Small molecule antagonists of melanopsin-mediated phototransduction

Kenneth A. Jones¹,*, Megumi Hatori²,*, Ludovic S. Mure²,*, Jayne R. Bramley³, Roman Artymyshyn¹, Sang-Phyo Hong¹, Mohammad Marzabadi¹, Huailing Zhong¹, Jeffrey Sprouse¹,5, Quansheng Zhu², Andrew T.E. Hartwick⁴, Patricia J. Sollars³, Gary E. Pickard³,6 & Satchidananda Panda²
Supplementary Information

Supplementary Results

Supplementary Figure 1. Melanopsin photosensitivity assay. (a) Schematic representation of various assays used in this study based on the melanopsin phototransduction mechanism. Melanopsin bound to cis-retinal forms a photopigment. Excitation by ~ 480 nm light source converts the retinal to trans retinal and activates Gq, which in turn activates phospholipase-C (PLC). PLC mediates conversion of PIP2 to IP3 and diacyl glycerol (DAG). IP3 triggers calcium release from intracellular stores. (i) The transient increase in cytosolic calcium in melanopsin-expressing CHO cells was measured in a fluorescent plate reader by fluorescence from a calcium-sensitive Fluo-4 based dye which itself has peak excitation ~480 nm. (ii) Photoexcitation of melanopsin ectopically expressed in Xenopus oocytes leads to rise in DAG, which in turn acts upon a co-expressed TrpC3 channel and results in membrane depolarization, which can be measured by whole cell recording. (iii) In the native rat ipRGCs, melanopsin photoactivation generates action potentials and opening of voltage-dependent calcium channels leading to rise in intracellular calcium which can be measured by Fura-2 calcium indicator dye. (b) Light-induced increase in cytosolic Ca2+ in CHO cells expressing melanopsin (CHOOpn4). Dark-adapted CHOOpn4 cells, but not the CHO cells, exhibited a light-induced transient increase in cytosolic Ca2+ measured by Fluo-4 based fluorescence. Exposing the CHOOpn4 cells to saturating levels of white light (1000 lx, 60 min) essentially abolished subsequent photoresponses, indicating inactivation of the melanopsin photopigment.

Supplementary Figure 2. Melanopsin photoresponse assay optimization. (a) Flow diagram of assay optimization and screen. For most of the experiments, the CHOOpn4 cells in serum-free medium were light-exposed on the day of the assay. All subsequent steps were carried out under darkness or dim red light. Relative temporal sequences of retinal or compound addition to light-exposed cells are shown. For acute addition of retinal, Ca2+ fluorescence from the cells were measured for 25 s and the retinal was acutely added inside the plate reader by a 384 pipette head. (b) Ca2+ fluorescence from light exposed CHOOpn4 cells in the first 25 s showed near complete inactivation of melanopsin. Photosensitivity was restored upon addition of retinal. (c) Ca2+ fluorescence from CHOOpn4 cells pre-incubated for 30 min with increasing concentrations of retinal. Note that light-evoked calcium responses occurred more quickly if 9-cis retinal was provided in the 30 min pre-incubation period, as compared to the acute addition protocol. (d) Difference spectra of purified bovine rhodopsin pre-incubated for 10 min with solvent, 3 μM or 6 μM opsinamide AA92593. After collection of dark spectrum, rhodopsin was light-exposed and another absorption spectrum was collected. Dark-light difference spectra are shown.

Supplementary Figure 3. Relationship between receptor binding and functional antagonism of opsinamides. (a) Linear correlation between binding affinity and functional antagonism for a series of sulfonamides. Binding affinity was measured by displacement of [3H]-AA41612. pA2 was calculated by measuring the rightward shift in the 9-cis retinal concentration response curve caused by a fixed concentration of antagonist (1 or 10 μM) applied after bright light exposure using the FLIPR (see Fig. 2c for example). (b) Structure of opsinamides.

