Brain-wide perception of the emotional valence of light is regulated by distinct hypothalamic neurons

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INTRODUCTION

Light as a sensory stimulus has biological effects on cognition and mood in addition to its effects on image-forming vision [1]. In humans, light therapy is proven effective for treating mood disorders, linking photic stimuli to emotional regulation [2–4]. Similar effects of light on mood-related behaviors have been observed in animals [5–7], suggesting evolutionarily conserved pathways that remain not well understood.

Neuromodulatory neurons play critical roles in behavioral regulation [8, 9]. How they exert brain-wide impacts remains unclear. The neuropeptide corticotropin releasing factor/hormone (CRF/CRH), first discovered in the early 1980s [10], is an important modulator of stress-associated physiology and behavior [11–15]. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is observed in mood disorders such as major depression [16–20]. CRF-expressing cells are mapped in the nervous system [21, 22]. Hypothalamic CRF is linked to aversive stimuli such as threat or danger [23–25] and their activity is negatively regulated by appetitive stimuli [26]. Despite these advances, the brain-wide effects of CRF neurons remain poorly understood. This is a challenging problem because brain circuits are distributed in distant locations and understanding the entire circuitry requires the ability to monitor neuronal activity throughout the brain.

Larval zebrafish are an attractive model organism for brain-wide circuit level studies [27–37]. As early as five days post fertilization (dpf), they are free-living and need to approach food and avoid predators. Thus, functional circuitry for exploratory reward approaching and anti-predatory avoidance behaviors exists in an accessible and relatively simple vertebrate brain composed of ~100,000 neurons. CRF neurons in larval zebrafish are present in multiple regions analogous to those in the mammalian brain [38–40]. Functionally, they regulate camouflage, a physiological survival reflex [41], and display sensitivity to ethanol [41], hypertonic solutions, and acidic stimuli [36, 42].

A behavior that is simple to characterize in mechanistic detail yet engages complex regulation is the light/dark preference behavior, which reflects the emotional valence of light and is...
observed across species [7, 43–48]. In mammals, light/dark preference is considered an anxiety-like trait and used to assess the anxiolytic properties of drugs [49]. Larval zebrafish, when presented with a choice for light or dark under well-controlled luminance, tend to spend less time in dark during behavioral tracking on the order of minutes. Intriguingly, treatment of larval zebrafish with anti-anxiety medications reduces dark avoidance [46, 50, 51], whereas stressors enhance the behavior [7, 46, 50, 51]. Dark avoidance also requires intrinsically photosensitive retinal melanopsin neurons (ipRGCs) and habenula function [52].

In this study, we recorded behavioral and brain-wide responses to light/dark stimuli and addressed the role of hypothalamic peptidergic neurons in regulating these responses. We previously gained genetic access to CRFHy neurons [36]. Here we employ this tool in combination with chemogenetic ablation, optogenetic stimulation, electrophysiology, and in vivo calcium imaging, and demonstrate in free-swimming animals that CRFHy neurons are necessary and sufficient to drive avoidance of the space where CRFHy neurons are activated. Inactivation of the cbrh gene or pharmacological inhibition of CRF receptor-1 activity reduced dark avoidance, indicating direct involvement of CRF neuropeptide. Intriguingly, the role of CRFHy neurons in promoting dark avoidance is not shared by other hypothalamic peptidergic neurons, even though they are previously shown to play a redundant role in a rapid defensive behavior in head-restrained larval zebrafish [36]. CRFHy neuronal processes were detected near sensory, motor, and ‘decision-making’ brain areas, with considerable heterogeneity observed across individual cells. In vivo calcium imaging also uncovered a heterogeneous response of individual CRFHy neurons to the light or dark stimulus, with a reduced overall sum of CRF neuronal activity in light. In addition to their sensitivity to sensory stimuli, CRFHy neurons were tuned to motor signals associated with struggle and turn. Finally, brain-wide calcium imaging revealed distinct photic response neuronal types that are distributed throughout the brain. CRFHy neurons regulated brain-wide photic perception by promoting a neural representation of dark. Brain-wide functional connectivity analysis further broadened our understanding of CRFHy neurons’ role in balancing brain states that potentially guide action selection between exploration and anti-predation in a context-dependent manner.

METHODS

Experimental model and subject details
All procedures were approved by the University of California San Francisco Institutional Animal Care and Use Committee.

