Deep brain photoreceptors control light seeking behavior in zebrafish larvae

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Summary

Most vertebrates process visual information using elaborately structured photosensory tissues including the eyes and pineal. However there is strong evidence that other tissues can detect and respond to photic stimuli [1, 2, 3]. Many reports suggest that photosensitive elements exist within the brain itself and influence physiology and behavior, however a long standing puzzle has been the identity of the neurons and photoreceptor molecules involved [4, 5]. We tested whether light cues influence behavior in zebrafish larvae through deep brain photosensors. We found that larvae lacking eyes and pineal perform a simple light-seeking behavior triggered by loss of illumination (`dark photokinesis'). Neuroanatomical considerations prompted us to test *orthopedia* (*otpa*) deficient fish which showed a profound reduction in dark photokinesis. Using targeted genetic ablations, we narrowed the photosensitive region to neurons in the preoptic area. Neurons in this region express several photoreceptive molecules, but expression of the melanopsin *opn4a* is selectively lost in *otpa* mutants, suggesting that *opn4a* mediates dark photokinesis. Our findings shed light on the identity and function of deep brain photoreceptors and suggest that *otpa* specifies an ancient population of sensory neurons that mediate behavioral responses to light.

Results

Evidence that deep brain photoreception influences physiology and behavior has accumulated in a variety of non-mammalian vertebrates including fish [1, 6, 7, 8]. We therefore looked for light-driven behaviors in larval zebrafish that persist after removal of the eyes. Responses to simple light flashes and optomotor stimuli were completely absent after enucleation (data not shown), however during phototaxis experiments [9], larvae showed a reduced but significant tendency to swim toward a weak light stimulus (Figure 1A). Light-seeking behavior differed from normal fish as it occurred without larvae orienting their body axis toward the illuminated area (Figure 1B), suggesting a different

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Supplemental Information Supplemental Information includes three figures and detailed Experimental Procedures and can be found with this article online.

navigational strategy. Non-directional `photokineses' have been described in many organisms, requiring only heightened locomotor activity in darkness [10]. Heightened locomotor activity drives individuals in a non-directional, stochastic fashion out of dark zones, and `traps' them in brighter zones due to reduced activity. Indeed upon loss of illumination, zebrafish larvae are known to show a transient period of hyperactivity before settling into a state of low baseline activity [11, 12, 13]. This hyperactive state has been termed the visual motor response (VMR) [14]. Zebrafish larvae enucleated at 5 dpf showed a robust VMR at 7 dpf as assessed by gross locomotor activity (Figure 1C). We obtained similar results from three independent genetic experiments rendering blind fish (Figure S1A–C). Kinematic analysis of swimming during the VMR showed specific defects in enucleated larvae. Control larvae initially perform an O-bend (large angle turn) in the first few seconds following abrupt loss of illumination then deploy routine turn (R-turn) maneuvers at high frequency for around 5 min (Figure S1 E and F). During subsequent dark adaptation, the magnitude and duration of swim bouts gradually increases (Figure S1G–H) while the frequency of R-turn initiations decreases after 10 min (Figure S1F). Enucleated fish lose O-bends (Figure 1C and S1E), indicating that these responses are mediated by the retina. However, consistent with photokinesis behavior, enucleated fish continue to show increased R-turns and longer swim bouts (Figure 1D and 1E). In blind cavefish larvae light extinction provokes a vertical swimming `shadow response'[15]. We therefore assessed vertical movement during the VMR. Under baseline conditions, larvae swim near the top of the tank. On loss of illumination, larvae rapidly swim downwards, then quickly return to the surface when the lights are switched back on (Figure S1K). This dive response was also intact after enucleation (Figure 1F), suggesting that the VMR and dive response occur as a part of a simple, extra-ocular driven strategy for photokinesis that relies on elevated locomotor activity in the dark. To exclude a contribution from the photoreceptive pineal gland, we used the transgenic line *Tg(tph2:NfsB-mCherry)y227* that expresses nitroreductase in pineal (Figure 1G, S1D). Metronidazole treatment ablated pineal photoreceptors but did not eliminate the VMR (Figure 1H) or dive response ($F_{1,6}$= 1.62, p=0.25). VMR and dive responses also persisted after combined pineal ablation and enucleation (Figure 1H and 1I), suggesting that a deep brain photosensor mediates photokinesis.

