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SUPPLEMENTARY INFORMATION

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A light-gated potassium channel for sustained neuronal inhibition

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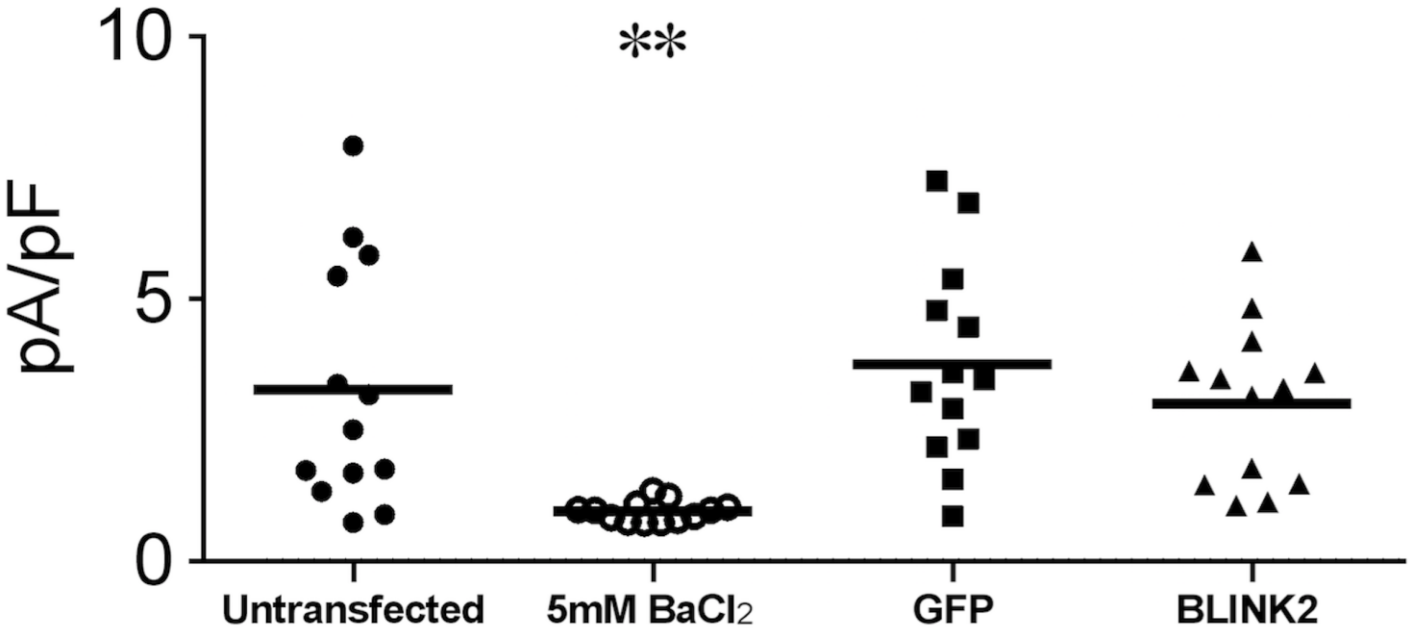
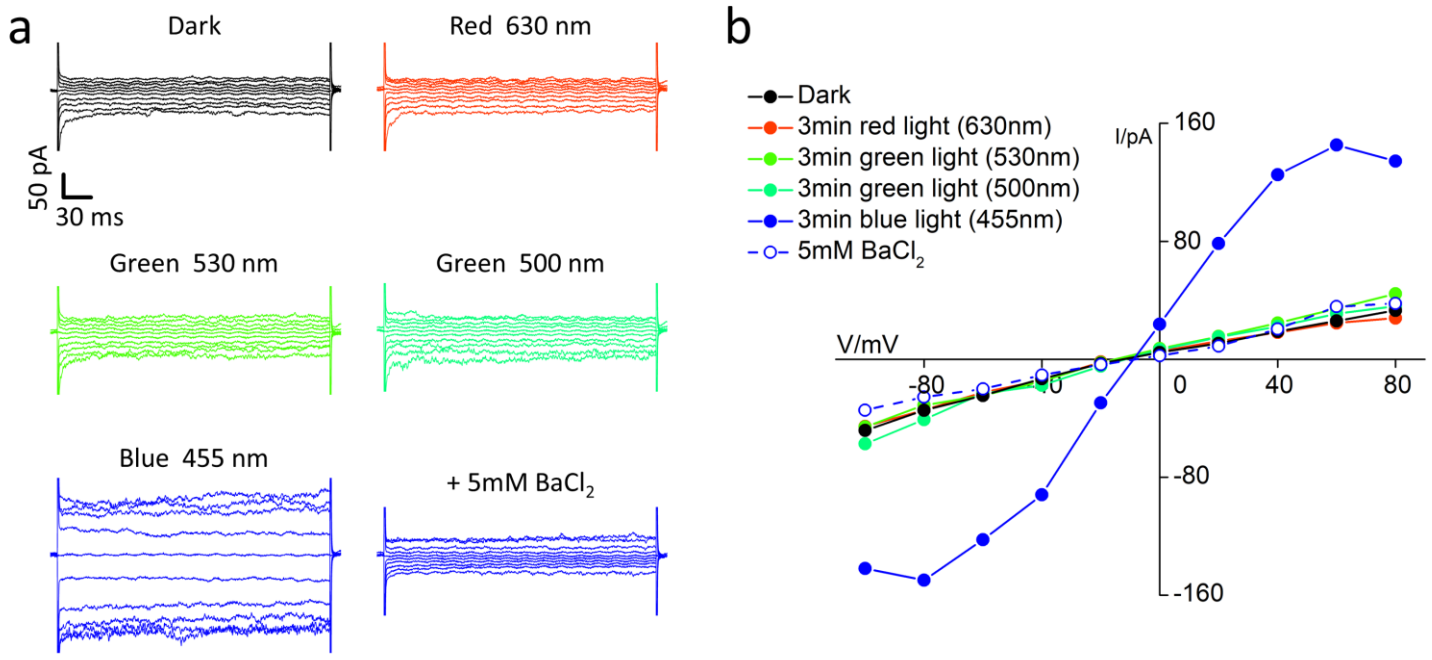


Supplementary Figure 1

Comparison of dark current levels in BLINK2-transfected and control COS7 cells.