Zebrafish
The AB-WT strain of zebrafish Danio rerio was used in this study. The transgenic lines developed and obtained for this study are detailed Resource Table. Embryos used for imaging experiments were treated with 0.003% of phenylthiourea (PTU) from 22 hpf. Embryos were incubated in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) at 28 °C. The drugs and chemicals were dissolved in the E3 medium as per the concentration mentioned for each experiment.

Quantification and statistical analysis
Data analysis was performed with custom code written in Python, using NumPy, SciPy, Matplotlib, Python, Seaborn, Statsmodels, Pandas, Scikit-image, Bokeh and Scikit-learn libraries (PMID). MATLAB was used for tracking the tail movement. GraphPad Prism 7 was used preparation of graphs and statistical analysis. All statistical details are described in the Figure captions and result sections, including the exact values of n, what n represents, the statistical tests used and p values for comparisons.

Detailed methods for molecular biology, genetics, CRISPR genome editing, pharmacology, behavior, cortisol measure, optogenetics, calcium imaging, and data analysis can be found in the Supplementary Methods.

RESULTS

CRFHy neuropeptide signaling is selectively required to promote dark avoidance behavior

Under well-controlled luminance history and surrounding, larval zebrafish of 5–7 dpf display a tendency to avoid dark in a light/dark preference behavioral paradigm (Fig. 1A; Video S1): Individual animals were monitored for ~8 min during free exploration of a half-light/half-dark arena. This behavior is sensitive to anxiolytics and environmental stressors [7, 51]. The extent of dark avoidance likely results from two competing drives: one is a natural tendency to explore, and the other is to avoid a potential threat (e.g., the dark side, which may signify coldness or predator shadow). The resulting outcome can be quantified using a light/dark choice index (C_{LD}): -1 denotes 100% time in light, 0 denotes no preference, and 1 indicates 100% time in dark. To investigate a possible role of CRFHy neurons in dark avoidance behavior, we gained genetic access to these neurons using a cross-species enhancer detection approach (Fig. S1) [36] and employed the nitroreductase/ metronidazole (NTR/MTZ) system [53, 54] for chemogenetic ablation. The genetically accessible CRFHy neurons are mostly located in the intermediate hypothalamus (Int-Hy), with minor populations detected in the preoptic nucleus (Po) and posterior tuberculum (PT); upon addition of MTZ, all three groups were effectively ablated (Fig. 1B, C). Consistent with a known role of CRF in regulating the hypothalamic-pituitary physiological stress axis, CRFHy-ablated animals showed a blunted cortisol level upon stress (Fig. 1D) and decreased camouflage response in dark or upon ethanol stimulation (Fig. 1E). These observations validate the efficacy of ablation and confirm the requirement of CRFHy neurons in physiological stress response.

We further observed that at the behavioral level, CRFHy-ablated animals displayed significantly increased population mean C_{LD} compared to sibling controls (Fig. 1F), indicating a decreased dark avoidance. CRFHy neurons, together with other peptidergic neurons in the hypothalamus, co-release the excitatory neurotransmitter glutamate that mediates a fast-timescale (within seconds) defensive behavioral response [36]. To determine whether the role of CRFHy neurons in dark avoidance behavior is mediated by the neuropeptide CRF, we carried out pharmacological inhibition of the CRF receptor 1 (CRF-R1) with the antagonist Antalarmin; we also performed genetic disruption of the cbrh gene that encodes the CRF neuropeptide (Fig. S2). In both cases, dark avoidance behavior was significantly attenuated compared to respective sibling controls (Fig. 1F–I), indicating that, different from the fast-timescale defensive response, the choice to avoid dark in an 8-minute timescale requires CRF neuropeptide signaling.

To address whether other hypothalamic peptidergic neurons play a role in dark avoidance, we chemogenetically ablated oxytocin (OXT) neurons using Tg[oxat:Gal4] [55] or somatostatin (SST) neurons using Tg[sst3:Gal4] [56]. A line with broad expression in hypothalamic Otpba neuroendocrine neurons including CRFHy neurons (Tg[otpba:Gal4]) [57, 58] was also used. We also tested whether the role of CRF in dark avoidance is mediated through its effect on proopiomelanocortin (POMC) neurons in the pituitary gland by ablating these neurons using Tg[pomc:Gal4FF]: Ablation of Otpba-expressing neurons had the same effect as ablating CRFHy neurons (Fig. 1G), while ablation of SST or POMC neurons had no effect (Fig. 1H, I). Intriguingly, ablation of OXT neurons significantly increased dark avoidance (Fig. 1J). Taken together, hypothalamic neuropeptide neurons play distinct roles in regulating dark avoidance.

