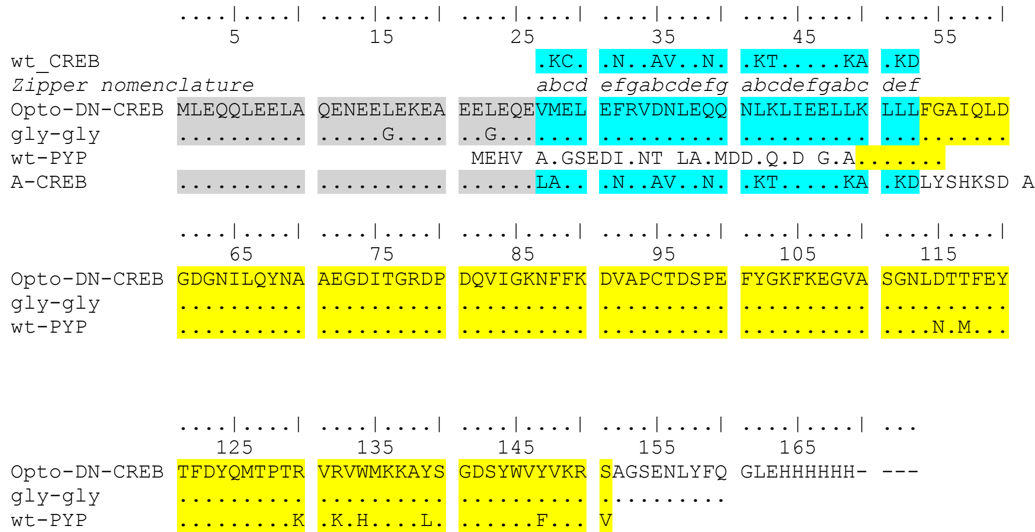
Supplemental Figures:



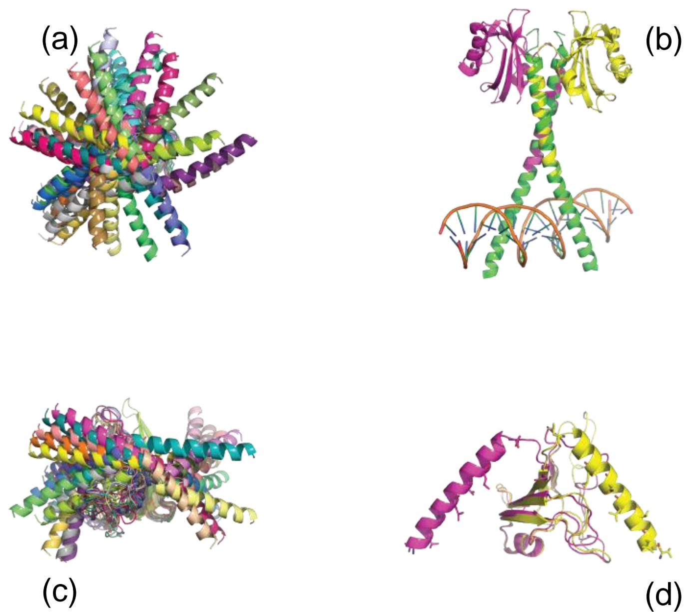


Figure S1 (refers to Figures 1, 3): Top (previous page): Sequence alignment of opto-DN-CREB with its parent proteins. ("**.**" Indicates identiy with the opto-DN-CREB sequence. The acidic extension section is highlighted in gray and is part or A-CREB, opto-DN-CREB and the opto-DN-CREB Gly-Gly muatant only. The zipper region is highlighted in cyan (corresponding to Figs 1,3). The PYP core region is highlighted in yellow (also corresponding to Figs 1,3). **Bottom (this page)**(a) Simulation of packing of the N-terminal A-CREB helix onto the PYP core domain in the dark state produced a large range of structures (32 representative structures in which the helix extends from the PYP core are shown). (b) A dimer model of opto-DN-CREB in dark state was built by aligning one of the simulated structures in (a) to the CREB bZIP domain (PDB: 1DH3). (c). Simulation of packing of the N-terminal A-CREB helix onto the PYP core domain in the light state showed only two structural clusters. Sixteen structures from each cluster are shown. (d) One representative structure from each cluster in (c) is shown. The residues corresponding to the *a/d* position of bZIP helix are shown in stick format. These residues point towards the PYP core, suggesting steric hindrance of dimer formation.