Several "non-visual" opsins are expressed in the zebrafish brain [16, 17, 18]. Evidence linking deep brain photoreception to physiological responses is strongest for the hypothalamus [1, 3]. Intriguingly, at least two opsins, multiple tissue opsin a (*tmtopsa*) and melanopsin 4a (*opn4a*) have been reported to be expressed within a domain in the hypothalamus specified by the Orthopedia transcription factor [18, 19]. We therefore tested the VMR and dive responses of *otpa* mutant larvae [20]. *otpa* homozygotes exhibit no obvious morphological abnormalities, possess normal baseline movement under constant illumination (Figure 2A), and normal retina mediated photic responses, evidenced by robust O-bends to the loss of illumination (Figure 2B). However mutants are severely affected in the VMR, failing to increase R-turn movements after loss of illumination (Figure 2A and 2C). Following enucleation, mutants completely lose the VMR response (Figure 2A), excluding a potential contribution from other photoreceptive areas. Nitroreductase ablation of orthopedia neurons in *Tg(otpb.A:Gal4)zc67; Tg(UAS:Nfsb-mCherry)c294* [21, 22] double transgenic larvae confirmed the deficit (Figure S2A and S2C). *otpa* mutants also showed an impaired dive response (Figure 2D). For larvae with mutations in both the *otpa* and *otpb* paralogous genes we found the VMR deficit not significantly distinct from *otpa* single mutants (data not shown). These results suggest that these extra-ocular light-driven behaviors are mediated by neurons within the *otp* expression domain. Mouse Otp specifies corticotropin-releasing hormone (CRH), somatostatin (SST), oxytocin/isotocin (OT/IT) and other neuroendocrine neurons in the hypothalamus [23]. However loss of *otpa* alone does not lead to a clear reduction in the number of neuroendocrine neurons in the zebrafish

hypothalamus (Figure S3B; for *crh* see [24]). *otpa* mutants show reduced differentiation of A11 type ventral diencephalic dopaminergic (DA) neurons [20] suggesting that reduced DA modulation of locomotor activity may contribute to the photokinesis phenotype. Nitroreductase ablation of DA neurons using Tg(BAC th:Gal4VP16) m1233 ; Tg(UAS:EGFPCAAX) m1230; Tg(UAS-E1b:NfsB-mCherry)c294 triple transgenic larvae did not produce a VMR deficit (Figure 2E–F), despite loss of more than 70% of A11 neurons in most larvae (Figure S2D,E).Therefore the VMR defect in *otpa* mutants is independent of DA signaling and is likely due to loss of neurons in the more rostral group of preoptic neurons in the *otpa* domain which do not co-express TH (Figure 3A).