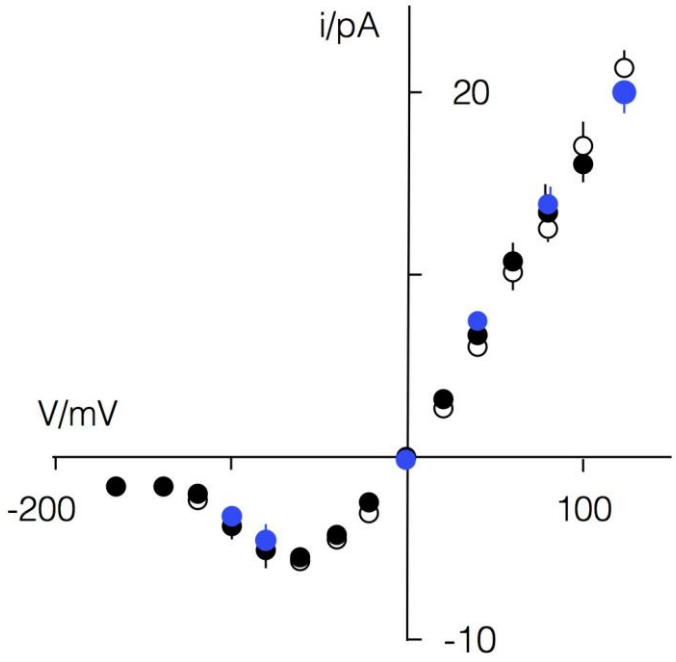
Currents recorded in the dark at –60 mV (N = 13 cells each) are shown normalized for cell capacitance (pA/pF) in the following conditions: untransfected, +5 mM $BaCl_2$, GFP-transfected and BLINK2-transfected cells. The mean values of the experimental groups Untransfected, GFP, and BLINK2 show no significant differences (P = 0.64). Only in barium-treated cells were the current values significantly smaller than in untransfected, GFP and BLINK2 (P = 0.001, 0.0001 and 0.003, respectively). Significance was calculated by one-way ANOVA and Tukey post hoc test.



Supplementary Figure 2

Spectral sensitivity of BLINK2 transfected in HEK293T cells.

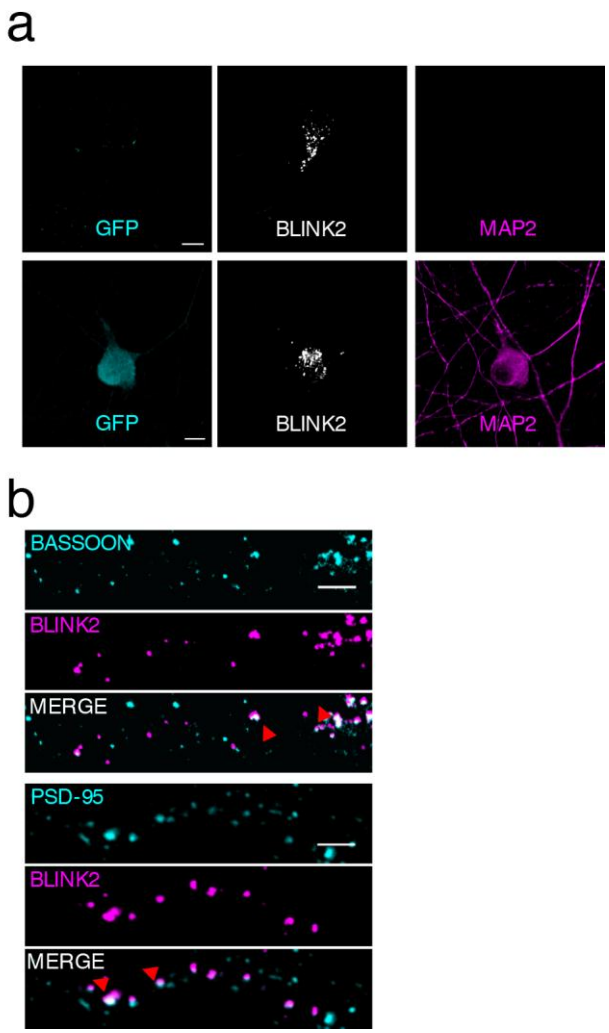
(**a**) Representative whole-cell current traces recorded in HEK293T cells expressing BLINK2 channel. Voltage steps from +80 to –100 mV, in the dark, 3 min after exposing the cell to 630-nm red light, 530-nm green light, 500-nm green light, 455-nm blue light and after the addition of 5 mM $BaCl_2$ in the external solution. (**b**) Current–voltage relationship of measurement in (**a**) in the dark (filled black circles), in 630-nm light (filled red circles), in 530-nm light (filled green circles), in 500-nm light (filled light-green circles), in 455-nm light (filled blue circles) and after the addition of 5 mM $BaCl_2$ in the external solution (open blue circles). Light of all wavelengths was provided at 90 $\mu W/mm^2$ intensity. The effect of each wavelength was tested versus the effect of blue light (455 nm) in 3 independent experiments.