While simple to quantify, dark avoidance is a complex behavior that results from a balance between exploration and anti-predation and involves moment-to-moment decision-making. To further understand the role of CRFHy in this complex behavior, we carried out kinematic analyses of several behavioral components, including zone transition probability at the border as a way of...
measuring “decision-making” (Fig. 1K–R). Disruption of CRF (due to ablation of CRF^{cyc} neurons, pharmacological inhibition of CRF-R1, or genetic mutation of the crhb gene) resulted in a decrease of speed in the dark zone (Fig. 1K–K”) but the zone entry number was unaffected (Fig. 1L–L”). While respective controls have a longer average entry duration in the light than the dark zone, CRF-disrupted groups did not (Fig. 1M–M”). Respective controls also showed a significantly shorter latency to enter the light than the dark zone, but CRF-disrupted groups did not (Fig. 1N–N”). Lastly, we analyzed how CRF might affect a decision at the border.
The border zone is defined as an 8 mm region that spans the light/dark boundary. The total border zone width is 1/5 that of the arena (40 mm) and twice the length of larval zebrafish (~4 mm). Transitions from both light->dark and dark->light were examined (Fig. 1Q, P). When entering the border zone from the light side, CRF-disrupted animals showed an increased probability of entering the dark zone (Fig. 1Q–Q'). However, when entering the border zone from the dark side, no significant differences were observed between CRF-disrupted and control groups (Fig. 1R–R'). These findings reveal a requirement of CRF{sup}Hy in promoting dark avoidance, by acting on multiple behavioral components, including decreasing the duration of dark entry, reducing the latency to enter the light side, and biasing a decision toward avoiding dark.

Optogenetic activation of CRF{sup}Hy neurons is sufficient to drive avoidance behavior

Next, we determined whether activation of CRF{sup}Hy neurons was sufficient to drive avoidance, using the transgenic line Tg[crf: Ga4FF; UAS:ChR2-mCherry]. We first verified by electrophysiology that CRF{sup}Hy neurons were activated by 473 nm blue light delivered for 10 s (100 ms pulses, 5 Hz) (Fig. 2A, B). We then constructed a behavioral chamber where half of the chamber was illuminated with 450 nm blue light and the other half illuminated with white light (Fig. 2C). We tuned the blue light intensity by adjusting the distance between the light source and the behavioral chamber. At 10 mW/cm{sup}2, non-transgenic siblings displayed a slight preference for the blue-light side but Tg[crf:Ga4FF; UAS:ChR2-mCherry] animals significantly avoided it (Fig. 2D). Further kinematic analysis uncovered that the transgenic animals made fewer entries into the blue-light side (Fig. 2F) and spent less time in it (Fig. 2H). Swim velocity and latency to enter zones were not significantly different between control and transgenic animals (Fig. 2E, G). Analysis of "decision-making" at the border zone uncovered a significantly reduced probability of transitioning from the white to the blue light (Fig. 2I) and increased probability of transitioning from blue to the white light in the transgenic animals (Fig. 2J). Together, optogenetic activation of CRF{sup}Hy neurons is sufficient to promote avoidance of the space where CRF{sup}Hy neurons are activated.

Single CRF{sup}Hy neuron projection analysis reveals broad yet heterogeneous connections near sensory, motor, and decision-making brain areas

How do CRF{sup}Hy neurons promote dark avoidance? To begin to address this question, we mapped the anatomical location of their cell bodies and neuronal processes. By registering images of CRF{sup}Hy transgenic animals to the Z-brain atlas [59] (Fig. S3), we found that most of the labeled CRF{sup}Hy neurons were in the intermediate hypothalamus (Int-Hy) near the diencephalic Otp Cluster 1 and the oxytocin-cluster 4 (Fig. S3A). This anatomical location suggests that Int-Hy CRF{sup}Hy neurons may correspond to the magnocellular population of CRF{sup}Hy neurons in the mammalian brain, which are also close to oxytocinergic cells [60]. Smaller numbers of labeled CRF{sup}Hy neurons were present in the preoptic (Po) and posterior tuberculum (PT) (Fig. S3A–S3B), also see Fig. 1B).