We next examined opsin genes as candidates to mediate the *otpa* photokinesis phenotype. Expression of several opsins was not affected in *otpa* mutants (Figure S3C; for *tmtopsa* see also Figure S3D,E). In contrast, *opn4a* was robustly expressed in the anterior and posterior preoptic area (aPO, pPO), posterior tuberculum (PT) and ventral hypothalamus (vH) (Figure 3A and 3B). We detected *opn4a* and *otpa* co-expression in PT and aPO but not in vH or pPO (Figure 3A). Surprisingly, we found that the Otp-dependent A11-type DA neurons designated groups DC2 and DC4 in the zebrafish PT also strongly coexpress *opn4a* (Figure 3A) suggesting that A11 DA neurons have the potential to sense light. However, our nitroreductase mediated ablation of these neurons did not eliminate the VMR response, thus A11 DA dependent light-driven behaviors remain to be identified. Consistent with *otpa* and *opn4a* coexpression, in *otpa* mutants, or after nitroreductase mediated ablation of Otp neurons, *opn4a* expression was lost in aPO and PT but remained intact in vH and pPO (Figure 3B and Figure S2B). Next we tested whether double transgenic *Tg(otpb.A:Gal4)zc67 ; Tg(UAS:GFP-v2a-opn4)y233* larvae, which overexpress melanopsin in Otp neurons, are sensitized to the VMR. We determined that 3-fold and 10-fold reductions in light intensity normally produce only a small VMR in enucleated larvae (Figure S3F). Enucleated larvae overexpressing *opn4* in the Otp domain showed a significantly enhanced response to small light decrements as compared to enucleated non-expressing siblings (Figure 3C). Our experiments suggest that *opn4a* neurons in aPO or PT mediate deep brain photoreception. The PT domain does not appear to contribute to the VMR as the response persisted after ablation of PT DA *opn4a* coexpressing neurons. In summary, our data reveals that photokinesis requires *opn4a* expression specifically in neurons of the preoptic region.

Discussion

We show that a simple light-seeking behavior in zebrafish in response to loss of illumination is mediated by deep brain photoreceptors, and not by the retina or pineal. Our findings strongly implicate *otpa* neurons of the preoptic region of the hypothalamus as photoreceptors for dark photokinesis (Figure 3D). Dark photokinesis is distinct from zebrafish phototaxis which is a robust directional navigation toward or away from target light cues that relies on retinal vision [9]. Instead, dark photokinesis is an undirected hyperactivity in darkness, which results in the aggregation of organisms into a lit area. This behavior may allow larvae to swim out of dark zones when they cannot visually detect illuminated areas of their environment. Our findings explain the puzzling observation that the VMR is robustly induced in 7 dpf *noir* mutants despite the absence of a detectable electroretinogram signal [25]. It has been reported that changes in illumination do not produce a VMR in eyeless *chokh* mutants [13]. In contrast, we observed a robust VMR in *chokh* mutants (Figure S1C). The difference may result from the longer duration of our VMR assay, or from our efforts to presort mutants for behavioral responsiveness (startle response) prior to VMR testing. In *chokh* mutants *opn4a* cells are lost in the vH (not shown), but present in both PT and aPO (Figure S3G), likely explaining why mutants retain the VMR response.

The teleost hypothalamus was first implicated as a site of extra-ocular photoreception in a classic study by von Frisch examining pigmentation control [1]. We show here that hypothalamic otp dependent cells in the preoptic region which express the photopigment melanopsin, may mediate dark photokinesis. Extra-ocular photoreception in other organisms has also been linked to acute behavioral responses. Loss of illumination triggers a pineal mediated vertical swim response in cavefish embryos [15]. Embryonic zebrafish respond to intense light by 30 hpf [26] which is before retinal ganglion cell axons have exited the retina. Intense light also elicits motor responses through deep brain photoreceptors in adult eels, suggesting that encephalic photoreception is not a phenomenon confined to early development [7]. In birds, photoperiodic gonadal growth is regulated by deep brain photoreceptors and recent reports have shown that *vertebrate ancient opsin* and *opsin5* are both expressed in the hypothalamus [27, 28]. In adult teleosts, reptiles and birds as much as 10% of short wavelength visible light reaches the hypothalamus [29]. However deep brain photoreception may also regulate behavior in neonatal mammals where significant amounts of light penetrate into the brain [30]. Extra-ocular sensors drive negative phototaxis in neonatal rats [31]and a recent report has shown that *opn4* is required for this behavior in newborn mice [32]. Melanopsin has been detected in the preoptic region in mice, but it is not clear whether this is due to intrinsic expression or derives from melanopsin positive retinal ganglion cells which are known to project to the hypothalamus [33]. An intriguing possibility is thus that hypothalamic melanopsin expressing neurons also drive locomotor responses to light in newborn mammals.