Supplementary Figure 3

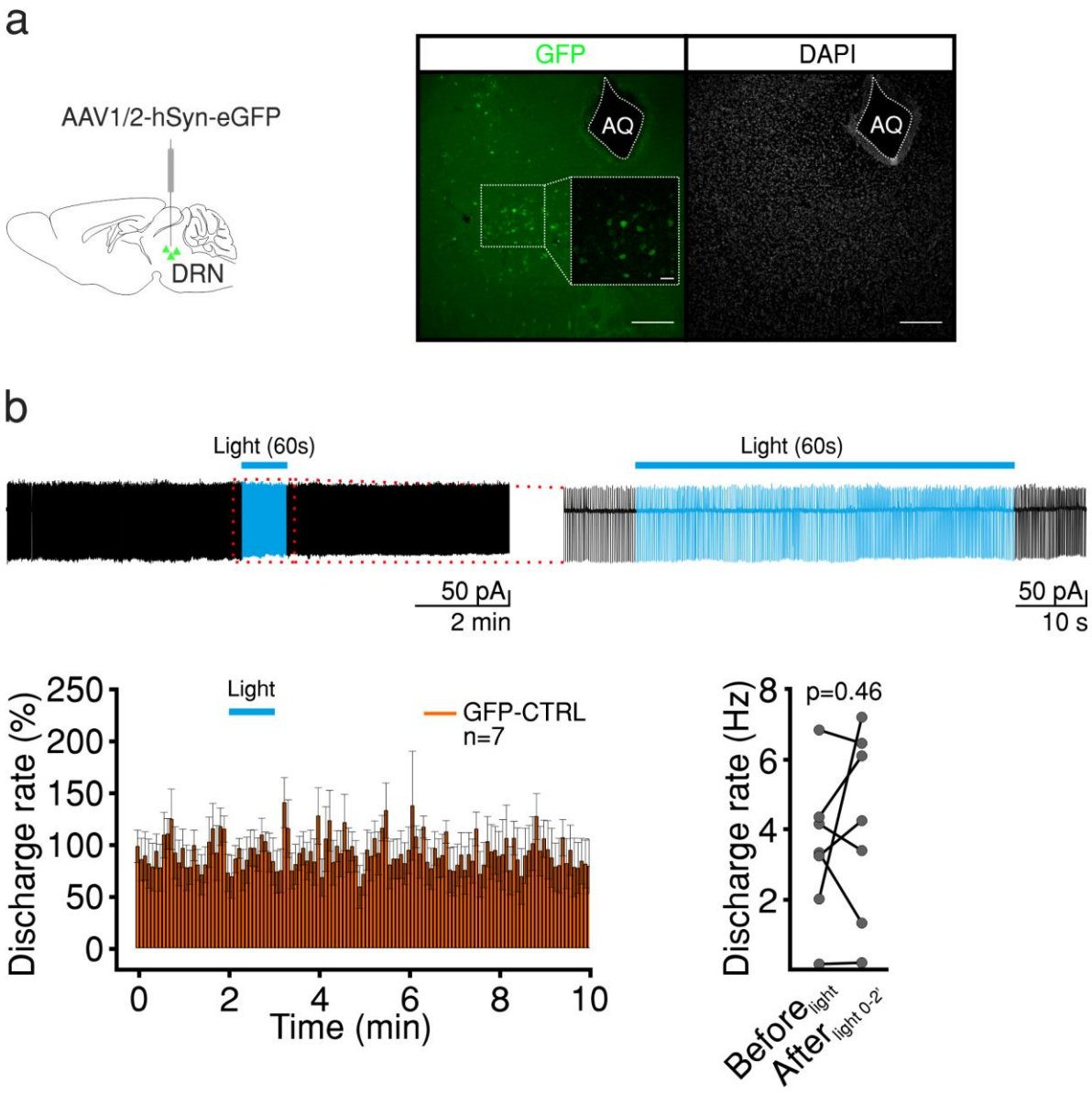
Single-channel *i*/*V* curve of BLINK2.

BLINK2 single-channel currents (blue symbols) plotted as a function of voltage. Currents for BLINK1 (white circle) and Kcv_{PBCV1} (black circle) are shown for comparison. Each data point is the average of n = 3 measurements performed in cell-attached configuration (BLINK2) or after insertion of purified proteins in planar lipid bilayers (BLINK1 and Kcv_{PBCV1}) in n = 3 independent experiments. Standard deviations are within the dimensions of the symbols. Recordings were performed in the following conditions: 103 mM K^+ in the pipette solution for BLINK2 and symmetrical 100 mM K^+ (BLINK1 and Kcv_{PBCV1}).