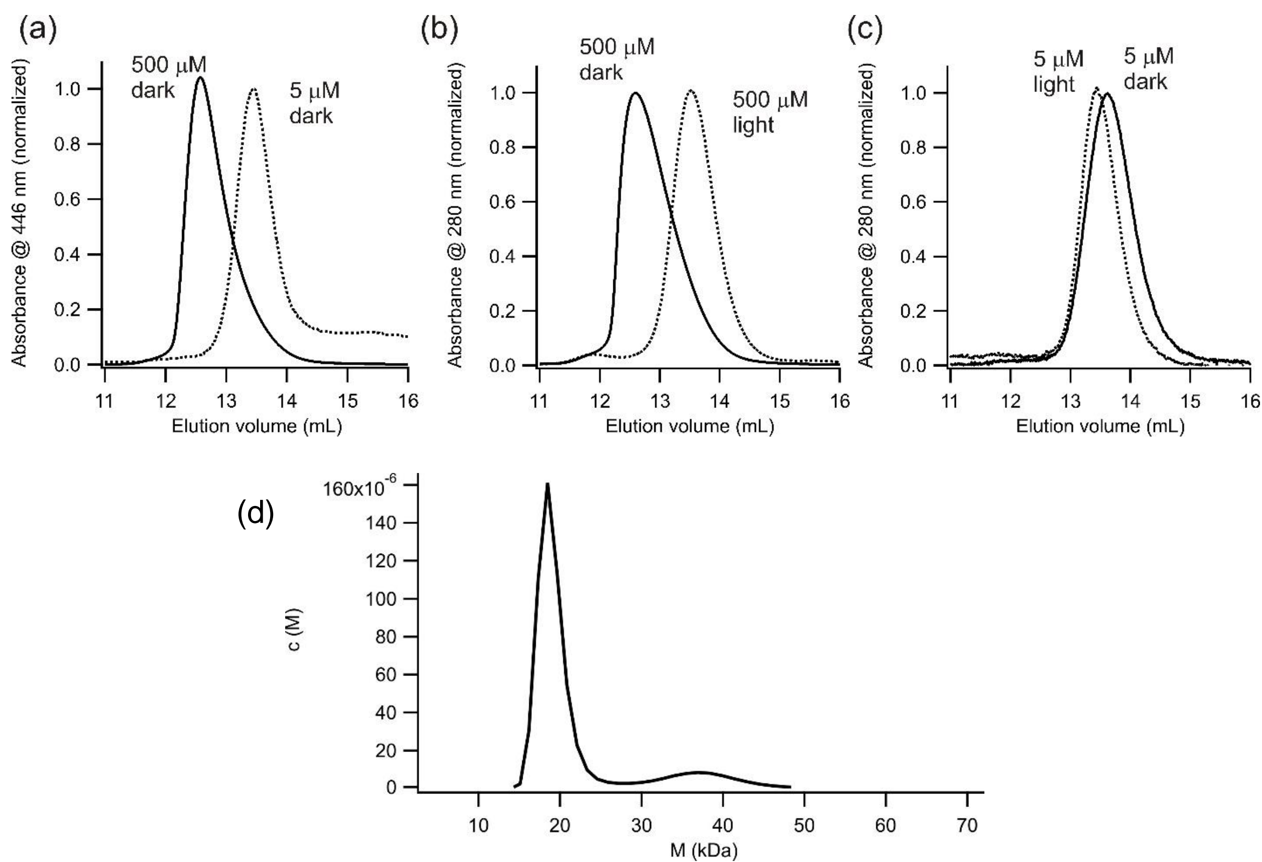


Figure S2 (refers to Fig. 2 and Experimental Procedures). Size exclusion FPLC chromatograms of opto-DN-CREB (a-c). (a). Size exclusion chromatography performed at 500 μM (solid line) and 5 μM (dashed line) shows a concentration dependent shift in elution volume. (b) At high concentrations (500 μM) dark adapted opto-DN-CREB (solid line) behaves as a larger species than blue light irradiated opto-DN-CREB (dashed line) (c) At low concentrations (5 μM) opto-DN-CREB elutes at the same volume in the light and dark. (d) Molecular weight distribution plot of dark adapted opto-DN-CREB derived from sedimentation velocity analysis at 14 μM. opto-DN-CREB behaves primarily as a monomer under these conditions.

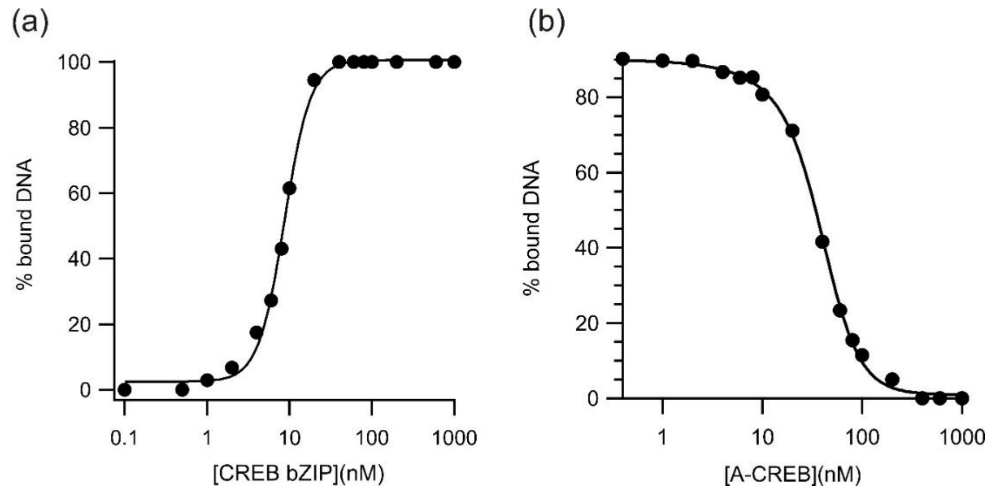


Figure S3 (refers to Figure 4). (a) EMSA analysis of CREB bZIP binding to CRE (Cy5-labeled oligo) performed as described in the materials and methods section. The apparent K_d is 10 ± 3 nM. (b) Inhibition of CREB bZIP binding to CRE DNA by A-CREB. The apparent K_i for inhibition is 15 ± 5 nM.

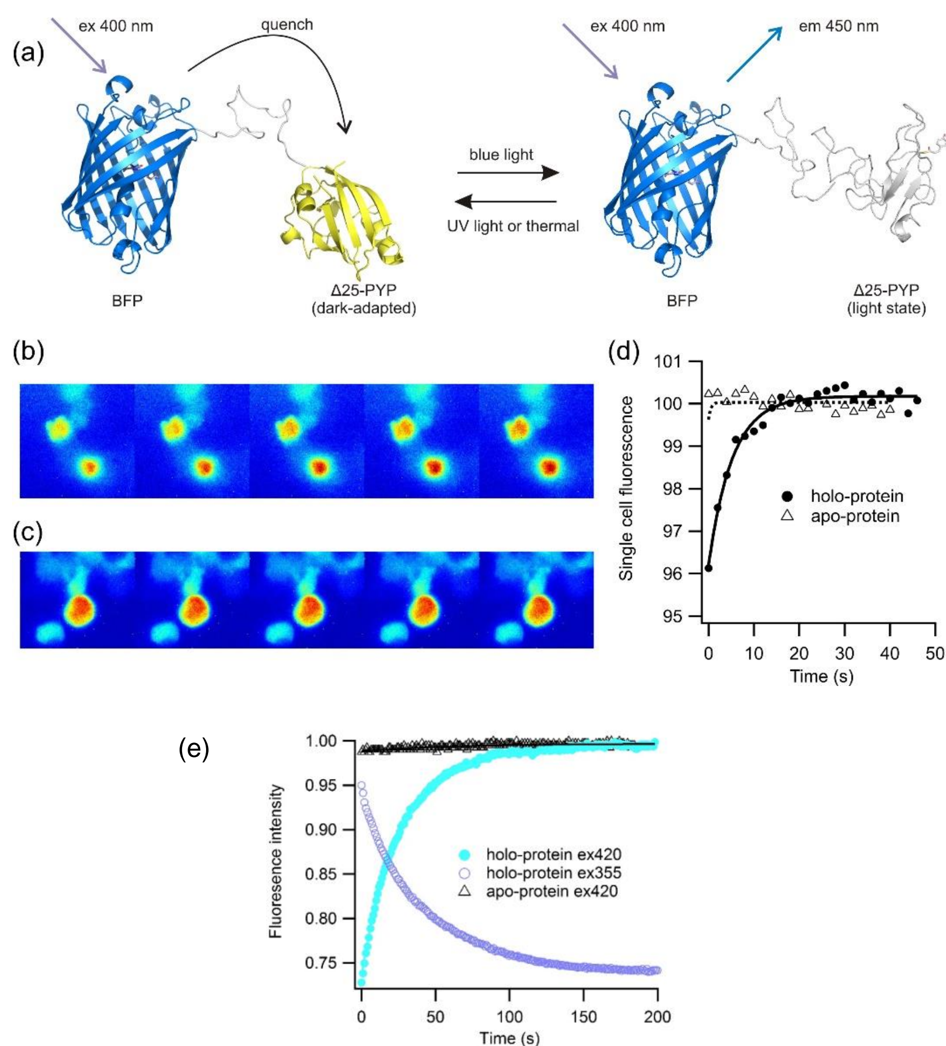


Figure S4 (refers to Experimental Procedures) . Photoswitching of PYP in living HEK293 cells. (a) Model of the reporter construct in which BFP is fused to the N-terminus of Δ25-PYP via a flexible linker. Excitation of BFP at 400 nm leads to fluorescence emission at 450 nm. Emission is partially quenched by dark-adapted PYP. The excitation wavelength also causes isomerization of PYP to its light state. This state does not quench BFP so effectively, thereby leading to a time dependent increase in fluorescence emission when dark-adapted BFP-Δ25-PYP is exposed to blue light. The dark state can be reformed by thermal relaxation or by UV (360 nm) irradiation. Fluorescence images of single HEK293 cells expressing BFP-Δ25-PYP with (b) or without (c) addition of chromophore. Images are acquired at 2 s intervals after exposure of dark-adapted cells to light (400 nm). Only those cells exposed to chromophore show a time dependent increase in fluorescence emission (d). (e) Similar fluorescence responses are seen in cell suspensions. Excitation at 420 nm causes a time dependent increase in emission at 450 nm due to relief of quenching by Δ25PYP as it is converted to its light state. Excitation of a holo-protein sample at 355 nm, after previous exposure to 420 nm, causes a time dependent quenching of fluorescence. Time dependent quenching is also observed during dark adaptation, but the rate is enhanced by UV excitation. Apo-protein (no chromophore added) shows no time dependent response at any wavelength.

