pupil constriction in agreement with previous report[29]. b, Quantification of PLR data of wild type (WT; n = 6; black squares) and *Opn4*$^{aDTA/aDTA}$ (red triangles; n = 7). The statistical comparisons were made by two-way ANOVA followed by Bonferroni post hoc test (**, $p < 0.01$; ***, $p < 0.001$).

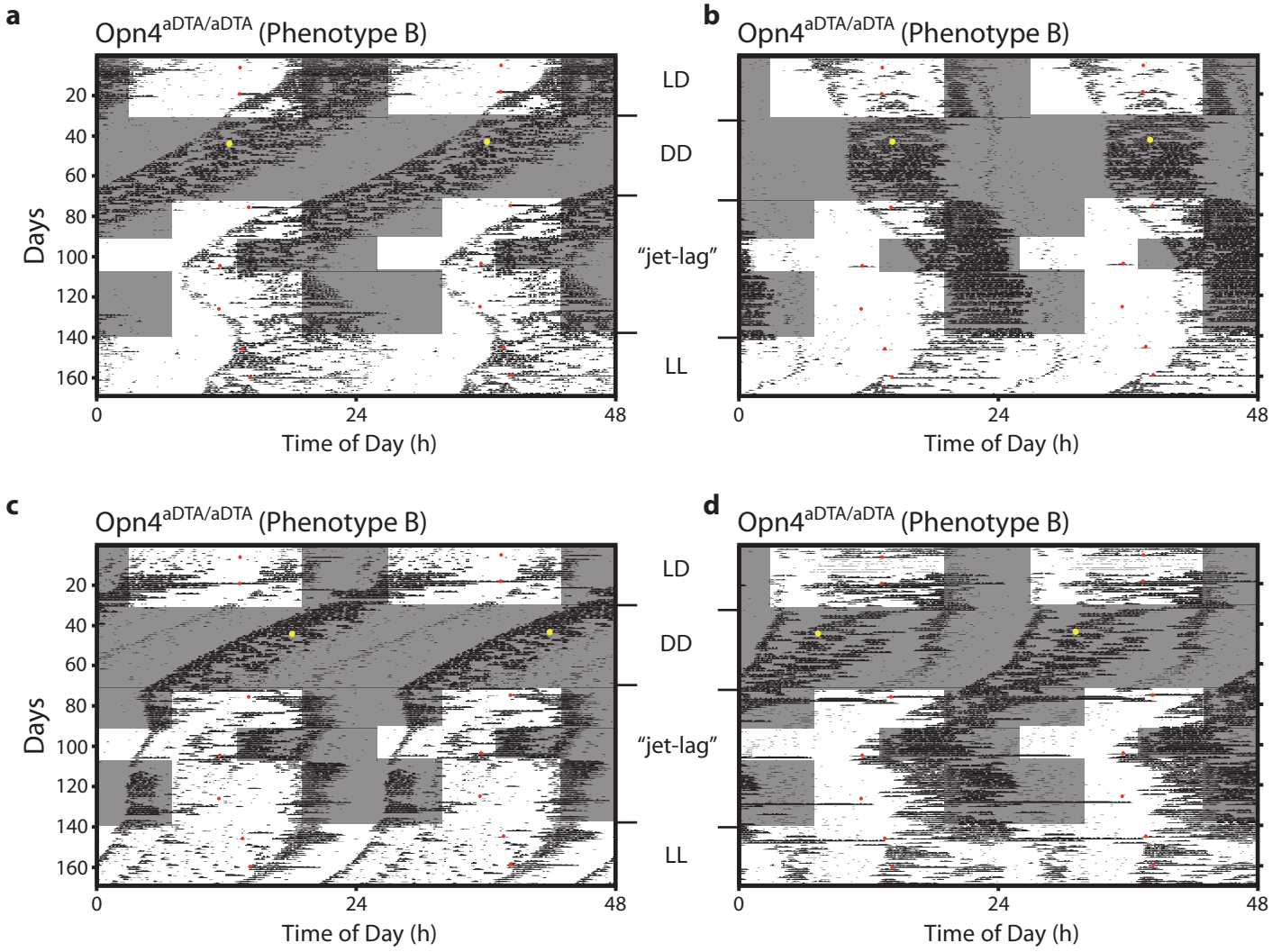
29. Prichard, J.R., Stoffel, R.T., Quimby, D.L., Obermeyer, W.H., Benca, R.M., Behan, M. Fos immunoreactivity in rat subcortical visual shell in response to illuminance changes. *Neuroscience* 114:781-93 (2002).



Supplementary Figure 6.

8 out of 12 *Opn4*$^{aDTA/aDTA}$ mice show inability to photoentrain to a 24-hour light dark cycle.

a, Wild-type animal shows normal photoentrainment and phase delays to a 15 minute 1500 lux white light pulse at CT16 (yellow dot). In addition, this animal is arrhythmic under constant light conditions, indicating that the ~700 lux light is high enough to disrupt the circadian oscillator. **b-f,** Five additional representative actograms of free-running *Opn4*$^{aDTA/aDTA}$ mice. All animals do not phase shift to 15-minute light pulse presented during DD (yellow dots). When animals are placed under the "jet-lag" paradigm, they free-ran with no phase association with the shifted light/dark cycle. Under LL the period of the circadian rhythm is shortened compared to DD period length. Refer to Supplementary Table 2 for statistical comparisons. Red dots indicate cage changes.