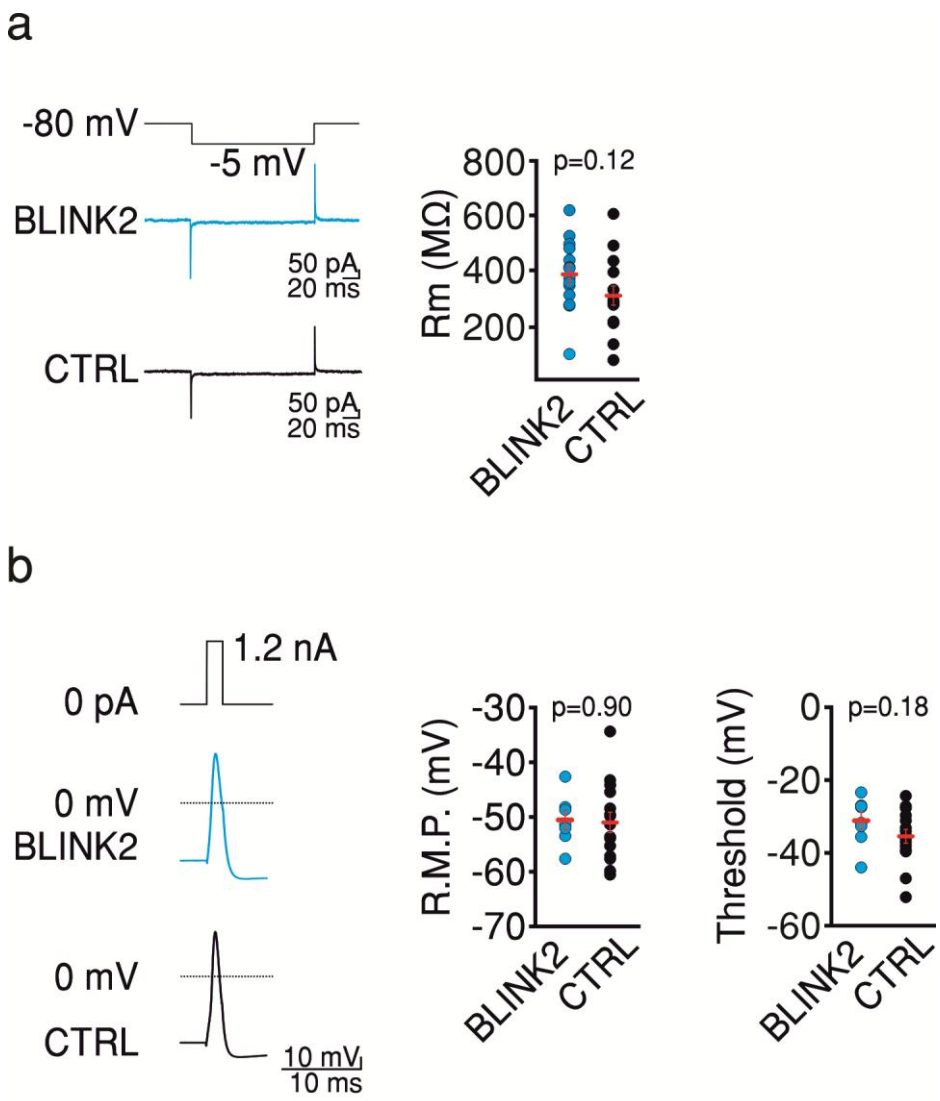
Supplementary Figure 4

Validation of the immunofluorescence-based antibody assay and targeting of BLINK2 to the synapses.
(**a**) Representative staining of BLINK2 surface and total expression. After fixation, rat hippocampal neurons, infected with AAV1/2-hSyn-BLINK2-IRES-eGFP expressing BLINK2 and GFP, were stained with the MAP2 (microtubule-associated protein 2) antibody and the 8D6 monoclonal antibody for BLINK2, without any permeabilization (upper panels). In the lower panels the staining of BLINK2 and MAP2 is shown after permeabilization with Triton X-100. Scale bar, 10 μm. (**b**) Representative dendrites showing the staining of BLINK2 and synaptic markers. BLINK2-expressing hippocampal neurons were stained with the BLINK2 8D6 monoclonal antibody (magenta) and Bassoon (turquoise), a presynaptic protein, or PSD-95 (turquoise), a postsynaptic marker. In merged images the red arrowheads indicate partial colocalization (white) of BLINK2 signal with the synaptic markers in a few synapses. Scale bar, 5 μm.



Supplementary Figure 5

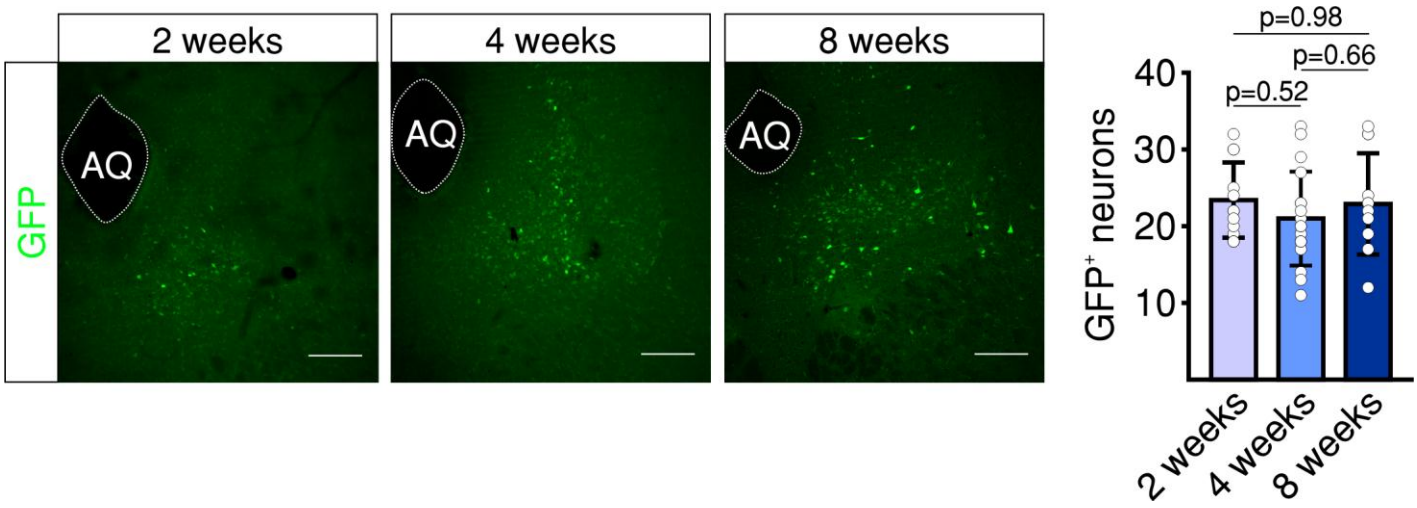
Light exposure did not silence tonic firing activity of neurons from mouse DRN injected with a GFP control virus.
(**a**) (Left) Diagram of injection site in the DRN. (Right) Sample confocal image showing expression of the AAV1/2-hSyn-eGFP virus in the DRN (green, GFP; gray, DAPI) (image representative of $n = 3$). Scale bars, 200 µm or 40 µm (inset). AQ, aqueduct. (**b**) (Top, left) Representative cell-attached voltage clamp recording of firing response before and after 60-s blue light illumination (blue bar) ($n = 7$ independent recordings). (Top right) Expanded view of the recording period defined by the red dotted box. (Bottom left) Time course of the effect of 60-s blue light stimulation (blue bar) on discharge firing rate (5-s binning). (Bottom right) Summary plots indicate mean firing discharge rate 2 min prior to light ($Before_{light}$) and 2 min after lights-off ($After_{light\ 0\text{-}2'}$) ($Before_{light}$, 3.4 ± 0.8 Hz; $After_{light\ 0\text{-}2'}$, 4.1 ± 1.0 Hz $n = 7$, $P = 0.46$, $t = 0.78$, df = 6, two-sided paired t-test). Data are presented as mean ± s.e.m.