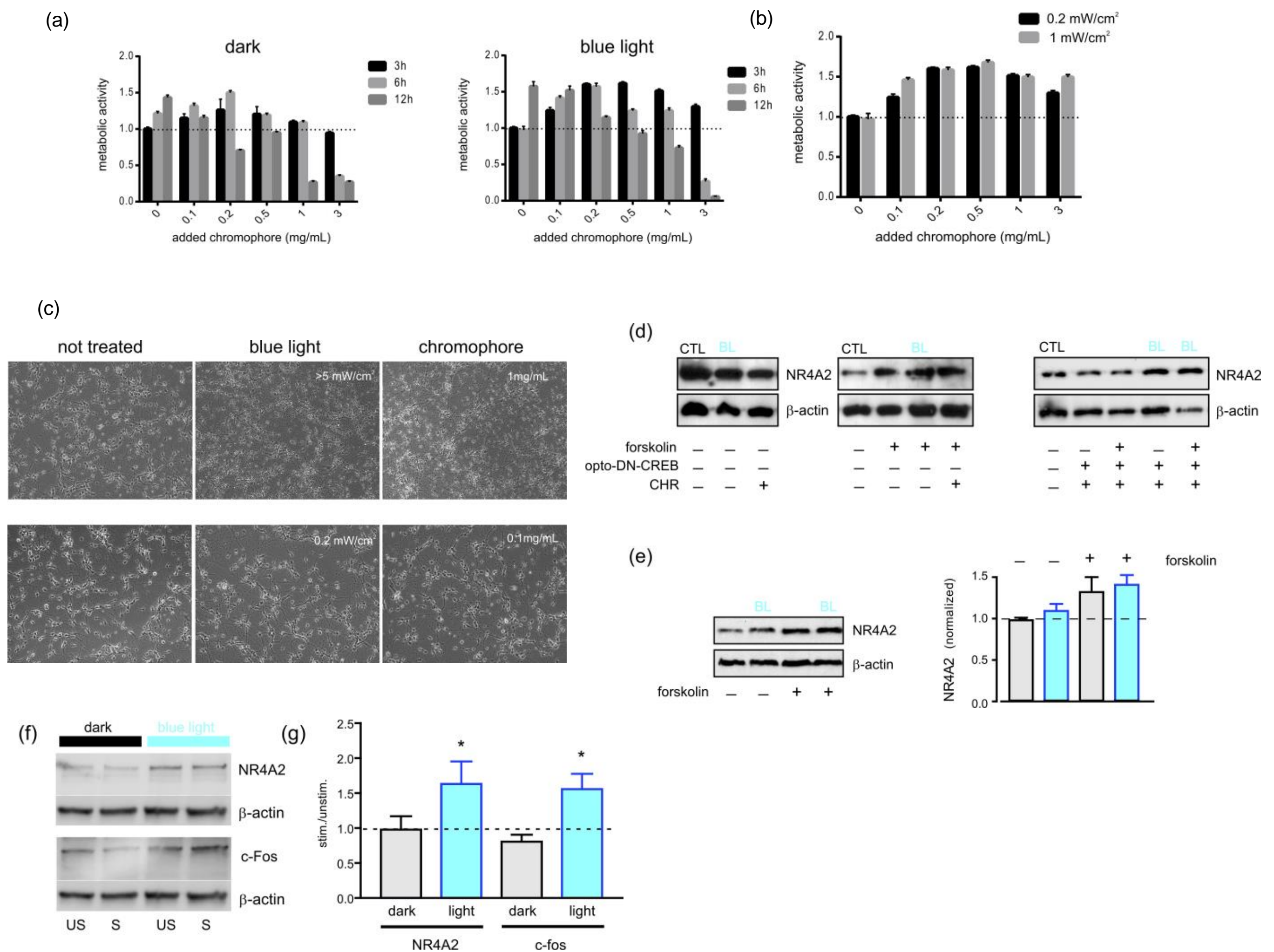


Figure S5 (refers to Fig. 5 and to Experimental Procedures). (a) HEK293T cell viability as a function of added chromophore concentration and time of incubation as measured using MTT assays (left – dark, right – under blue light (0.2 mW/cm^2)) . (b) HEK293T cell viability vs light level (3h incubation) as measured using MTT assays. (c) Light microscopy images of primary cultured neurons under the conditions indicated

Forskolin treatment of HEK293 cells (d,e) produces an increase in NR4A2 that is blocked by opto-DN-CREB (oCR) in the dark, but not under blue light. (d) Western blots**:** *left:* cells were transfected with control (empty) vector (CTL) and illuminated with blue light (BL) or incubated with chromophore (CHR). *center*: cells were stimulated with forskolin along with blue light or chromophore treatment. Neither blue light nor chromophore alone affect cellular levels of NR4A2 under basal conditions or after forskolin stimulation. *right*: in cells transfected with opto-DN-CREB (oCR) and incubated with chromophore in the dark, NR4A2 levels under basal conditions were decreased and the forskolin-mediated increase seen in controls was prevented. Under blue light illumination, however, this inhibition by opto-DN-CREB was relieved. Levels of NR4A2 were quantified relative to β-actin and normalized to levels under control conditions (*i.e.* no treatment, lane 1 in each blot). (e) The opto-DN-CREB mutant in which two Leu residues are mutated to Gly residues in the A-CREB domain (2Gly) is not able to act as a dominant negative inhibitor in the dark, or under blue light; in both cases a forskolin stimulated increase in NR4A2 is seen. **Opto-DN-CREB (oCR) inhibits forskolin and KCl mediated NR4A2 or c-Fos production in primary cortical neurons (f,g). This inhibition is relieved by blue light.** (f) Representative Western blots for NR4A2, c-Fos and β-actin (loading control) without stimulation (US) and after stimulation with forskolin and KCl (S) (g) Bar graphs of data. Bands were quantified and data represented as [stimulated/ β -actin]/[unstimulated/ β -actin] (*, significant difference between S and US, $p<0.05$, n=8-10 for each condition). With a control vector, NR4A2 increases in response to forskolin and KCl stimulation were observed in both dark and under light illumination (not shown).