Supplementary Figure 4. General procedure for the preparation of substituted aryl-sulfonamides 1-6 and tritiation of 1-((2,5-dichloro-4-methoxyphenyl)sulfonyl)-1,2,3,6-tetrahydropyridine (compound 7) to yield 1-((2,5-dichloro-4-methoxyphenyl)sulfonyl)piperidine-3,4-t2 (Radio ligand [3H]-AA41612) (compound 8).
Supplementary Figure 5. Electoretinogram (ERG) of C57BL/6J mice treated with vehicle or opsinamides. Representative (out of 5 individual mice) ERG showing rod response, maximal combined rod and cone response and oscillatory potential from mice treated with vehicle or 30 mg kg⁻¹ body weight of mouse treated with AA92593 20 min prior to the assay. The rod and cone responses from opsinamide treated retina were similar to those from vehicle treated retina.

Supplementary Figure 6. Opsinamide does not affect rod/cone photoresponse to successive light pulses of increasing intensity. (a) Electoretinograms of Opn4⁻/⁻ mice in responses to 5 successive light pulses (0.006, 0.04, 0.25, 1.6 and 10 cd s/m², 250 ms each, separated by 2 min of darkness) were recorded. As expected, both the a and the b waves showed and increased amplitudes and reduction in onset latency with increasing light pulses intensity. The injection of compound 20 min before the first light pulse did not alter any of these 4 ERG parameters (average + SEM, n = 5, P > 0.05, Student’s t test) at any of the 5 light intensities tested demonstrating an absence of adverse acute effect of the opsinamide AA92593 on rod or cone functions. (b) Opsinamide did not affect rod/cone photoreceptor recovery after photobleaching. Mice electoretinograms (ERGs) in response to identical successive light pulses delivered 5 and 15 min after an intense 5 min saturating light exposition were recorded and normalized to a reference ERG recorded in response to a similar light flash delivered just before the bleaching exposure. 5 and 15 min after an extensive 5 min bleaching (e.g. 10 and 20 min after the reference ERG and the injection of the compound or the vehicle), the animals had only partially recovered the positive deflection of the ERG (15 - 20% and 25 - 30% after 5 and 15 min, respectively). The resulting waves maximum amplitudes and their delays were not different whether the animal was treated with the compound or vehicle alone (average + SEM, n = 5, P > 0.05, Student’s t test) showing the absence of adverse effect of the opsinamide AA92593 on rod or cone functions in these conditions.

Supplementary Figure 7. Multielectrode array (MEA) recordings of the light-evoked responses during wash out. Examples of light-evoked (blue shading) responses over 30 min of 4 ipRGCs recorded from mice pretreated with vehicle (a) or mice pretreated with opsinamide (b). Note that discharge rates do not change over time in vehicle-treated mice whereas the light-evoked responses of ipRGCs pretreated with opsinamide gradually increase over time as opsinamide is washed out.

Supplementary Figure 8. Opsinamide affects the rate of pupil constriction in response to light and relaxation after cessation of light pulse. (a and b) A reproduction of Fig. 4A showed changes in pupil constriction in response to light has three major features which were influenced by the photopigment; constriction speed after the first 2 s, maximum constriction and relaxation rate. (c and d) In rd mice, opsinamides affected the rate of constrictions (c) and relaxation rate (mean constriction 60 s after lights off) (d) in response to light within 30 min of drug administration. As the drug was gradually cleared during the next hour, these response properties also returned to values as in vehicle-treated mice. (e) Average pupil diameter during the first 5 sec in response to bright light (10¹³ ph.cm⁻².s⁻¹). Dark adapted pupil diameter is normalized to 1. Data reproduced from Fig. 4c. Light pulse begins at t = 0 sec. Note, the initial constriction speed (first ~1 sec), previously shown to be independent of melanopsin, is similar in solvent and compound- treated WT mice.