Supplementary Figure 6

Passive and active membrane properties in mouse DRN BLINK2-expressing and GFP-expressing neurons.

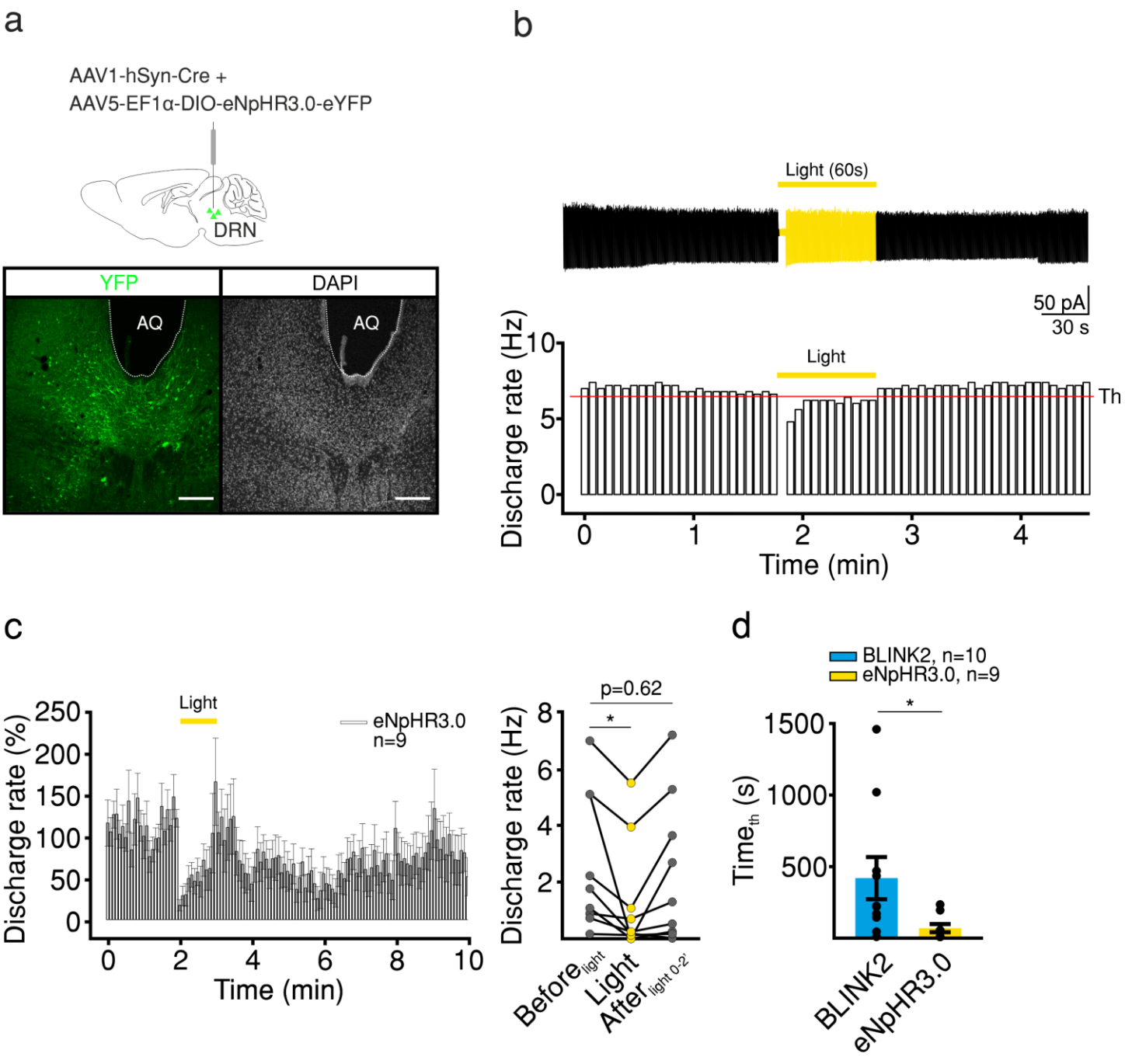
(**a**) (Left) Representative current traces. (Right) Membrane resistance (Rm) was measured in response to –5-mV steps in voltage clamp configuration, and did not significantly change between BLINK2 and CTRL group (Rm, BLINK2 386 ± 35 MΩ, n = 14; CTRL, 308 ± 35 MΩ, n = 15; P = 0.12, t = 1.59, df = 27, two-sided unpaired t-test). (**b**) (Left) Representative current clamp traces in response to fixed current injections. Action potential firing was generated by 2-ms current pulses at 1.2 nA. (Right) resting membrane potential (R.M.P.) and the action potential threshold did not differ between BLINK2 and CTRL groups (R.M.P.: BLINK2, –50.6 ± 1.8 mV n = 7; CTRL, –50.9 ± 1,8 mV, n = 15, P = 0.90, t = 0.13, df = 20, two-sided unpaired t-test; Threshold: BLINK2, –31.1 ± 2.3 mV, n = 8; CTRL, –35.4 ± 1.9 mV, n = 15, P = 0.18, t = 1.39, df = 21, two-sided unpaired t-test). Action potential threshold was determined from the second derivative of the spike waveform. Data are presented as mean ± s.e.m. obtained from: BLINK2, independent recordings, n = 14, n = 6 mice; CTRL, independent recordings, n = 15, n = 4 mice.