CRF{sup}Hy neuronal processes were detected in the visual (e.g., Arborization Fields (AF) AF4, AF9), auditory (e.g., Tectorial Membrane (Tm)) (Fig. 3A2, A3), pre-motor (e.g., nMLF) and motor areas (e.g., reticulospinal motor neurons), as well as the pituitary (Fig. 3A4). The observations of CRF processes near visual and motor areas were verified by DiI injection into the retina and dextran backfilling from the spinal cord (Fig. S4A–S4E). The presence of CRF processes near the pituitary was verified by adrenocorticotropin hormone (ACTH) antibody labeling (Fig. S4F). CRF neuronal processes were also detected in the interpeduncular nucleus (IPN) (Fig. S3A3), which receives direct inputs from the habenula (Hb), an important decision-making brain area [61].

The finding that CRF{sup}Hy neurons extend processes to sensory, motor, and decision-making areas are in line with the notion that neuropeptides such as CRF can act at different sites within a circuit to coordinate functional outputs [8]. Does each CRF{sup}Hy neuron connect to multiple target areas, or alternatively, is such broad connectivity a collective feature of the CRF{sup}Hy neuronal group? To differentiate these possibilities, we performed single neuron projection analysis using sparse labeling in transgenic larvae followed by whole brain registration and reconstruction (Fig. 3B, and Video S2). We found that most CRF{sup}Hy neurons projected to multiple target areas (Fig. 3C). For instance, the processes of a single Int-Hy CRF neuron were detected in ten different brain regions (Fig. 3C1, C2). When comparing the three subgroups of CRF{sup}Hy neurons (i.e., Po, PT, and Int-Hy), we found that processes of each of these sub-groups were detected in the hypothalamus, the auditory area Ts, reticulospinal neurons, and pituitary. Intriguingly, neuronal processes from Int-Hy and PT but not Po were detected in the visual fields, the pre-motor nMLF, and IPN. Taken together, our data indicate that most CRF{sup}Hy neurons project to a broad set of brain regions, albeit heterogeneity exists across individual neurons.

Finally, to determine the pre- vs. post-synaptic identity of CRF{sup}Hy neuronal processes, we performed co-labeling with
synaptophysin-mRFP (to mark presynaptic terminals) (Fig. 3D) and PSD95-GFP (to mark post-synaptic terminals) (Fig. 3E). Presynaptic terminal co-labeling was detected in most CRF<sup>Hy</sup> neuronal processes near visual, motor, and “decision-making” areas (Fig. 3D1–D2, selected 10-Z). Post-synaptic terminal co-labeling was detected in CRF<sup>Hy</sup> neuronal processes within the hypothalamus, on CRF<sup>Hy</sup> neuronal soma, and intriguingly also near the IPN (Fig. 3E1–E2, selected 10-Z), suggesting possible reciprocal connections between CRF<sup>Hy</sup> and IPN. Together, these analyses uncover at single-cell resolution that CRF<sup>Hy</sup> neurons extend processes near sensory (e.g.,
both visual and auditory), motor (e.g., pre-motor nMLF and reticulospinal MNS), and decision-making (e.g., IPN) brain areas.

In vivo calcium imaging uncovers a heterogeneous response of individual CRF<sub>Hy</sub> neurons to light/dark stimuli, with a reduced overall sum of CRF neuronal activity in light

For CRF<sub>Hy</sub> neurons to promote dark avoidance, they must receive photic information, either directly or indirectly. To determine whether light/dark stimuli impact CRF<sub>Hy</sub> neuronal activity, we performed in vivo calcium imaging in head-restrained and muscle-paralyzed Tg[crf:Gal4FF; UAS:GCaMP6s] animals. Alternating periods of dark or light (100 seconds each) were presented during 2-photon imaging: To avoid tripping the photomultiplier tube (PMT), a pulsed light at 40 Hz was delivered (Fig. 4A, B). Possibly due to the relative weaker fluorescence of GCaMP6s than GFP, only CRF<sub>Hy</sub> neurons in the Int-Hy were visible and therefore recorded in the transgenic line. Six distinct light/dark response CRF types were detected (using a custom method as described in next section), suggesting heterogeneity in their activity profiles. Most neurons displayed high activity in dark (including all dark phases, first dark phase, and transition from light to dark, n = 59/75 neurons, from FIVE animals) (Fig. 4C–E). By calculating the sum of neuronal activity in dark vs. light, we uncovered a significantly higher activity of CRF neurons in dark than in light (Fig. 4F, G). These results indicate that light reduces the overall sum of CRF<sub>Hy</sub> neuronal activity.