Supplemental Experimental Procedures:

Protein Design

A sequence alignment shows that *a/d/e/g* positions of the first two heptads of CREB align best to the PYP N-terminal cap via the register shown in Fig. S1(top). This mapping involves aligning the 1st position of the PYP sequence to the "a" position in the CREB heptads (alignment of M1V, E2K, H3C ….), and was expected to serve as a less disruptive starting point than any other potential alignment.

Computational design was carried out using the Rosetta software suite (Leaver-Fay et al., 2011). Rosetta is freely available for academic use (www.rosettacommons.org).

Using X-ray structural data for the dark state of PYP (PDB: 1NWZ) and the DNA bound structure of CREB bZIP (PDB: 1DH3), we used the "fixbb" application in Rosetta to guide design of the chimera. We enforced the CREB sequence at each of the *a/d/e/g* positions of the heptad, while redesigning *b/c/f* positions of the heptad and residues on the PYP core that make contact with the N-terminal cap in the dark state structure. The designed structure with the lowest (most favorable) Rosetta score was selected.

The Rosetta command line used to carry out the "fixbb" step is as follows:

```
fixbb -s XXX.pdb -resfile XXX.resfile -nstruct 20 -packing:ex1 -packing ex2
        -packing:extrachi_cutoff 0 -packing:use_input_sc
```

Subsequently, iterative structural refinements were carried out using the "Rosetta Holes"(Sheffler and Baker, 2009) and "backrub" applications (Smith and Kortemme, 2008). In this design stage, we inspected designed structures to identify potential steric clashes, exposed hydrophobic patches, and buried unsatisfied polar groups. "Rosetta Holes" was used to identify

void cavities in the designed structure, and “backrub” was used in further refinements to solve these potential structural defects. For example, residue N41 (N15 in wtCREB, at the "a" heptad position) is critical for CREB function (Gonzalez et al., 1996), but Rosetta predicts replacing L41 with N would destabilize the dark state opto-DN-CREB structure. This prediction is based on the fact that the (polar) side chain of N15 points into the core of (dark state) opto-DN-CREB, without forming any compensatory hydrogen bonds. Simulations using Rosetta showed that an F147Y mutation (F121Y in wtPYP) would help accommodate N41 by forming a new hydrogen bond to N15. As another example, Rosetta Holes identified a buried void created by incorporating the I37L mutation (position 11 in wtCREB, at the "d" heptad position); our design resolved this void by introducing a compensatory H134W mutation in the opto-DN-CREB core.

The Rosetta command line used to carry out the “Rosetta Holes” step is as follows:

```
holes -s XXX.pdb -dalphaball PATH_TO_DALPHABALL -holes:make_pdb
    -holes:make_voids
```

The Rosetta command line used to carry out the “backrub” step is as follows:

```
backrub -s XXX.pdb -resfile XXX.resfile -nstruct 20 -packing:ex1 -packing:ex2
    -packing:extrachi_cutoff 0 -packing:use_input_sc -pivot_residues
    RESIDUES_SURROUNDING_TARGET_AA
```

The final design incorporates the CREB identity at all *a/d/e/g* positions except for two. In contrast, at *b/c/f* positions, which are not expected to contribute to binding specificity of the coiled coil) the final design incorporates the CREB identity at only 3 of 12 residues. Using the “Talaris2013” score function in Rosetta, the final designed structure is predicted to stabilize the dark adapted state structure by 7.6 Rosetta energy units relative to the wild type.

The “loopmodel” application in Rosetta was next used to explore packing of the (A-CREB derived) N-terminus of opto-DN-CREB onto its (PYP derived) protein core. Structures from the NMR ensembles of wildtype PYP that lack the N-terminal cap (Δ25PYP) in either dark-adapted (PDB: 1XFN) or light-adapted states (PDB: 1XFQ) were used as a starting point for the PYP core domain, the sequence was adjusted to that of opto-DN-CREB, then the N-terminus was added. Fragment files for loop modeling were generated using the ROBETTA server (http://robetta.bakerlab.org/)(Kim et al., 2004).

The Rosetta command line used to carry out the “loopmodel” step is as follows:

```
loopmodel -s XXX.pdb -loops:loop_file XXX.loop -loops:build_initial 1
        -loops:remodel quick_ccd -loops:refine refine_ccd -loops:intermedrelax
        fastrelax -loops:relax fastrelax -loops:extended -in:file:fullatom
        -nstruct 3000 -loops::frag_sizes 9 3 -loops::frag_files frag9 frag3
        -out:file:silent_struct_type binary -out:file:fullatom
```

Output structures from these “loopmodel” simulations were combined into a single file (in “silent file” format), then clustered, and representative structures from each cluster were selected. The Rosetta command line used to carry out the clustering step is as follows:

```
clustering.py --silent=all_decoys.out
        --rosetta=PATH_TO_ROSETTA_CLUSTER_EXECUTABLE cluster_summary.txt
        cluster_histogram.txt
```

To build a model of dimeric opto-DN-CREB (Fig. 1b), we used the “remodel” application in Rosetta (Huang et al., 2011). The twenty-five amino acids from the N-terminal

designed sequence were threaded onto the corresponding structural segment of the CREB helix (PDB: 1DH3), and the "remodel" application was used to sample the degrees of freedom of the assembly between this designed helix and the core domain of PYP. In essence, this approach treats this as a "domain assembly" problem: the N-terminal helix and the PYP core were fixed as two rigid body domains in the simulation, and only the conformation of the linker region connecting these two domains was varied in the sampling. The Rosetta command line used to carry out the "remodel" step was as follows:

```
remodel -s XXX.pdb -remodel:blueprint XXX.bp -run:chain X -no_jumps
        -nstruct 3000 -in:file:fullatom -out:file:fullatom
```

The simulation of opto-DN-CREB in the dark adapted state exhibited extensive structural variation in the position of the N-terminal helix (Figure S1a). Further, many of these models exposed the dimer interface of the coiled coil, which may allow formation of "canonical" CREB bZIP helices: we illustrate this possibility by generating a potential opto-DN-CREB dimer by aligning a low-energy simulated structure from the dark adapted state ensemble onto the wildtype CREB dimer (Figure S1b).

In contrast, the corresponding simulation of opto-DN-CREB in the light-adapted state showed far less structural diversity in the position of the N-terminal helix (Figure S1c). In these models, the N-terminal helix preferentially docks to one of two surfaces on the PYP core (Fig. S1d), and residues corresponding to *a/d* positions of helix domain point towards hydrophobic patches on the PYP core. In these models, the surface that mediates CREB bZIP homodimerization is thus occluded, suggesting the structural basis for light-adapted opto-DN-CREB preferentially existing as a monomer.