Supplementary Figure 7

Effect of long-term expression of BLINK2 in neurons.

(Left) Representative confocal images from coronal brain sections obtained from AAV1/2-hSyn-BLINK2-IRES-eGFP injected animals and immunoreacted with anti-GFP antibody. (Right) Plot indicates the number of GFP-expressing neurons scored in the region of the DRN surrounding the injection site 2 (n = 12), 4 (n = 18), and 8 (n = 12) weeks after injection. Points represent the number of GFP-expressing neurons in each volume analyzed. Quantification analysis was performed in a blinded manner and sample identity was not revealed until correlation was completed. The number of virus-infected BLINK2-expressing cells was scored assessing the number of GFP-positive (GFP^+) cells in 10× confocal image sections. GFP-positive neurons were counted on 3 consecutive 50-μm coronal sections within two distinct volumes of 200 μm × 200 μm × 15 μm chosen in the DRN region surrounding the injection site (n = 2 mice at 2 and 8 weeks post-injection; n = 3 mice at 4 weeks post-injection). Statistical significance was calculated with one-way ANOVA with multiple comparison and Tukey's P value correction (ns: $P > 0.05$). Scale bar, 200 μm. AQ, aqueduct. Data are presented as mean ± s.d.

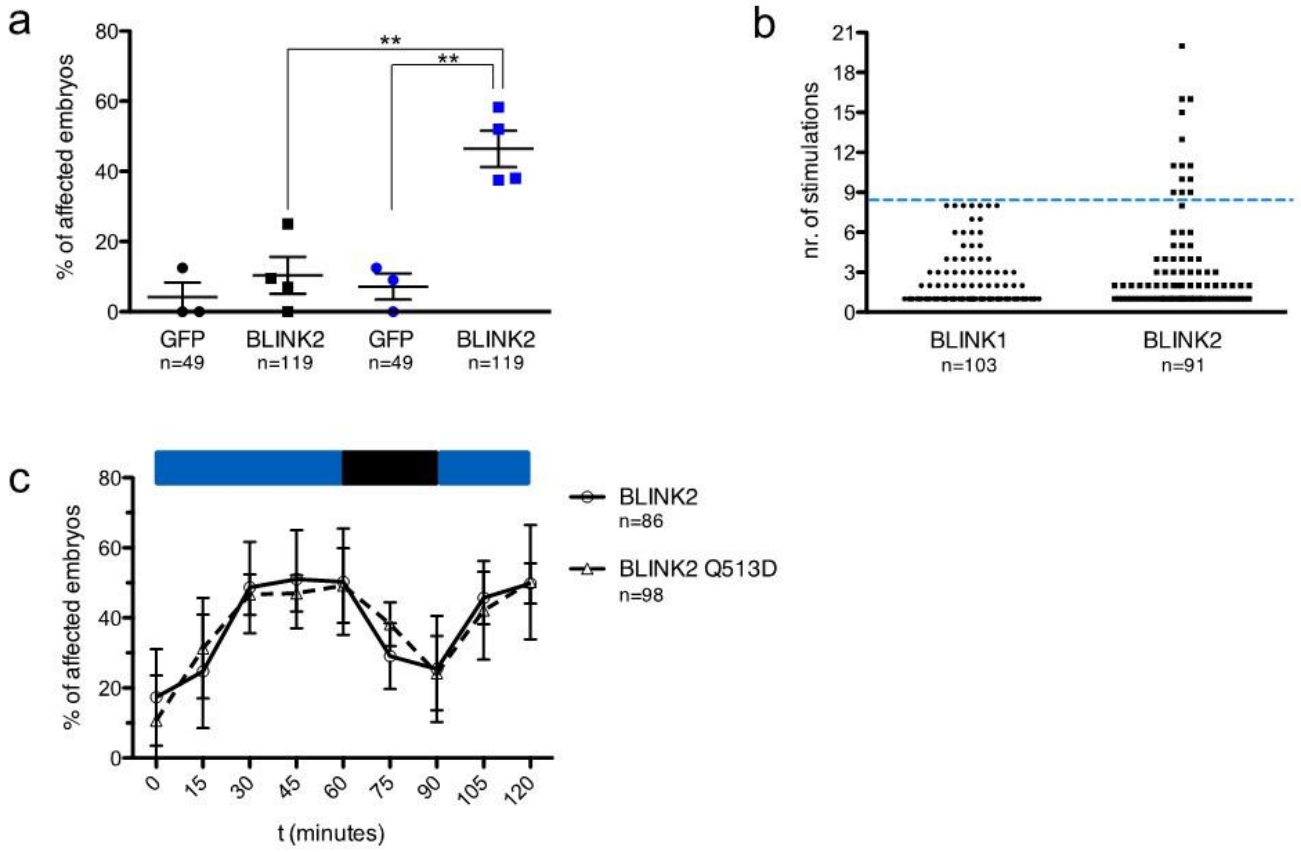


Supplementary Figure 8

Optogenetic activation of eNpHR3.0 silences tonic firing activity of mouse DRN neurons.

(Top) Diagram represents virus injection site (AAV1-hSyn-Cre + AAV5-EF1α-DIO-eNpHR3.0-eYFP). (Bottom) Confocal image showing expression of eNpHR3.0-eYFP in the mouse DRN (green, YFP; gray, DAPI; scale bar, 200 µm; AQ, aqueduct) (*n* = 3 mice). (**b**) (Top) Representative cell-attached voltage clamp recording of firing response before and after 60 s of yellow light illumination (yellow bar) (independent recordings, *n* = 9, *n* = 3 mice). (Bottom) Time course of the effect on the discharge rate of 60 s of yellow light stimulation (yellow bar) on this representative recording; red horizontal bar represents the threshold (Th) defined as the mean discharge rate minus two times the s.d.; mean firing rate is calculated on values (5-s binning) computed over 1 min prior to light illumination (see also Methods). (**c**) (Left) Average time course of the effect of 60 s of yellow light stimulation (yellow bar) on firing