Supplementary Figure 9. Central projections of ipRGCs in neonatal (P8) mouse. Serial coronal brain sections (100 μm thick) from a P8 Opn4Cre/++;Z/AP mouse (33). These mice express Cre recombinase from the melanopsin locus, which activates Cre-inducible expression of human
alkaline phosphatase (AP) from β-actin promoter. AP present in the ipRGC axons can be visualized by a chromogenic dye. Coronal brain sections stained for AP (33) showed major ipRGC projections to various brain regions including the suprachiasmatic nucleus (SCN), hypothalamus, ventral and dorsal lateral geniculate nucleus (vLGN and dLGN), thalamus, intergeniculate leaflet (IGL), olivary pretectal nucleus (OPN), and superior colliculus (SC) are nearly complete by P8. OC, optic chiasma. OT, optic tract.

**Supplementary Figure 10.** Opsinamides inhibit negative phototaxis behavior of neonatal mice. Example of a WT pup injected with vehicle (a), WT pup injected with AA92593 (b), and *Opn4−/−* pup (c). Their activities are also shown in Supplementary movies 1-3.

**Supplementary Table 1.** Small molecule screening summary.

**Supplementary Table 2.** Binding and inhibition potency of several opsinamides against human melanopsin.

**Supplementary Table 3.** Potency and microsomal clearance rate of opsinamide AA92593.

**Supplementary Table 4.** Biological targets inhibited <30% by AA92593 at 10 μM concentration. The biological targets in appropriate assay conditions were tested for inhibition of specific radioligand binding by opsinamide AA92593 at 10 μM concentration. Less than 30% displacement of radioligand was observed for all 74 targets.

**Supplementary Movie 1.** Negative phototaxis of WT neonatal (P8) mouse treated with vehicle. The first 2 min of the movie showed the pup’s activity inside a plexiglass tube under complete darkness. The next 2 min showed response to bright blue light illuminated from the left (shown as an arrow) of the plexiglass tube. Movie is sped 4X.

**Supplementary Movie 2.** Evaluation of negative phototaxis in WT neonatal (P8) mouse treated with AA92593. The first 2 min of the movie showed the pup’s activity inside a plexiglass tube under complete darkness. The next 2 minutes showed response to bright blue light illuminated from the left (shown as an arrow) end of the plexiglass tube. Movie is sped 4X.

**Supplementary Movie 3.** Evaluation of negative phototaxis in neonatal (P8) *Opn4−/−* mouse. The first 2 min of the movie showed the pup’s activity inside a plexiglass tube under complete darkness. The next 2 min showed response to bright blue light illuminated from the left (shown as an arrow) end of the plexiglass tube. Movie is sped 4X.
Supplementary Figure 1

(a) Schematic diagram of intracellular calcium signaling in photoreceptors.

(i) Fluo-4 binds to free calcium ions (Ca$^{2+}$).
(ii) TrpC and VDCC channels.
(iii) Fura-2 binds to calcium ions.

(b) Graph showing relative fluorescence units over time.

- Red line: CHO$^{388}$ Dark adapted
- Blue line: CHO$^{388}$ + Light exposed
- Light green line: CHO Dark adapted
- Grey line: CHO + Light exposed

Time (s) range from 0 to 50.
Supplementary Figure 2

### a

```
CHO<sup>transf</sup> cells in 384 well plates

| Light exposure | + Fluo-4 Dye | Incubate |
|----------------|-------------|----------|

Acute addition

Pre-incubation

Ca<sup>2+</sup> transient measured at 0.5-2 Hz

488 nm Laser

Screening & Validation

```

### b

**Acute addition**

Relative fluorescence units

| Time (s) | 0 | 25 | 50 | 75 | 100 | 125 | 150 |
|----------|---|----|----|----|-----|-----|-----|
| 9-cis retinal | 0 | 200 | 400 | 500 | 600 |

Relative fluorescence units

| Time (s) | 0 | 25 | 50 | 75 | 100 | 125 | 150 |
|----------|---|----|----|----|-----|-----|-----|
| 9-cis retinal | 0 | 200 | 400 | 500 | 600 |