To predict the specificity of coiled coil formation, we used the "bZIP Coiled-Coil Scoring Form" server (http://compbio.cs.princeton.edu/bzip/) that provides a frontend to the bZIP-score program (Fong et al., 2004).The opto-DN-CREB sequence and the wild-type CREB sequence were entered with the expected heptad registers. The server uses three different score functions to calculate the specificity between these two sequences, and outputs the predicted percentile rank using each sequence as the reference. The output is shown in the table below. For instance, the first row of the table reads: "Base-optimized weights   37.423   99.70   100.00" This means that using the "Base-optimized weights" parameters, the raw score of the specificity between the design sequence and WT CREB sequence is 37.423. This score is at 99.7 percentile in the score distribution for designed sequence (opto-DN-CREB). In other words, for all of the pairwise specificity scores between opto-DN-CREB sequence and known bZIP sequences, the specificity score between opto-DN-CREB sequence and WT CREB sequence is higher than 99.7% of them. The last number (100.00) means that for all of the pairwise specificity scores between WT CREB sequence and known bZIP sequences, the specificity score between WT CREB sequence and the design sequence is the highest. The rest of the rows can be interpreted in the same way, but using different parameters for the score function.

```
Scored sequence alignment:
opto-DN-CREB = VMELEFRVDNLEQQNLKLIEELLKLLLFGAIQ
registers    = abcdefgabcdefgabcdefgabcdefgabcd
wt CREB      = VKSLENRVAVLENQNKTLIEELKALKDLYSHK
```

Model	Actual score of opto-DN-CREB + wt CREB	Percentile among opto-DN-CREB scores	Percentile among wt CREB scores
Base-optimized weights	37.423	99.70	100.00
Base + Human bZIPs	4.718	96.95	95.43
Simple electrostatic weights	4.000	95.12	88.11
Coupling energy weights	5.000	100.00	97.26

Protein Expression *in vitro*

Opto-DN-CREB bacterial protein expression and purification.

The expression and reconstitution of the opto-DN-CREB protein was adapted from the work of Morgan *et al* (Morgan et al., 2010). The same protocol was followed for all opto-DN-CREB constructs. The pET24b plasmid containing the target DNA (0.2 ng) was transformed into *E. coli* BL21*(DE3) competent cells and plated onto an agar plate containing 30 μg/mL of kanamycin. The following day, a single colony was inoculated into 25 mL of LB broth media containing the same concentration of kanamycin and allowed to shake overnight at 37°C. Subsequently, the 25 mL culture was used to inoculate 1 L of LB media containing 30 μg/mL of kanamycin. Cells were grown at 37°C until an O.D. of 0.6 was reached then protein expression was induced with 1 mM IPTG. The temperature was adjusted to 25°C, and the culture was allowed to shake for one hour before 25 mg of activated chromophore (*p*-coumaric acid, thiophenylester) dissolved in 1 mL of ethanol was added to the medium. The culture was allowed to grow for a further 5 hours at 25°C before the cells were harvested by centrifugation.

The cell pellets were subsequently resuspended in lysis buffer (50 mM sodium phosphate (pH 8.0), 300 mM sodium chloride, and 5 mM magnesium chloride) and were lysed by sonication for 5 min on ice. After centrifugation, the cleared lysate was passed through a 0.45 μm filter and loaded onto a Ni-NTA column that was equilibrated with lysis buffer. After addition of the protein-containing supernatant, the resin was washed with 10 column volumes (CV) of the lysis buffer. The resin was subsequently washed with 5 CV of high-salt buffer (i.e., lysis buffer supplemented with 2 M NaCl) followed by a further 5 CV of lysis buffer. To elute nonspecifically bound proteins, the resin was washed with 5 CV of lysis buffer supplemented

with 5 mM imidazole. The protein was eluted by increasing the imidazole concentration to 200 mM.

The eluted protein was dialyzed extensively against 40 mM Tris-OAc, 1 mM EDTA, and 100 mM NaCl (pH 7.5) [1× TAE, 100 mM NaCl (pH 7.5)] at 4ºC. The dialyzed protein solution was concentrated to ~1.5 mL using an Amicon ultracentrifugal device (10000 Da NMWL) (Millipore). The concentrated protein was treated with protease solution by adding 500 µL of 20× ProTEV Buffer, 100 µL of 100 mM DTT, and 1000 unit (200 µL) of ProTEV Plus (Promega Corp), with a final reaction volume of 10 mL. The protein protease mixture was incubated at 30ºC for 5 hours before it was passed slowly through a Ni-NTA column that was equilibrated with 1× TAE, 100 mM NaCl buffer (pH 7.5). The resin was washed with an additional 5 mL of the latter buffer to completely elute the tag-free protein, and the eluted protein was concentrated using an Amicon ultracentrifugal device. Subsequently, the protein was applied to a Superdex 75 10/300 GL column (GE Healthcare) running in 1× TAE, 100 mM NaCl buffer (pH 7.5). UV−Vis absorbance spectroscopy was used to determine which eluted fractions had the highest ratios of absorbance at 446 nm to that at 278 nm (~1.4). The purity and identity of samples were confirmed using 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis and electrospray ionization mass spectrometry (ESI-MS).

CREB bZIP protein expression and purification.

The pET24b plasmid containing the target DNA (0.2 ng) was transformed into *E. coli* BL21*(DE3) competent cells and plated onto an agar plate containing 30 µg/mL of kanamycin. The following day, a single colony was inoculated into 25 mL of LB broth media containing the same concentration of kanamycin and allowed to shake overnight at 37ºC. The overnight culture

was used to inoculate 1 L of LB containing 30 μg/mL of kanamycin. Cells were grown at 37°C and - protein expression was induced using 1 mM IPTG when an O.D. of 0.6 was reached. After shaking for 4 hours, cell were harvested by centrifugation, resuspended in buffer A (6 M of guanidine HCl solution, 10 mM of imidazole, 100 mM of phosphate at pH 8.0), and lysed by shaking at room temperature for one hour. The cell debris was removed by centrifugation. The supernatant was passed through a 0.45 μm filter and was loaded onto a Ni-NTA column pre-equilibrated with buffer A. After washing several times with buffer A, the protein was eluted from the column using buffer B (6 M of guanidine HCl solution, 200 mM of sodium acetate at pH 4.5), and dialyzed against buffer Z (25 mM MES, 1 mM EDTA at pH 6.0). The concentrated protein solution was further purified using HPLC (Zorbax Rx-C8 semi-preparative column) applying a linear gradient of 5% to 60% acetonitrile–H_2O (+0.1% trifluoroacetic acid) over 30 min (t_R= 21 min). The protein identity was confirmed by ESI-MS. The calculated mass for $C_{386}H_{645}N_{129}O_{112}S_2$ is 8949.2 Da; the observed mass was 8948.0 Da.

The expressed sequence was: MEAARKREVR LMKNREAARE SRRKKKEYVK SLENRVAVLE NQNKTLIEEL KALKDLYSHK SDKLAAALEH HHHHH

A-CREB protein expression and purification.