discharge rate (5-s binning). (Right) Summary plots indicate mean firing discharge rate 2 min prior to light ($Before_{light}$), during 1 min of light (Light) and 2 min after light ($After_{light\ 0\text{-}2'}$) ($n$ = 9; Repeated Measures 1 Way ANOVA (RM1WA), $F_{8,2}$ = 6, P = 0.019; post hoc, $Before_{light}$ versus Light, P = 0.047; $Before_{light}$ versus $After_{light\ 0\text{-}2'}$, P = 0.62; with multiple comparison and Dunnett's P value correction). (**d**) Bar graph indicating the duration of neuronal silencing (time below threshold; $Time_{th}$) induced by eNpHR3.0 or BLINK2 activation ($Time_{th}$: BLINK2 versus eNpHR3.0, P = 0.03, Mann–Whitney U =18, two-sided Mann–Whitney test). The $Time_{th}$ of BLINK2 has been calculated from the dataset included in Fig. 3c. Data are presented as mean ± s.e.m.



Supplementary Figure 9

Light controls the behavior of zebrafish embryos transiently expressing BLINK2: comparison with BLINK1 and BLINK2 Q513D.

(**a**) Altered escape response in 2-d-old zebrafish embryos expressing BLINK2 (squares) or GFP (circles) (both RNAs injected at 200 pg/embryo), measured in embryos kept in the dark (black) or after 60 min of exposure to blue light (465 nm, 85 $\mu W/mm^2$) (blue). The escape response was considered altered when one or two touches were not sufficient to elicit it. BLINK2 Q513D RNA was used for the experiments shown in this graph. Similar results were obtained when BLINK2 RNA was injected (data not shown); mean and s.e.m. were calculated on three (GFP) and four (BLINK2) different experiments. Total number of embryos (*n*) is 49 and 119, respectively. (**b**) BLINK1[20] versus BLINK2. The graph shows the number of stimulations required in order to elicit an escape response in each embryo, injected with either BLINK1 (dots) or BLINK2 (squares) RNA. BLINK2 Q513D RNA was used for the experiments shown in this graph. Measurements were performed after 30 min of exposure to blue light. The blue dashed line highlights the maximum number of tactile stimuli (8 touches) required to elicit an escape response in BLINK1 injected embryos. (**c**) BLINK2 versus BLINK2 Q513Q. Reversibility in dark and kinetics of the light effect on the escape response of 2-d-old embryos expressing BLINK2 (continuous line) and BLINK2 Q513D (dashed line) Blue light was turned on at time 0, after the first measurement. After 60 min of exposure, the light was turned off for 30 min and then turned on again (as indicated by blue and black bars above the graph). The response to mechanical stimulation was checked and recorded every 15 min. The total number of embryos in each group was $n = 86$ (BLINK2) and $n = 98$ (BLINK2 Q513D) from 3 independent experiments. For **a**, we performed two-tailed *t*-tests comparing GFP versus BLINK2 in the dark, GFP versus BLINK2 in blue light, GFP dark versus blue light, BLINK2 dark versus blue light. Statistically significant results are shown with asterisks ($P = 0.028$ and 0.0023 for BLINK2 dark/light and GFP/BLINK2 light, respectively).