To simultaneously track CRF<sub>Hy</sub> neuronal activity and behavioral output upon light/dark stimuli, we performed in vivo calcium imaging in head-restrained tail-free larval zebrafish (Fig. 5A). To characterize motor patterns, we first analyzed tail movement tracking videos using a previously described Matlab script [62]. The tail tip angles calculated from this script were further analyzed to detect the activity of CRF<sub>Hy</sub> neurons during brain-wide calcium imaging. Areas including the forebrain, midbrain, and anterior hindbrain were imaged. We established a data processing pipeline that obtained single-cell resolution neuronal activity data of over 14,000 neurons per subject (14,972 ± 357, n = 10 subjects) and detected neuronal correlates to both photic stimuli and tail movements (Fig. S6A) (Video S3). We also used the image registration pipeline (Fig. S3) that enabled us to assign each neuron to an anatomical mask in the Z-brain atlas and compare neuronal activity profiles of each anatomical area across different individuals. We found that CRF<sub>Hy</sub> neurons, as described earlier, showed distinct photic responses, and were also activated during vigorous motion (marked by red dots in Fig. S8–I, right). These findings further validate our brain-wide analytical approach.

We next aimed to classify neurons brain-wide based on their tuning properties to photic stimuli. While it is possible to use regression analysis, one limitation of such method is its bias toward cells with sustained stimulus responses; cells transiently activated at the onset of the stimulus will be masked. Here we employed a different algorithm known as the heatwave algorithm [63], which not only enabled the identification of transiently active cell types but also reduced computation complexity. We computed the largest values (Fig. S6B–D) to detect neuronal activity associated with light/dark phases. By classifying each neuron based on their top 10% z-scored activity values in each light or dark phase (Fig. S8–H), we uncovered seven different photic response classes: (1) activated in light (Fig. S8B, 1940 ± 171); (2) activated in dark (Fig. S8C, 2553 ± 232); (3) activated in first light phase (Fig. S8D, 1683 ± 177); (4) activated in first dark phase (Fig. S8E, 5787 ± 428); (5) activated during transition from dark to light (Fig. S8F, 190 ± 40); (6) activated during transition from light to dark (Fig. S8G, 174 ± 21); (7) unclassified (Fig. S8H, 2643 ± 197), which contains neurons not responsive to light or dark, or having mixed properties [64]. Neurons activated during light/dark transitions were notably fewer than other classes. All classes of photic responsive neurons are present throughout the brain (Fig. S8–H, right), suggesting distributed coding of photic information.

It is worth noting that light-tuned (Fig. S8B) and first dark phase-tuned (Fig. S8E) neuronal types were also strongly activated during vigorous motion, whereas other photic response types are weakly activated during vigorous motion (Fig. S8C, D, F, G). Using the same algorithm for classifying photic response types, we classified...
neurons based on their correlation with vigorous tail movements and observed that most neurons in the brain were activated during vigorous motion. Among them, the top 1,000 neurons were shown distributed across brain regions (Fig. 5I). Thus, both photic and motor variables are encoded in a distributed fashion in the brain; specific neuronal response types are identified that are activated in light, dark, first light, first dark, or transitions between light/dark.
CRF<sup>Hy</sup> neurons suppress light representation in selective and distributed brain areas

Given that CRF<sup>Hy</sup> neurons promote dark avoidance in free-swimming animals, we asked whether they might do so in part by altering photic perception. Brain-wide calcium imaging was carried out in head-restrained/tail-free control and CRF<sup>Hy</sup>-ablated subjects. By assigning each neuron to a distinct photic responsive class and anatomical area, we compared photic representation at cellular resolution between control and CRF<sup>Hy</sup>-ablated animals (n=10 per group) (Fig. 6A). Among 165 anatomical areas examined, we uncovered 14 that had significant changes in their photic tuning properties (Fig. 6B–D). The total number of recorded neurons in these anatomical areas were not different between control and CRF<sup>Hy</sup> neuron-ablated conditions (Fig. S7A, B).