A pTXB1 vector expressing the A-CREB-intein fusion was transformed into *E. coli* BL21*(DE3) competent cells and cells were plated onto an agar plate containing 100 μg/mL of ampicillin. The next day, a single colony was inoculated to 25 mL of LB media containing 100 μg/mL of ampicillin and allowed to shake at 37°C for 6 hours. Subsequently, the 25 mL culture was used to inoculate 1 L of LB media containing 100 μg/mL of ampicillin. Induction of protein expression was performed using 0.4 mM IPTG when an O.D. of 0.6 was reached. The culture

was allowed to grow for a further 4 hours at 37°C before the cells were harvested by centrifugation. The cell pellets were subsequently resuspended in column buffer (20 mM HEPES, 500 mM NaCl, pH 8.5) and were lysed by sonication for 5 min in an ice bath. After centrifugation, the lysate was passed through a 0.45 μm filter and loaded onto a chitin column. The beads were washed several times with column buffer. Intein cleavage was performed by incubating the chitin bound fusion protein with column buffer containing 50 mM of DTT overnight at room temperature. The eluted protein was concentrated and further purified by HPLC (Luna® 5μ C18(2) analytical column, Phenomenex) applying a linear gradient of 5% to 60% acetonitrile–H_2O (+0.1% trifluoroacetic acid) over 30 min (t_R= 24 min).

UV-Vis spectra and photoisomerization

Spectral and kinetic measurements were acquired using a PE Lambda 35 spectrophotometer or using a diode array UV−Vis spectrophotometer (Ocean Optics Inc., USB4000). In each case, the spectrophotometer was coupled to a temperature-controlled cuvette holder (Quantum Northwest, Inc.). Protein concentrations were determined using an extinction coefficient at λ_{max} (~446 nm) of 45×10^3 M^{-1} cm^{-1} for opto-DN-CREB constructs and 2980 M^{-1} cm^{-1} at λ_{max} (280 nm) for CREB bZIP. For thermal relaxation measurements, a final protein concentration of 10 μM in 1× TAE and 100 mM NaCl (pH 7.5) or in dilution buffer (20 mM Tris (pH 7.5), 100 μg/mL BSA, and 0.1% Triton X100) were used, and CREB bZIP protein was added in the same buffer with or without 100 μg/mL of sheared salmon testes DNA. Irradiation of the protein sample was conducted using a Luxeon III Star LED Royal Blue (455 nm) Lambertian operating at approximately 10 mW/cm^2 until no change in the irradiated state was observed. Changes in the absorbance spectrum at 350 nm were used to determine the rate constants for thermal relaxation

at 20°C. Data were fit using the global fitting process implemented in Igor Pro with double exponential functions used to describe the thermal decay process.

Circular dichroism (CD) studies

CD studies were performed using an Olis RSM 1000 circular dichroism spectrophotometer with a Quantum Northwest Peltier accessory. The measurements were carried out in a 1 mm cuvette using a protein concentration 15 µM in 1× TAE and 100 mM NaCl (pH 7.5). In samples containing CREB bZIP, CREB bZIP was added in the same buffer to a final concentration of 15 µM. Samples were fully dark adapted before they were scanned in the far-UV region from 260–190 nm (1 nm step) at 20°C with an integration time of 2 s at each wavelength and 3 scans were averaged, smoothed, and baseline subtracted. For CD spectra of the irradiated state, samples were irradiated using a Luxeon III Star LED Royal Blue (455 nm) Lambertian operating at approximately 20 mW/cm^2 for 5 min, and three individual sets were averaged to obtain the final spectrum. Buffers were also scanned under the same conditions and subtracted from the protein spectra.

Size exclusion chromatography

Size exclusion chromatography was carried out on a Superdex 75 10/300 GL column (GE Healthcare) running in 1× TAE, 100 mM NaCl buffer (pH 7.5) with a flow rate of 0.4 mL / minute. Absorption at 280 nm was monitored to determine elution volumes. Dark samples were incubated at 25°C for 3 h in the dark prior and then separated in a dark room on an aluminum foil protected column. For light irradiation, samples were irradiated with a RoyalBlue (450 nm) LED

(25 mW/cm^2) for 1 minute and the column was irradiated with an array of 20 RoyalBlue (450 nm) LEDs (approx. 0.5 mW/cm^2)

Sedimentation velocity measurements

Sedimentation velocity experiments were carried out on a Beckman ProteomeLab XL-1 analytical ultracentrifuge at the Macromolecule and Vaccine Stabilization Center (University of Kansas). Opto-DN-CREB was prepared at a concentration of 14 μM in 1× TAE (pH 7.5) 100 mM NaCl and was dark adapted inside the instrument for 3 hours prior to acquisition. 160 scans were collected over 12 hours, using a rotor speed of 48,000 rpm. Data were fit to a continuous c (M) model using SEDFIT.

Chemical cross-linking of opto-DN-CREB

Solutions (20 μL) of dark-adapted or blue-light-irradiated opto-DN-CREB (~10 mW/cm^2) at a concentration of 250 μM in sodium phosphate buffer, pH 7.0 were incubated in a water bath at 25ºC in the presence of bis(sulfosuccinimidyl) suberate (BS3)(spacer arm 11.4 Å) (ThermoScientific) for 30 min. The reactions were quenched by adding 5 μL of 1 M Lysine (~ 200 mM final conc). Samples were then analyzed by SDS-PAGE (10% polyacrylamide, 1 M Tris, 10% (w/v) glycerol). The upper chamber buffer was 100 mM Tris (pH 8.9), 100 mM tricine, 0.1% (w/v) SDS and the lower chamber was 100 mM Tris (pH 8.3) Gels were run for 1 hour at 130 V.

Electrophilic Mobility Shift Assays (EMSA):

Target DNA: a 28 base-pair DNA duplex containing the CRE site (underlined) was annealed from single stranded cyanine labelled DNA: 5` (Cy5) GTCAGTCAGA<u>TGACGTCA</u>TATCGGT-CAG 3`and unlabeled DNA: 5` CTGACCGATA<u>TGACGTCA</u>TCTGACTGAC 3`. To anneal DNA, 100 µL of annealing buffer (10 mM Tris.HCl, 50 mM NaCl, 1 mM EDTA, pH 7.6) containing 100 µM of labeled DNA and 120 µM of unlabeled DNA was heated in a water bath at 80ºC for 10 min. Subsequently, - the solution was allowed to cool down slowly to room temperature.

CREB bZIP CRE binding assay:

5 µL of each CREB bZIP dilution (1 nM, 5 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM, 100 nM, 200 nM, 400 nM, 600 nM, 800 nM, 1 µM, 4 µM, 6 µM, and 10 µM) was incubated with 45 µL of EMSA cocktail for 1 hour at 25ºC. The cocktail contained 25 µL of 2x EMSA buffer (1× EMSA buffer contains 20 mM Tris (pH 7.5), 40 mM KCl, 3 mM $MgCl_2$, 0.1% Triton X-100, 5% glycerol, and 1 mM DTT), 1 µL of 5 mg/mL BSA, 2.5 µL of 2 mg/mL of sheared salmon testes DNA, 11.5 µL H_2O, and 5 µL of 40 nM target DNA. The first lane contained the negative control, where 5 µL of dilution buffer (20 mM Tris (pH 7.5), 100 µg/mL BSA, and 0.1% Triton X-100) was used instead of 5 µL of CREB bZIP. After the indicated incubation time, 20 µL of the sample was run on an 8% polyacrylamide gel containing 0.5× TBE (1× TBE contains 90 mM boric acid, 2 mM EDTA and 100 mM Tris, pH 8.3). The running buffer was 0.5× TBE. Gels were run at 25°C for 105 min at 300 V using an Emperor Penguin Water cooled dual-gel electrophoresis system. The gels were scanned with a red-light laser on Pharos FX® plus molecular imager (Bio-Rad) and the images were recorded using QualityOne software (Bio-

Rad). The extent of CREB bZIP CRE binding was quantified by analysis with Image Lab®

software (Bio-Rad). Using Igor Pro software, three sets of data were averaged and fit to the Hill equation to determine the apparent K_d, i.e., the concentration of protein required for 50% binding to the CRE site:

$$\%CRE\ bound = base - \frac{(\max - base)}{\{1 + \left(Kd_{app}/[CREB]\right)^{n}\}}$$

Where [CREB]is taken to be the total concentration of CREB bZIP added. Data are shown in Figure S3 (a).