Supplementary Notes

Supplementary Table 1- List of constructs and details on their sequences

	#	NAME	SEQUENCE DETAILS
	0	BLINK1	
ER export/ PM trafficking	1	BLINK-EYFP	BLINK1::eNpHR3.0 (291-559)
	2	BLINK-ER	BLINK1::mKir2.1 (374-380)
	3	BLINK-TS	BLINK1::mKir2.1 (233-252)
	4	BKir	BLINK1::mKir2.1 (180-428)
	5	BKir2	BLINK1::mKir2.1 (233-428)
	6	BKir3	BLINK1::mKir2.1 (233-380)
14-3-3 binding site	7	BT1D	BLINK1::mTASK1 (245-394) S393D
	8	BT3D	BLINK1::mTASK3 (245-402) S401D
	9	BK6D/BLINK2	BLINK1::KAT1 (506-677) S676D
14-3-3 binding site/ER export	10	BLINK2.1	BLINK1::KAT1 (506-677) S676D, ^{524}DIDAE^{528}
	11	BLINK2.2	BLINK1::KAT1 (506-677) S676D, ^{547}DIDAE^{551}

Constructs were obtained by adding at the C terminus of BLINK1 (Cosentino, C. et al. Optogenetics. Engineering of a light-gated potassium channel. *Science* **348**, 707-710 (2015)) amino acid sequences from the following proteins: eNpHR3.0; mKir2.1 (Gene ID: 16518); mTASK1 (NCBI gene: 16527); mTASK3 (NCBI gene: 223604); KAT1 (Gene ID: 834666). The numbers in brackets refer to the amino acid numbering in the original sequence. Phospho-mimetic mutations S/D were introduced for constructs 7-11 as indicated. In constructs 10 and 11 additional residues in position 524-528 and 547-551 have been mutated to DIDAE, to introduce the ER export sequence of KAT1 (residues 392-396). Construct #9 has been renamed BLINK2.

Supplementary Methods

Planar lipid bilayer recordings

Kcv_{PBCV1} and BLINK1 were produced by in vitro translation into nanodiscs (MembraneMax HN Protein Expression Kit (Invitrogen, Carlsbad, CA, USA)) and reconstituted into planar lipid bilayers from 1,2- diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC, from Avanti Polar Lipids, Alabaster, AL, USA) as described (Braun, C.J., Baer, T., Moroni, A. & Thiel, G. Pseudo painting/air bubble technique for planar lipid bilayers. *J Neurosci Methods* **233**, 13-17 (2014)).

Rat neurons

Antibodies. PSD-95 (NEUROMAB, cod 75-028), Bassoon (Assay Designs mab7f). Alexa fluorescent-labeled antibodies were purchased from ThermoFisher.

Viral injection and immunofluorescence in mice

C57BL/6J male mice were maintained in standard cages with food and water ad libitum at 22±1°C under an artificial 12/12h light/dark cycle. Animals have been deeply anesthetized (Avertin 250 mg/kg, intraperitoneal injection) and firmly stabilized on a stereotaxic apparatus (Stoelting Inc.). Craniotomy were performed with a microdrill (Cell Point Scientific Inc.) in correspondence of Dorsal Raphe Nucleus (DRN) coordinates (AP: -4, 36; ML: 0,5; DV: -2,8). A volume of 0.5 µL of AAV1/2-hSyn-Blink2-IRES-eGFP (or AAV1/2-hSyn-GFP control virus) was injected through a Nanofil syringe mounted on an UltraMicroPump UMP3 with a 4-channel Micro4 controller (World Precision

Instruments), at a speed of 0.1 μL/min. Mice were sacrificed 2, 4 or 8 weeks after the injection. Anesthetized mice were transcardially perfused with Phosphate Buffered Saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were dissected and post-fixed in PFA 4% O/N at 4°C. 50μm coronal sections were obtained with a vibratome (Leica Microsystems). Antigen retrieval was performed as follows: sections were incubated 30 min at 80°C in sodium acetate 50mM solution. Then, the slices were washed 3 times in a PBTriton 0.1% solution. Sections were incubated with chicken anti-GFP (Abcam 1:500) primary antibody for two O/N at 4°C and then rinsed in PBTriton 0.1%. Alexa Fluor 488 goat anti chicken IgG (1:500, Life Technologies) was used O/N at 4°C as secondary antibody. Next day, sections have been washed 3 times with PBTriton 0.1% solution and counter stained with DAPI.High power confocal images in the injection site of the DRN region were obtained on a Nikon A1 confocal microscope with a 10x plan-apochromat.

Quantification and statistical analysis.

Quantification analysis was performed in blind and sample identity was not revealed until correlation was completed. The number of viral-infected BLINK2-expressing cells was scored assessing the number of GFP positive (GFP+) cells analysing 10x confocal images sections. GFP-positive neurons were counted on 3 consecutive 50 μ m coronal sections within two distinct volumes of 200 μ m x 200 μ m x 15 μ m chosen in the DRN region surrounding the injection site (n=2 mice at 2 and 8 weeks post injection; n=3 mice at 4 weeks post injection). A group mean and a SD was

generated. Data were plotted with GraphPad Prism 6.0 software. Comparison between time points has been statistically validated by one-way ANOVA with Tukey's correction for multiple comparison. Statistical significance occurred with p-value < 0.05.

Zebrafish

pCS2+GFP, pCS2+BLINK1 and pCS2+BLINK2 constructs were linearized with NotI and transcribed with Sp6 RNA polymerase, using the mMESSAGE mMACHINE® SP6 Transcription Kit (Ambion) and following manufacturer's instructions. RNA microinjection and touch-evoked escape response were performed essentially as previously described (Cosentino, C. et al. Optogenetics. Engineering of a light-gated potassium channel. *Science* **348**, 707-710 (2015)), except for E3 buffer substituted here with Fish Water (0.1 g/l Instant Ocean Sea Salt, 0.1 g/l NaHCO3, 0.19 g/l CaSO4, 0.2 g/l methylene blue).