### c

**Pre-incubation**

Relative fluorescence units

| Time (s) | 0 | 25 | 50 | 75 | 100 | 125 | 150 |
|----------|---|----|----|----|-----|-----|-----|
| 9-cis retinal | 0 | 200 | 400 | 500 | 600 |

Relative fluorescence units

| Time (s) | 0 | 25 | 50 | 75 | 100 | 125 | 150 |
|----------|---|----|----|----|-----|-----|-----|
| 9-cis retinal | 0 | 200 | 400 | 500 | 600 |

### d

```
Relative absorbance

| Wavelength (nm) | 425 | 450 | 475 | 500 | 525 | 550 | 575 | 600 | 625 | 650 | 675 | 700 | 725 | 750 |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Rhodopsin | 0.05 | 0.10 | 0.15 | 0.20 | 0.25 |
| Rhodopsin + AA92593 0 mM (solvent) | 0.05 | 0.10 | 0.15 | 0.20 | 0.25 |
| Rhodopsin + AA92593 3 mM | 0.05 | 0.10 | 0.15 | 0.20 | 0.25 |
| Rhodopsin + AA92593 6 mM | 0.05 | 0.10 | 0.15 | 0.20 | 0.25 |
```
Supplementary Figure 3

a

Ki (nM)

10,000

100

10

1

0.01

0.1

1

10

100

1,000

1,000

100

10

1

AA41612

AA92593

AA73920

AD83947

AD96765

AE51310

b

1  
AA92593

2  
AA41612

3  
AE51310

4  
AD83947

5  
AD96765

6  
AA73920
Supplementary Figure 4

Supplementary Figure 4

**Chemical Structures**

- **7** (CAS #: 433690-62-1)
  - Reactions:
    - a. piperidine, triethylamine, dichloromethane;
    - b. 1,2,3,6-tetrahydropyridine, triethylamine, dichloromethane;
    - c. T$_2$, Pd/C, ethyl acetate

**[³H]-AA41612**

- a. piperidine, triethylamine, dichloromethane;
- b. 1,2,3,6-tetrahydropyridine, triethylamine, dichloromethane;
- c. T$_2$, Pd/C, ethyl acetate

* 2,5-dichloro-4-methoxybenzene-1-sulfonyl chloride (7) as well as the other arylsulfonyl chlorides / piperidines / piperazines used herein are commercially available.
Supplementary Figure 5

Vehicle

Rod response

Maximal combined response

Oscillatory potential

AA92593
Supplementary Figure 6

(a) Relative amplitude and latency for a wave. (b) Relative amplitude and latency for b wave. The graphs show data for Vehicle and AA92593 treatments at different light intensities (cd s m⁻²).
Supplementary Figure 7

a  Vehicle

AA92593

Washout
Supplementary Figure 8

(a) Vehicle
(b) AA92593
(c) % constriction/s
(d) Mean pupil constriction (%)
(e) rd (melanopsin only), Opn4^- (rod/cone only), WT (rod/cone and melanopsin)
Supplementary Figure 10