CREB bZIP CRE binding inhibition assay:

Concentrations of 40 nM, 60 nM, 80 nM, 100 nM, 200 nM, 400 nM, 600 nM, 800 nM, 1 µM, 2 µM, 4 µM, 6 µM, 8 µM, 10 µM, 20 µM, and 40 µM were used for dark adapted opto-DN-CREB, dark adapted opto-DN-CREB-2Gly, and dark adapted and irradiated opto-DN-CREB (W134H). Concentrations 200 nM, 400 nM, 600 nM, 800 nM, 1 µM, 2 µM, 4 µM, 6 µM, 8 µM, 10 µM, 20 µM, 40 µM, 60 µM, 80 µM, and 100 µM were used for irradiated opto-DN-CREB and opto-DN-CREB-2Gly. For A-CREB concentrations of 0.4 nM, 1 nM, 2 nM,
4 nM, 6 nM, 8 nM, 10 nM, 20 nM, 40 nM, 60 nM, 80 nM, 100 nM, 200 nM, 400 nM, and 600 nM.

A volume of 5 µL of each dilution of opto-DN-CREB protein was incubated with 40 µL of EMSA cocktail for 3 hours. The cocktail contained 25 µL of 2× EMSA buffer (1× EMSA buffer contains 20 mM Tris (pH 7.5), 40 mM KCl, 3 mM $MgCl_2$, 0.1% Triton X-100, 5% glycerol, and 1 mM DTT), 1 µL of 5 mg/mL BSA, 2.5 µL of 2 mg/mL of sheared salmon testes DNA, 6.5 µL of H_2O, and 5 µL of 200 nM CREB bZIP. Subsequently, 5 µL of 40 nM annealed CRE DNA was added to each dilution and the samples were incubated further for 1 hour. After

the incubation period, 20 µL of binding complexes were resolved on 8% polyacrylamide gels containing 0.5× TBE (1× TBE contains 90 mM boric acid, 2 mM EDTA and 100 mM Tris at pH 8.3). The running buffer was 0.5× TBE. Gels were run at 25°C for 105 min at 300 V using an Emperor Penguin Water cooled dual-gel electrophoresis system. To assay under blue light irradiation, the sample preparation, incubation, and gel run were performed under cycles of 450 nm light illumination composed of 3 minutes of irradiation followed by 2 minutes in the dark. An array of Luxeon III Star LED Royal Blue LEDS (455 nm), (overall ~50 mW/cm^2), was used as the light source. During the 4 hour incubation period, the incubator was set at 25°C. A temperature increase of 1-3°C, originating from LED generated heat, was observed during this period. Under dark adapted conditions, all the samples were prepared under red light (which is the source of light during gel loading and run). The 4 hour incubation was done at dark at 28°C to correct for the temperature increase under irradiation condition. To determine the reversibility of CREB bZIP DNA binding inhibition, the first 3 hours of opto-DN-CREB protein EMSA cocktail incubation were performed in the presence of cycles of blue light irradiation, 3 min on/2 min off, followed by an additional 3 hours of incubation in the dark, and then the assay was completed following the same steps adopted for the assay under dark adapted condition. During all trials, negative and positive controls were loaded in the first two lanes respectively. For the negative control, 10 µL of dilution buffer (20 mM Tris (pH 7.5), 100 µg/mL BSA, and 0.1% Triton X-100) was used instead of 5 µL of dominant negative and 5 µL of CREB bZIP. Only 5 µL of dilution buffer was used to replace the 5 µL dominant negative in the case of positive control. After the run time, the gels were scanned with a red-light laser on Pharos FX® plus molecular imager (Bio-Rad) and the images were recorded using QualityOne software (Bio-

Rad). The extent of CREB bZIP CRE binding inhibition was quantified by analysis with Image Lab® software (Bio-Rad). Data were fit to a modified Hill equation:

$$\%CRE\ bound = base - \frac{(\max - base)}{\{1 + (Kd_{DNA}/[CREB])^n\}}$$

Where Kd_{DNA} is determined in the CREB-bZIP/CRE titration described above and [CREB]is given by

$$[CREB]_{free} = [CREB]_{total} - \frac{(K_i + [CREB]_{total} + [I]_{total}) - \sqrt{(K_i + [CREB]_{total} + [I]_{total})^2 - 4[CREB]_{total}[I]_{total}}}{2}$$

Where $[CREB]_{total}$ and $[I]_{total}$ are the total concentrations of CREB bZIP (fixed) and opto-DN-CREB (variable) and K_i is the dissociation constant for the CREB/opto-DN-CREB complex.

Construction of a reporter to test photoswitching and chromhore incorporation in cells.

A reporter construct was created by cloning the Δ25 domain of PYP into the blue fluorescent protein (BFP) reporter vector mTagBFP (Evrogen). Detailed characterization of this protein construct will be reported separately. The plasmid was transformed into HEK293 cells as described below. After two days, cells were either treated with chromophore (*p*-coumaric acid thiophenyl ester) as described below or left untreated. For bulk fluorescence measurements, cells were harvested by trypsin treatment and fluorescence of the cell suspension was measured using a Tecan fluorescence plate reader (Tecan M1000). For single cell imaging an Olympus X71 fluorescence microscope was used together with a mercury burner and custom filter sets (S395/10x ex (Chroma), T400lp (em)).

Assays of opto-DN-CREB function in living cells

Plasmids

The cDNAs for mammalian expression of A-CREB and opto-DN-CREB were synthesized (Biobasic, Inc.) and subcloned into the vector pHSV. This vector, normally used to generate replication-defective Herpes virus amplicons, can also be used as an expression vector in mammalian cells. Expression of genes inserted into the multicloning site in this vector is driven by the constitutive HSV promoter IE4/5.

Cell culture

HEK 293T cells were grown in Dulbecco's modified eagle medium (DMEM, Life Technologies, Inc.) with 10% fetal bovine serum (FBS) at 37oC in the dark. Primary cortical neurons from E14 mice were generated as previously described (Cole et al., 2012) and used after 8-10 days of growth *in vitro*.