In 11 out of the 14 brain areas, more neurons were tuned to light in CRF<sup>Hy</sup>-ablated animals (Fig. 6B). These included six diencephalic regions [hypothalamic dopaminergic cluster 6 (#111), eminentia thalami (EmT, #14), hypothalamic hcrTr-cluster (#18 and #19), diencephalic islet1 cluster 3 (#38), left habenula vlglut2 cluster (L-Rhdb, #39)], one mesencephalic region [A8F (#103)], and four rhombencephalic regions [Gad1b cluster 7 (#152), oculomotor nucleus nIV (#201), rostral motor neurons in Rol1-R1 (#44), and reticular spinal motor neurons in Rol1-R1 (#226)]. The evolutionarily conserved retina-EmT-L-tor nucleus nIV (#201), olig2-enriched area in the cerebellum (#19), four rhombencephalic regions (#15, #44, #50, #67), and one diencephalic islet1 cluster (#36), rostral motor neurons in Rol1-R1 (#233), and reticular spinal motor neurons in Rol1-R1 (#226). These observations highlight the complexity of neural circuitry mediating photic perception and the regulation by CRF<sup>Hy</sup> neurons.

Intriguingly, in 3 out of the 14 brain areas, more neurons were tuned to dark in CRF<sup>Hy</sup>-ablated animals (Fig. 6C). The total number of recorded neurons in these anatomical areas were not different between control and CRF<sup>Hy</sup>-ablated conditions (Fig. S7C, D). The fact that our unbiased brain-wide analysis uncovers them further validated our analytical methods. Together, these observations suggest that CRF<sup>Hy</sup> neurons suppress light tuning in these experimentally identified brain areas.

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CRF<sup>Hy</sup> neurons regulate functional connectivity among selective and distributed brain areas

Brain-wide neuronal activity data at cellular resolution present a salient opportunity to investigate neuronal interactions in an unbiased manner. Functional connectivity (FC) measures correlations between time series of individual neurophysiological events [65], and is not limited to direct anatomical connections. Analysis of FC is particularly relevant in the context of head-restrained tail-free larval zebrafish dataset, since only limited behavioral repertoire can be assessed in this setting. By analyzing FC, it is possible to examine neuronal ensembles that act concertedly in information processing.

Using the activity of ~14,000 individual neurons per subject as input data, we calculated the neuron-to-neuron connectivity via Pearson correlation previously described [66], resulting in ~225 million neuronal pairs per subject. We next calculated neuroanatomical mask-to-mask connectivity. A total of 135 anatomical masks and 9,045 mask pairs were examined. For each mask pair, both Pearson correlation values and number of neuronal pairs in the masks above the threshold value (0.5) were computed for control and CRF<sup>Hy</sup>-ablated animals (n=10 per group) (Fig. 7A).

Through such analyses, we found 14 mask pairs with increased FC in CRF<sup>Hy</sup>-ablated subjects (Fig. 7B, D, and Table S1). Strikingly, seven out of 14 pairs involved the mask “Mes. -Pft1a” (#101), which represented ~15 neurons in our recorded dataset. These neurons, likely derived from the upper rhombic lip progenitors expressing the proneural gene ptf1a [67], are in the dorsal tegmental area (DTA) bordering the cerebellum. Its FC to diencephalic (#4, #9, #31) and hindbrain areas (#130, #131, #134, #146) were increased in CRF<sup>Hy</sup>-ablated subjects. Interestingly, DTA, which is homologous to mammalian periaqueductal gray (PAG), dorsal tegmental nucleus and nucleus incertus, is known to receive projections from the IPN [68]. IPN is directly innervated by
the L-dHb, which, as described earlier, receives retina-EmT input [52]. Other anatomical areas worth noting are the hindbrain locus coeruleus (LC) (#206), whose FC to one hindbrain (#205) and two diencephalic areas (#15, #73) was increased; the right habenula vglut2 cluster (#73) with increased FC to two hindbrain areas (#226 and #206), and the rhombencephalic Otpb cluster 1 (#205) with increased FC to one diencephalic area (#16) and the hindbrain LC (#206).

Twelve mask pairs showed decreased FC in CRF Hy-ablated conditions (Fig. 7C, D, and Table S2). Four out of twelve involved the anatomical mask named "Mes. -Vmat2 clst. of paraventricular organ" (#112), which has ~27 neurons in our recorded dataset and are in the ventral tegmental area close to the diencephalon. Its FC to one diencephalic region (#49), one midbrain region (#104), and two hindbrain regions (#124 and #130) was significantly decreased. Other anatomical regions worth noting were Mes. -medial tectal band (#96) and Di DA clst. 6 (#11), whose FC to two anatomical areas were decreased. Together, CRFHy neurons critically regulate the FC of distributed brain areas.