The hyperactivity that drives dark photokinesis is homeostatic, as it enables larvae to return to an illuminated environment. Interestingly a recent study has shown that *otpa* also regulates hormonal responses to homeostatic challenge [24]. Moreover, the similarities in projection behaviors of neuroendocrine and A11-type DA neurons led to the hypothesis that the Otp-dependent DA system may be involved in setting basic behavioral states [34]. Our findings strengthen the notion that a fundamental role of the Orthopedia system is to drive a coordinated physiological response to environmental challenges.

Experimental Procedures

Behavioral testing

Behavioral tests were carried out at 5–7 dpf. Danio rerio strain Tuebingen long fin (TL) and AB were used as wild type for photokinesis and VMR characterization. Two different apparatus were used to measure VMR, resulting in different measures of locomotor activity (displacement and proportion of time moving).

Ablations

Ablations were accomplished using nitroreductase mediated cell ablation. Nitroreductase is a bacterial enzyme that converts the pro-drug metronidazole (Sigma, St. Louis, MO) into a cell-impermeable cytotoxin enabling cell specific conditional ablation [35, 36].

Statistical analysis

For all experiments, repeated measures ANOVA with simple main effects contrasts were used for within subject pairwise comparisons between baseline and `off' stimulus time points. Analyses were performed using SPSS (ver 17.0) or Microsoft Office Excel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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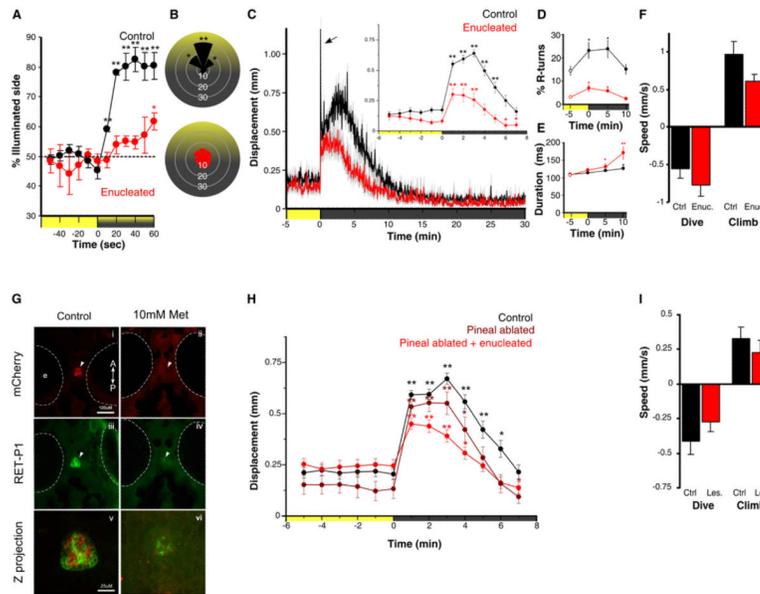
Highlights

- Larvae aggregate in the light even after loss of retinal and pineal photoreception.

- Dark driven photokinesis is absent in eyeless *otpa* mutant larvae.

- The melanopsin *opn4a* is co-expressed with *otpa* and selectively lost in *otp* mutants.

- *otpa* neurons of the preoptic area are deep brain photoreceptors for dark photokinesis.

Figure 1. Light-driven behavior in larval zebrafish without eyes or pineal

(A) Attraction of control and enucleated larvae to a phototaxis stimulus, measured by the percent of larvae observed on the illuminated side of the testing arena over time. Enucleated larvae exhibit a gradual shift to the illuminated side of the arena (symbols show one sample t-test to 50%; N=4 groups of 15 larvae). Larval positions were recorded every second and then averaged over 10 s for each time point. Color along X-axis indicates light condition.