a
WT + Vehicle

b
WT + AA92593

c
Opn4−
Supplementary Table 1. Small molecule screening summary

| Category         | Parameter               | Description                                                                 |
|------------------|-------------------------|-----------------------------------------------------------------------------|
| Assay            | Type of assay           | Intracellular Ca\(^{2+}\) release using CHO-OPN4                           |
|                  | Target                  | Human and mouse OPN4                                                        |
|                  | Primary measurement     | Light-stimulated increase in intracellular Ca\(^{2+}\)                     |
|                  | Key reagents            | Fluo-4 indicator dye, CHO cells stably expressing OPN4, 9-cis retinal      |
|                  | Assay protocol          | See Methods section                                                        |
|                  | Additional comments     |                                                                             |
| Library          | Library size            | 80,000                                                                      |
|                  | Library composition     | Drug-like small molecules                                                   |
|                  | Source                  | Lundbeck library (commercial and proprietary)                               |
|                  | Additional comments     |                                                                             |
| Screen           | Format                  | 384-well plate                                                              |
|                  | Concentration(s) tested | 10 \(\mu\)M in 0.1% DMSO                                                   |
|                  | Plate controls          | Positive controls ATP, 9-cis retinal; negative control buffer alone         |
|                  | Reagent/ compound dispensing system | FLIPR\(^{384}\)                                                             |
|                  | Detection instrument and software | FLIPR\(^{384}\)                                                             |
|                  | Assay validation/QC     | \(Z' > 0.7\)                                                                |
|                  | Correction factors      | Not necessary                                                               |
|                  | Normalization           | Baseline subtraction                                                        |
|                  | Additional comments     |                                                                             |
| Post-HTS analysis| Hit criteria            | > 50% inhibition at 10 \(\mu\)M, sigmoidal concentration-effect curve, confirmation of compound ID and purity, activity in oocyte secondary assay |
|                  | Additional assay(s)     | Radioligand binding using \(^{[3]}\)H-AA41612, oocyte voltage clamp        |
|                  | Confirmation of hit purity and structure | LCMS                                                                         |
**Supplementary Table 2. Binding and inhibition potency of several opsinamides against human melanopsin**

| Compound ID | Human OPN4 Ki (nM) | Human OPN4 Kb (nM) | Compound class |
|-------------|-------------------|-------------------|----------------|
| AA73920     | 1200              | 13000             | Sulfonamide    |
| AD96765     | 710               | 3000              | Sulfonamide    |
| AD84080     | 74                | 790               | Sulfonamide    |
| AD83947     | 24                | 260               | Sulfonamide    |
| AA92593     | 13                | 110               | Sulfonamide    |
| AA51307     | 1.9               | 89                | Sulfonamide    |
| AA41612     | 0.48              | 6.4               | Sulfonamide    |
| AE51310     | 0.096             | 1.6               | Sulfonamide    |
Supplementary Table 3. Pharmacological and pharmacokinetic properties of AA92593