DISCUSSION
Here we investigate brain-wide perception of the emotional valence of light and show that these processes are regulated by distinct hypothalamic neuronal types in larval zebrafish. We demonstrate both a necessity and a sufficiency of CRFHy neurons in promoting avoidance of the space that activate these neurons, including the dark. We further show that individual CRFHy neurons extend processes to diverse brain areas including sensory, motor, and decision-making, and their activity is regulated by the light/dark stimuli in a complex way, with the overall sum of activity being reduced by light. Brain-wide calcium imaging during alternating phases of light/dark stimuli reveal distributed coding of photic features that are regulated by CRFHy neurons in selected anatomical clusters. Overall, our study illustrates the brain-wide impact of alternating light/dark photic stimuli at cellular resolution. It uncovers, for the first time, the role of CRFHy neurons in the perception and emotional valence of light as well as in regulating brain-wide functional connectivity. It also provides a plausible mechanism for the effectiveness of light in treating mood disorders.

Role of hypothalamic peptidergic neurons in mediating the emotional valence of light
Photic stimuli carry distinct emotional valence information in diverse species across their life stages [7, 43, 69]. Larval zebrafish display positive phototaxis, a short-burst behavior (on the order of seconds) that actively orients the animals toward a light source and involves stereo-visual comparison [70], spatiotemporal...
sampling [71], and a self-oscillating hindbrain population (HBO) [72]. Positive phototaxis suggests that light carries a positive valence (e.g., associated with warmth or food) whereas dark has a negative valence (e.g., associated with coldness or shadows of predators).

The light/dark preference behavior, measured on the order of minutes and assessing total time spent in dark vs. light territories, engages more complex patterns of neuromodulation and a process of “decision-making” super-imposed onto a phototactic reflex. The molecular and cellular mechanisms underlying this
Breadth and heterogeneity of CRFHy neuron projections revealed by single-cell imaging in an intact vertebrate brain

Neuronal morphology is a distinguishing feature of cell type and associated functionality [73]. Larval zebrafish, with its small brain containing few cells per neuronal class (estimated to be ~1000 fold less than the mammalian brains) [74], offers an excellent opportunity to classify single-neuron projection patterns in an intact vertebrate brain. Here, we applied such analysis to the genetically accessible CRFHy neurons, estimated to be a total of ~28 (±3) in the larval Int-Hy and ~50 (±3) in Po, PT, and Int-Hy combined. Previous bulk labeling has shed light on the distribution and morphological features of CRF neurons in vertebrate brains [60, 75], but these studies are not able to discern the breadth of each neuron’s projection.

CRFHy neurons are classically thought to only regulate physiological stress response, whereas another CRF neuronal population in the lateral central nucleus of the amygdala (CeAL) is extensively studied in the context of conditioned fear [76–78]. Recent studies, however, have uncovered a crucial role of CRF neurons in innate fear-related behaviors, in both mice [23–25, 79] and zebrafish (this study). Consistent with this notion, we observed CRFHy neuronal processes and varicosities in behaviorally relevant brain areas, including non-image forming visual fields (e.g., AF4, AF9), pre-motor and motor (e.g., nMLF, Rol1-R1), and decision-making areas (e.g., IPN, and DA clusters). Moreover, single cell analysis of 34 individual (representing over 60% of total cells scored in each photic response class) neurons showed the position of CRFHy neurons, red dots show the position of CRFHy neurons.

Brain-wide photic perception through distinct, distributed, and tunable neuronal response types

Significant advances have been made in understanding how physiochemical stimuli such as light, sound, odorants, and temperature/touch are transformed into electrical impulses in sensory neurons, but how these stimuli are perceived by the brain remains poorly understood. Photic perception is an organism’s ability to perceive the surrounding light or dark information. While brain-wide activity in response to patterned visual stimuli have been observed [28, 80], how light dark information is represented in the brain has not been systematically analyzed. Other tissues such as pineal or preoptic area are photosensitive [81] in addition to retina. Since light/dark preference behavior is largely abolished in retinal RGC-deficient larval zebrafish, RGCs are the most prominent cell types that transduce photic information to the brain. By brain-wide calcium imaging upon alternating light or dark stimuli, we were able to classify most neurons in the brain into distinct photic response types, including those repeatedly activated in light or dark, those activated during first periods of light or dark, and those transiently activated during light–dark transitions. Several features of photic perception are worth noting. First, distinct photic response types are distributed in an intermingled manner in the brain. Most photic response types are likely present in most anatomical areas. Second, photic response types are functionally rather than anatomically defined, as the response types are tunable brain-wide (e.g., by CRFHy neurons). Third, response types that are “activated in light” or “activated in first dark period” are also robustly activated by vigorous motion, more so than other photic response types. How photic perception as described in this study is established in the brain is an important question for future investigation. Neurons activated at light–dark transitions are notably few in numbers and may represent a good entry point for further research.