(B) Larval body orientation during exposure to a phototaxis stimulus. A significant proportion of control larvae exhibit a `head-on' orientation towards the spotlight (one way ANOVA; F7, 24=51.21, p<0.001; comparisons are Tukey post-hoc), whereas enucleated larvae show no bias in body orientation (ANOVA; F7, 24=1.73, p=0.15; N=4 groups of 15 fish). Data represents mean proportion of larvae oriented relative to the target light over 1 min.

(C) Locomotor activity during dark-induced VMR. Arrow indicates O-bend spike only observed in controls. Inset: enucleated larvae significantly increase activity following light extinction (repeated measures ANOVA; $F_{2.5, 88.7}$=16.57, p<0.001) (N=36 larvae). Data represents the mean activity for the preceding minute. Color along X-axis indicates light condition. Pairwise comparisons are to the baseline time point at -5 min.

(D and E) Kinematic analysis of VMR. Enucleated larvae (red) retain elevated (D) R-turn initiation frequency (repeated measures ANOVA; $F_{2.1, 56.6}$=4.63, p=0.013) and (E) swim bout duration (repeated measures ANOVA; $F_{3, 40}$=6.41, p=0.001) as seen in controls. Data represents the mean of observations during the first 16 s following each time point. Pairwise comparisons are to the baseline measurement at -5 min (empty circles)(control: N=18 groups of 10 larvae; enucleated: N=28 groups of 10 larvae).

(F) Diving and climbing speed during VMR. In either response, enucleated larvae were not significantly different from controls (dive: t-test, p=0.27; climb: t-test, p=0.07) (N=6 groups of 5 larvae). Additionally, speed in all conditions is significantly different from 0 (one sample t-test, p<0.005). Data represents mean vertical swim speed over the first 20s of dive and ascent.

(G) Nitroreductase mediated ablation of the pineal. Epifluorescent image of dorsal view of pineal (arrow) in untreated and metronidazole (Met) treated *Tg(tph2:NfsB-mCherry)y227* larvae with (i-ii) anti-mCherry (red) and (iii-iv) anti-RET-P1 (green) (6 dpf). Scale bar is 100 µm. (v) Confocal z projection (mCherry + RET-P1) showing concurrent nitroreductase and opsin expression in the pineal. Scale bar is 25 µm.

(H) VMR in enucleated, pineal-ablated larvae. Both pineal ablated and pineal ablated-enucleated larvae show a robust VMR following light extinction (repeated measures ANOVA; $F_{3.8, 131.3} = 32.44$, $p<0.001$) (control and double lesioned: N=36 larvae; pineal ablated: N=26 larvae). Data represents mean activity for the preceding minute. Pairwise comparisons are to baseline time point -5 min.

(I) Diving and climbing speed of enucleated, pineal-ablated larvae during VMR. In either response, lesioned fish were not significantly different from controls (t-test: dive: $p=0.26$; climb: $p=0.42$). Mean speed of dive and ascent for both groups is significantly different from zero (one sample t-test, $p<0.005$) (N=14 groups of 5 larvae). Data represents mean swim speed over the first 20 s of dive and ascent. For all panels, error bars show SEM and * $p<0.05$, ** $p<0.01$.
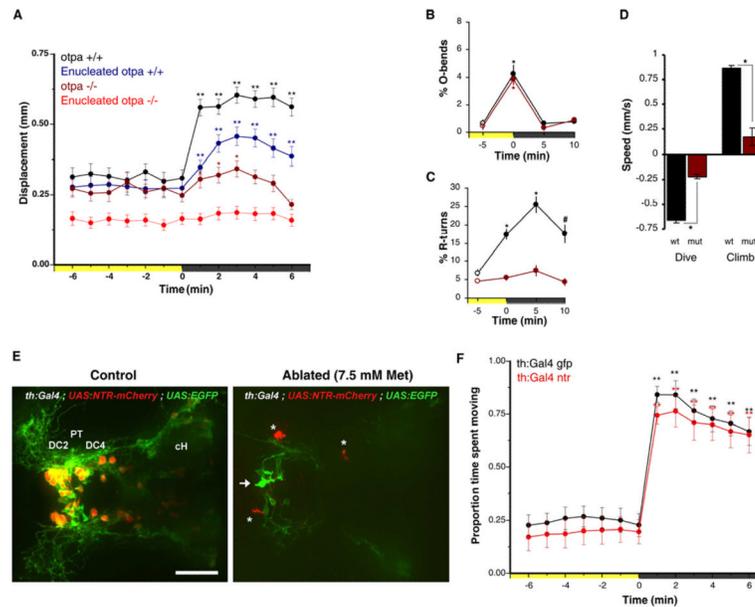
Figure 2. Reduction of VMR in *otpa* mutants and lack of dopaminergic contribution