| Opn4 Ki (nM) | Opn4 Kb (nM) | In vitro clearance (liver microsomes) | Plasma protein binding (% bound) |
|-------------|--------------|--------------------------------------|----------------------------------|
| Human       | Mouse        | Human (l/min) | Mouse (ml/min) | Human | Rat |
| 13          | 47           | 110          | 160           | 250   | 49  | 39  | 95.8 | 94.9 |
| No. | Description                                      | Human gene symbol |
|-----|-------------------------------------------------|-------------------|
| 1   | Adenosine A1 Receptor (Human)                   | ADORA1            |
| 2   | Adenosine A2A Receptor (Human)                  | ADORA2A           |
| 3   | Adenosine A3 Receptor (Human)                   | ADORA3            |
| 4   | Adrenergic Alpha1A receptor                     | ADRA1A            |
| 5   | Adrenergic Alpha2A receptor                     | ADRA2A            |
| 6   | Adrenergic Beta1 receptor                       | ADRB1             |
| 7   | Adrenergic Beta2 receptor                       | ADRB2             |
| 8   | Angiotensin II Receptor, Type 1                 | AGTR1             |
| 9   | Angiotensin II Receptor, Type 2                 | AGTR2             |
| 10  | Arginine vasopressin receptor 1A                | AVPR1A            |
| 11  | Arginine vasopressin receptor 2                 | AVPR2             |
| 12  | Bradykinin Receptor B1                          | BDKRB1            |
| 13  | Bradykinin Receptor B2                          | BDKRB2            |
| 14  | Cannabinoid Receptor 1                          | CNR1              |
| 15  | Cannabinoid Receptor 2                          | CNR2              |
| 16  | Cholecystokinin A Receptor                      | CCKAR             |
| 17  | Cholecystokinin B Receptor                      | CCKBR             |
| 18  | Corticotropin Releasing Hormone Receptor 1       | CRHR1             |
| 19  | Dopamine Receptor D1                            | DRD1              |
| 20  | Dopamine Receptor D2                            | DRD2              |
| 21  | Dopamine Receptor D3                            | DRD3              |
| 22  | Dopamine Receptor D4                            | DRD4              |
| 23  | Endothelin Receptor Type A                      | EDNRA             |
| 24  | Endothelin Receptor Type B                      | EDNRB             |
| 25  | Histamine Receptor H1                           | HRH1              |
| 26  | Histamine Receptor H2                           | HRH2              |
| 27  | Histamine Receptor H3                           | HRH3              |
| 28  | Imidazoline I1 Receptor (Nischarin)              | NISCH             |
| 29  | Imidazoline I2 Receptor                         |                   |
| 30  | Leukotriene D4 Receptor (Cysteinyl Leukotriene Receptor 1) | CYSLTR1 |
| 31  | Melanocortin 4 Receptor                         | MC4R              |
| 32  | Muscarinic Receptor (non-selective) (cholinergic receptor, muscarinic 2) | CHRM2 |
| 33  | Neurokinin (Substance P) Receptor                | TACR1             |
| 34  | Neuropeptide Y Receptor Y1                      | NPY1R             |
| 35  | Opiate Receptor Like 1                          | OPRL1             |
| 36  | Trachykinin (Neurokinin A) Receptor              | TACR2             |
| 37  | Trachykinin (Substance B) Receptor               | TACR3             |
|   | Name                                                                 |   |
|---|----------------------------------------------------------------------|---|
| 38| AMPA receptor                                                        | AMPAR |
| 39| NMDA receptor                                                        | NMDAR |
| 40| 5-hydroxytryptamine Receptor                                        |   |
| 41| Thyrotropin-Releasing Hormone Receptor                               | TRHR |
| 42| Nuclear Receptor Subfamily 3, group C, member 1 (glucocorticoid receptor) | NR3C1 |
| 43| Estrogen Receptor 1                                                 | ESR1 |
| 44| Progesteron Receptor                                                 | PGR  |
| 45| Androgen Receptor                                                   | AR   |
| 46| Phosphodiesterase I                                                 | PDE1 |
| 47| Phosphodiesterase II                                                | PDE2 |
| 48| Phosphodiesterase III                                               | PDE3 |
| 49| Phosphodiesterase IV                                                | PDE4 |
| 50| Phosphodiesterase V                                                 | PDE5 |
| 51| Adenylly cyclase (basal)                                            |   |
| 52| Guanylyl cyclase (basal)                                            |   |
| 53| GABA A Receptor                                                     | GABAAR |
| 54| Kainate Receptor                                                    |   |
| 55| Purinergic Receptor P2X, Ligand-gated Ion Channel                    |   |
| 56| Purinergic Receptor P2Y, Ligand-gated Ion Channel                    |   |
| 57| Voltage Dependent Calcium Channel (L type, 1,4-dihydropyridine sensitive or N binding site) | CACNA1C |
| 58| ATP-sensitive potassium channel                                      | KCNJ11 |
| 59| Small conductance calcium-activated potassium channels               |   |
| 60| Sodium channel                                                      |   |
| 61| Chloride channel                                                    |   |
| 62| Norepinephrine transporter                                          | SLC6A2 |
| 63| Dopamine transporter                                                | SLC6A3 |
| 64| Sodium- and Chloride-dependent GABA transporter 1                   | SLC6A1 |
| 65| High-affinity Choline Transporter                                    | SLC5A7 |
| 66| Serotonin Transporter                                               | SLC6A4 |
| 67| Protein kinase C                                                    | PKC   |
| 68| Acetylcholinesterase (h)                                            | AChE  |
| 69| Catechol-O-methyltransferase                                         | COMT  |
| 70| 4-aminobutyrate aminotransferation                                  | ABAT  |
| 71| Monoamine oxidase A                                                 | MAOA  |
| 72| Monoamine oxidase B                                                 | MAOB  |
| 73| Phenylethanolamine N-methyltransferase                              | PNMT  |
| 74 | Tyrosine hydroxylase | TH |