Effects of blue light exposure and chromophore on cell viability

(i) Trypan blue assay

As an initial test of cell viability under blue light illumination, HEK293 cells were grown in 10 cm plates to 80% confluency in the dark followed by blue light illumination for different lengths of time (2-12 hrs)(0.2 mW/cm^2). Cells were then removed from the plates by pipetting and, to an aliquot of these cells, trypan blue (0.4% stock solution in PBS) was added to 10% final concentration. The proportion of viable cells were determined by loading a hemacytometer and

counting the number of blue staining (dead) cells and the total number of cells under a microscope at low magnification.

(ii) MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide) assays (Vybrant MTT Cell Proliferation Assay Kit, Life Technologies Inc., cat# V13154) were used to investigate cell viability under blue light illumination and/or incubation with chromophore. HEK cells were grown in 96-well plates to 80% confluency and then incubated with different concentrations of chromophore (0, 0.1, 0.2, 0.5, 1 and 3 mg/mL) for different times (3h, 6h, 12h) under blue light illumination (0.2 mW/cm^2) or in the dark, along with MTT (0.12 mM final concentration). Media was then replaced with media without phenol red and DMSO. After 10 min, absorbance at 540 nm was measured using a plate reader. At least four samples for each condition were quantified and normalized to the 3h condition without chromophore. There was a decrease in cell viability with higher concentrations of chromophore (1 or 3 mg/mL), but only with 6-12 h of incubation (Fig. S6). There was no change in cellular activity/viability when using 0.1 mg/mL chromophore for 3h, the conditions used in assays for Cre-mediated transcription (Fig. S6). We then tested HEK293T viability when the light intensity was increased five-fold from 0.2 mW/cm^2 to 1 mW/cm^2. When measured at the 3h time point, no additional effect of light was seen (Fig. S6). Since these light levels were higher than those required to see complete relief of inhibition in the transcripton assays, we did not explore higher light intensities using this approach.

(iii) Morpholgical analysis of primary cultured neurons.

To check that similar working ranges of added chromophore and blue light intensity were suitable for use with primary cultured neurons we carried out morphological analysis of cultures under using light microscopy (Fig. S6).

Modulation of Cre activity by opto-DN-CREB: qPCR

To examine modulation of Cre-mediated transcription by opto-DN-CREB, qpcr was performed to detect transcript levels of *nurr1* (NR4A2) and *hprt1* (hypoxanthine-guanine phosphoribosyltransferase) as a control. HEK cells were grown in 96-well plates and transfected with 0.5 μg of either pHSV-opto-DN-CREB or empty pHSV using lipofectamine 2000. Transfection media was changed after 4-5 hrs and replaced with normal growth media. Approximately 40-48 hrs after transfection, cells were washed once in serum-free media and chromophore was added (0.1 mg/mL in 2 -2.5 ml serum free media, diluted from 200 mg/mL stock in DMSO). All samples were incubated for 15 min in the dark and a subset of plates were subsequently moved to an incubator with blue light illumination (0.2 mW/cm^2) for 60 min. Cells under both dark and illuminated conditions were then treated with forskolin (50 μM) for 2 hrs in serum free media. Media was then aspirated and cells were flash frozen on dry ice / isopentane, and stored at -80 until RNA could isolated. RNA was isolated using the Biobasic EZ RNA miniprep kit (cat# BS82322) following the manufacturer's protocol. Total RNA was quantified and 500-1000 ng of RNA was made into cDNA using a high capacity cDNA reverse transcriptase kit (Life Technologies Inc., cat#4374966). Quantitative real-time PCR (qPCR) was performed on 2 ng of cDNA using the Evagreen mastermix (ABM, #mastermix-S) in a Biorad CFX96 real time detection system. CtThresholds were automatically determined, and compared

using the ΔΔCt method. The following primers were added to each reaction (final concentration 500 nM): nurr1 F: 5' CAACTACAGCACAGGCTACGA 3' ; nurr1 R: 5' GCATCTGAATGTCTTCTACCTTAATG 3' ; hprt F: 5' TGGGAGGCCATCACATTGT 3' ; hprt R: 5' AATCCAGCAGGTCAGCAAAGA 3'. Between 4-6 replicates were measured for each condition and levels of *nurr1* transcript were normalized to that of *hprt* within each sample. Final data were normalized to untransfected cells without chromophore, forskolin or light incubation.

Modulation of Cre activity by opto-DN-CREB: Western blots

HEK293T cells were grown in 6 cm dishes to 80% confluency in DMEM with 10% FBS. Each dish was then transfected with 4 µg of either pHSV-A-CREB, pHSV-opto-DN-CREB or empty pHSV using lipofectamine 2000. Transfection media was changed after 4-5 hrs and replaced with normal growth media. Approximately 40-48 hrs after transfection, cells were washed once in serum-free media and chromophore was added (0.1 - 1 mg/mL in 2-2.5 mL serum free media, diluted from 200 mg/mL stock in DMSO). All dishes were incubated for 15 min in the dark and a subset of plates were subsequently moved to an incubator with blue light illumination for 60 min (0.2 mW/cm^2). Cells under both dark and illuminated conditions were then treated with forskolin (50 µM) for 2 h in serum free media before collection and lysis for Western blotting. For lysis, cells were removed from the dishes and collected by centrifugation (250*g*, 5 min). The resulting cell pellet was resuspended in 150 µL ice-cold lysis buffer (150 mM NaCl, 20 mM HEPES, 1% Triton-X-100, 0.1% SDS, 2 mM EDTA, pH 7.4, protease inhibitors) and cells were sonicated for 5.5 min (50% power, cycles of 1 min on, 30 sec off) before quantification by Bradford assay.

To examine light dependent inhibition of CRE activity by opto-DN-CREB in neurons, replication-deficient Herpes virus amplicons were generated and used to infect primary cortical neurons from E14 mice as previously described (Cole et al., 2012). After 8-10 days of growth in vitro, neurons were incubated with HSV-ACREB-PYP or HSV-GFP (control vector) viral particles. Five hours later the medium was replaced. One day later, cells were loaded with chromophore (0.1 mg/mL) and then incubated for 1 hr in the dark or under light illumination (0.2 mW/cm^2) before stimulating with 30 μM forskolin and 50 mM KCl for 2h at 37ºC. Cells were then harvested and processed for Western blotting as described for the HEK293T cell experiments.

For Western blotting, lysates were subjected to polyacrylamide gel electrophoresis (1μg per lane) and electroblotted onto PVDF membranes. Membranes were blocked for 2 h in 10% skim milk in Tris-buffered saline and then incubated overnight at 4ºC with antibodies against NR4A2 (rabbit, 1:1000; Sigma-Aldrich, Cat# N6413) and β-actin (rabbit, 1:1000; Cell Signaling Inc., Cat# 4967) as a loading control. With neurons, blotting was also performed to detect protein levels of c-Fos (1:1000; rabbit polyclonal, sc-52, Santa Cruz). For quantification, optical densities of bands associated with NR4A2 or β-actin were measured using Image J software (NIH). NR4A2 levels were measured relative to β-actin levels in each lane and then normalized to controls indicated in each blot.

Supplemental References

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