(A) Locomotor activity during dark-induced VMR of intact and enucleated *otpa* mutants and sibling larvae. Intact mutants show a response to light extinction (repeated measures ANOVA; $F_{3.0,175}=8.5$, $p<0.01$; N=59 larvae) that is greatly reduced relative to controls (intact siblings: N=47 larvae; enucleated siblings: N=97 larvae). Without eyes, mutants lose any response to light extinction (repeated measures ANOVA; $F_{3.2,317}=1.72$, $p=0.16$; N=101 larvae). Data represents mean activity for the preceding minute. Color along X-axis indicates light condition. Pairwise comparisons to baseline time point 0 min: *$p<0.05$, **$p<0.01$.

(B and C) Kinematic analysis of photokinesis in intact *otpa* mutants. *Otpa* mutants retain O-bend responses to light extinction (B) (repeated measures ANOVA ; $F_{3,9}=53.7$; $p<0.001$; N=4 groups of 10 larvae) but do not show characteristic increases in R-turn initiation (C ; $F_{3,9}=2.1$; $p=0.17$; N=4 groups of 10 larvae). # $p<0.05$, * $p<0.01$ for pairwise comparisons to baseline at -5 min (empty circles). Data represents the mean and SEM of observations during the first 16 s following each time point.

(D) Diving and climbing speed of intact *otpa* mutant larvae. Compared to siblings, *otpa* mutants exhibit significantly reduced diving speed during and climbing speed following a 60 s dark flash (t-test: dive: $p<0.001$; climb: $p<0.005$; N=3 groups of 8 larvae). Data represents mean and SEM swim speed over first 20 s of dive and ascent.

(E) Nitroreductase mediated ablation of dopaminergic (DA) neurons in Tg(BACth:Gal4VP16) m1233; Tg(UAS:EGFPCAAX); Tg(UAS-E1b:NfsB-mCherry) triple transgenic larvae. The asterisk indicates mCherry aggregates remaining from ablated cells. The arrow indicates GFP expressing non-ablated cells. PT-posterior tuberculum; DC2, DC4 - Otp-dependent dopaminergic groups 2 and 4; cH - caudal hypothalamus. Dorsal view. Scale bar is 50 μm.