Supplementary Figure 7.

4 out of 12 *Opn4*$^{aDTA/aDTA}$ mice show light responsiveness, but do not form stable photoentrainment. a-d, Representative actogram of all weakly light responsive *Opn4*$^{aDTA/aDTA}$ mice. In the 16:8 light/dark portion, all animals run with no phase association with the imposed light dark cycle and do not phase shift to 15-minute light pulse presented during DD. However, when the animals are placed under the "jet-lag" paradigm, a weak association with the shifted light/dark cycle and a split in the period in 12:12 light/dark that was maintained in LL is observed. Note that under LL the period of the circadian rhythm is not lengthened (as measured by using either phase of the split rhythms) despite the weak light responsiveness observed under the "jet-lag" paradigm. Refer to Supplementary Table 2 for statistical comparisons. Red dots indicate cage changes.

	Wild-type	TKO	rd/rd cl	Mel. KO	**Mel. aDTA**
% PLR at low intensity	50%[8,12]	NC[6]	NC [8,12]	50%[8]	**NC**
% PLR at high intensity	95%[8,12]	NC[6]	95%[8,12]	85%[8]	**42%**
Photoentrainment to 24 hr LD cycle	Yes [9,10,11]	No[6]	Yes [4]	Yes [9,10,11]	**No**

Supplementary Table 1:

Summary of results from different retinal mutants for PLR and photoentrainment obtained from several published reports[4,6,8-12] in comparison to animals lacking ipRGCs (Mel.aDTA). We selected time-points from irradiance curves that are comparable to the intensities we used in this report. % PLR low intensity; represents the light intensity at which the pupil constriction is at half maximal response point (50%). % PLR high intensity; represents the light intensity at which the pupil is fully constricted (95%). Note that in the melanopsin knockout (Mel.KO) animals the maximum pupil response is only 85% compared to 95% wild-type and rodless/coneless (rd/rd cl) animals. The triple knockout animals (TKO), which lack the phototransduction pathways of rods, cones and ipRGCs, have no light-dependent pupil constriction (NC). For photoentrainment, Mel.KO, *rd/rd cl* and wild-type animals always photoentrain to a 24-hour light dark cycle. TKO animals never show any photoentrainment.

	Wild-type	Opn4$^{aDTA/aDTA}$		
		Phenotype		Combined
	(n = 11)	A (n = 8)	B (n = 4)	(n = 12)
DD Period	23.3±0.1	23.8±0.1^{c}	23.7±0.1^{b}	23.8±0.1^{c}
LL Period	25.5±0.1§	23.4±0.1^{c,†}	23.8±0.1^{c,†}	23.5±0.1^{c}
LD 12:12 Phase angle	19.0±0.1	n.a.	17.3±1.0^{a}	n.a.
Phase shift at CT16 (hrs)	-1.66±0.23	-0.08±0.13^{c}	-0.02±0.05^{c}	-0.06±0.09^{c}
Ultradian percent activity in dark (%)	83.6±3.7	61.9±4.1^{c}	67.2±2.6^{a}	63.7±2.9^{c}

Supplementary Table 2.

Summary of circadian and ultradian associated values and statistical comparisons for *Opn4*$^{aDTA/aDTA}$ and wild-type animals. As mentioned in the text, two phenotypes in the aDTA-expressing animals were observed. The non-photoentrainable animals (free-running; 8 out of 12) and the weakly entrained animals (4 out of 12) are referred to as phenotypes A and B, respectively. Statistical comparisons were also performed for all 12 mutant animals combined (final column). Free running periods in DD and LL were calculated from 7 continuous days of actograms during days 35 – 41 and 161 – 167, respectively. Phase angles were determined during days 117 – 123 for mice that had a stable phase relationship to the 12:12 light/dark cycle. All phase shifting experiments were completed between days 42 – 45. 7 days after the beginning of the 3.5:3.5 light/dark cycle, the percent activity in the dark portion of the ultradian cycle was calculated from 24 consecutive ultradian days. The symbol § indicates the average period of 7 out of 11 wild-type animals with rhythmic periods in LL. Comparisons between wild-type and combined *Opn4*$^{aDTA/aDTA}$ animals as well as between *Opn4*$^{aDTA/aDTA}$ phenotypes A and B are made with Student's *t* test. One-way ANOVA with Tukey's post test was utilized to compare wild-type animals to each of *Opn4*$^{aDTA/aDTA}$ phenotypes. The symbols ^{a}, ^{b}, ^{c} indicate statistical differences relative to wild-type animals with *p* values less than 0.05, 0.01, 0.001, respectively. The symbol † indicates $p < 0.05$ in comparison between phenotype A and B.