(F) VMR in DA neuron ablated larvae. Control *Tg(th:Gal4VP16); Tg(UAS:EGFPCAAX)* (black line) and DA neuron ablated larvae *Tg(th:Gal4VP16); Tg(UAS-E1b:NfsB-mCherry)* (red line) show similar, robust VMR following light extinction (repeated measures ANOVA; $F_{12, 564}=148.29$, $p<0.001$) (N=48 larvae). Data represents mean and SEM activity as in A. Pairwise comparisons to baseline time point at -5 min *$p<0.05$, **$p<0.01$.
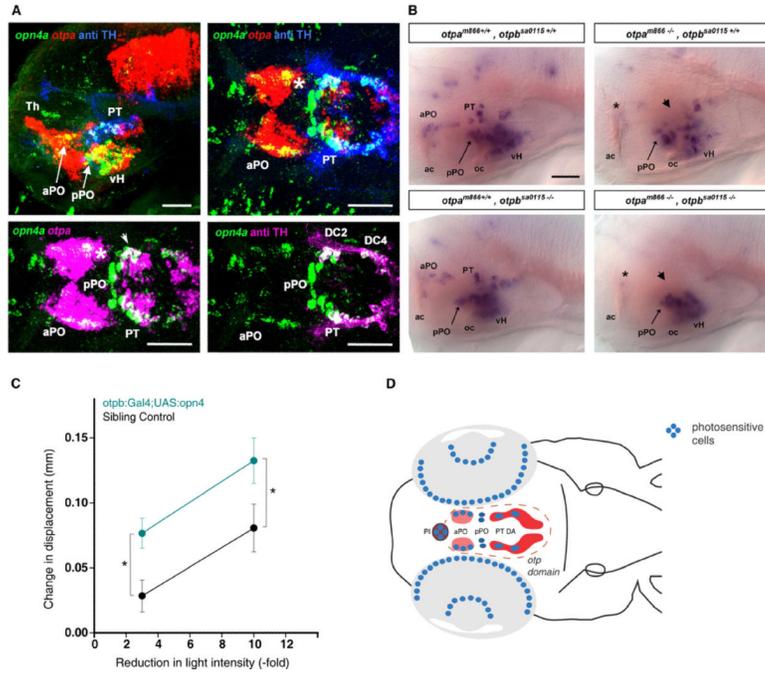
Figure 3. *opn4a* expression depends on Otp activity in areas of co-expression with *otpa*
(A) Analysis of expression domains of *opn4a*, *otpa*, and TH (anti-TH) in 3 dpf wild type larvae. Top row shows z-projections of combined channels (see labels) of confocal stacks recorded from lateral (left) and dorsal (right) views of the brain. Anterior at left, dorsal at top for lateral view. Bottom row shows z-projections of channel combinations of a confocal stack showing dorsal views of the brain. Scale bars are 50 μm. *opn4a* is co-expressed with *otpa* in the anterior preoptic area (aPO) (asterisk) and posterior tuberculum (PT)(arrowhead).
(B) Expression of *opn4a* in *otpa* and *otpb* mutants. Whole-mount in situ hybridization reveals loss of *opn4a* expression in the aPO (arrow) and PT (arrowhead) of *otpa* and *otpa, otpb* double mutants (3 dpf). *otpb* mutants alone did not significantly affect *opn4a* expression, which is in line with the previously reported compensation of *otpb* knockdown by *otpa* activity in A11 DA neuron differentiation [20]..Anterior at left, dorsal up. Scale bar is 50 μm.
(C) Increase in activity following decrements in light intensity in enucleated *Tg(otpb.A:Gal4)zc67; Tg(UAS:GFP-v2a-opn4)y233* larvae. Data shows the difference in mean activity between 2 min after light change and 1 min prior to light change (mean displacement at t2 – mean displacement at t-1). Enucleated *opn4* overexpressing larvae show an increased response to decrements in light intensity (repeated measures, 2 way ANOVA; $F_{1, 100} =8.84$, $p<0.005$) (non-expressing control: 28 larvae; GFP positive in *otpb* domain: N=42 larvae). * $p<0.001$
(D) Schematic summarizing our results suggesting preoptic *opn4a* expressing neurons are deep brain photoreceptor driving dark photokinesis. We eliminated all depicted photoreceptive regions except the PO and found the VMR response remained intact. As *otpa* mutants lack aPO but not pPO *opn4a* expression, and as mutants without eyes do not show a VMR, the behavior must originate in the *opn4a* positive cells in the aPO (pinkfill). The illustrated DA domain (dark red color fill) only comprises the diencephalic groups 2–6 in the posterior tuberculum (PT DA).