A model depicting the role of CRFHy neurons in regulating photic perception and brain state

To uncover brain-wide photic perception at cellular resolution, we needed to use head-restrained animals. One limitation with such preparation is the impact of restraining stress. At the behavioral level, we observed periodic, vigorous tail movements that are accompanied by robust brain-wide neuronal activation. The frequency of vigorous motion was not different in light vs dark, suggesting head restraining, a much stronger threat, can mask the valence of photic stimuli in behavioral expression. Despite this,
**A Data analysis pipeline**

- HucH2B-GCaMP
- CRF-NTR-mCherry/GCaMP larvae

**Classification of neurons based on photic coding**
- Light tuned
- Dark tuned
- Unclassified

**Sorting activity plots**
- Z-score

**3 types of response for light and dark phase**
- Not correlated with photic stimuli

**B Increased light tuning in CRF-ablated subjects**

**Color code for photic response class:**
- **Light** First Light Phase Transition from Dark to Light
- **Dark** First Dark Phase Transition from Light to Dark
- **Not correlated** with Photic stimuli

**#11: Di - Dopaminergic clst. 6 - hypothalamus**
- Ctrl
- Abl

**#14: Di - Eminentia Thalami**
- Ctrl
- Abl

**#18: Di - Hy. 6.7Frhcrtr-gal4 clst. 1**
- Ctrl
- Abl

**#19: Di - Hy. 6.7Frhcrtr-gal4 clst. 2**
- Ctrl
- Abl

**#38: Di - Isl1 clst. 3**
- Ctrl
- Abl

**#43: Di - Left Habenula Vglut2 clst.**
- Ctrl
- Abl

**#103: Mes - Retinal Arborization Field 8 (AF8)**
- Ctrl
- Abl

**#152: Rh - Gad1b clst. 7**
- Ctrl
- Abl

**#201: Rh - Oculomotor Nucleus nIV**
- Ctrl
- Abl

**#204: Rh - Olig2 enriched areas in cerebellum**
- Ctrl
- Abl

**#220: Rh - RoL-R1**
- Ctrl
- Abl

**D Anatomical position of selected regions**

- **Increased light tuning in CRF<sup>Ty</sup> ablated subjects**
- **Increased dark tuning in CRF<sup>Ty</sup> ablated subjects**

Comparing photic representation in each anatomical region between control and CRF<sup>Ty</sup> ablated subjects.

- Overall change in photic coding confirmed by χ² test, p<0.1.
- Unpaired t test, p<0.05.
brain-wide activity tuned to photic stimuli were still robustly observed. Disruption of CRFHy neurons did not alter the frequency of vigorous tail movement in head-restrained subjects. This is consistent with previous observations in mice [76] and in zebrafish [36], which suggest that strong threats likely engage broader neuronal groups than CRF alone. However, disruption of CRFHy neurons did alter photic tuning in selected brain areas, with most (11/14) having increased tuning to dark in the CRFHy-ablated subjects. Anatomical regions with increased tuning to dark response type whereas anatomical regions with decreased tuning was further carried out with \( \chi^2 \) test. B Color codes are used to represent each photic class. Bar graphs showing comparison of proportion of cells belonging to each photic response class in control vs CRFHy-ablated subjects. Increased tuning to the light was observed in CRFHy-ablated subjects for 11 brain areas, with ID number 14, 39, 103, 226 having a significant increase of “light” response type, ID number 11, 18, 201, 204 having significant increase of “First light” response type, ID number 152 having a shift from First light to light response type, whereas ID number 19 and 38 having a significant decrease in “dark” response type. C Bar graphs for anatomical regions showing increased tuning to dark in the CRFHy-ablated subjects. Anatomical region with ID 36 showed a significant increase in “dark” response type whereas anatomical region with ID 230 and 247 showed a significant decrease in “First dark” response type. For (B) and (C) \( \chi^2 \) test, \( p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, n = 10 larvae per group. D Schematic showing the position of anatomical regions presented in (B) and (C). The anatomical mask outlines are shown unilaterally (except for #14 and #39).

**Fig. 6** CRFHy neurons decrease the representation of light in a highly selective, distributed set of brain areas. A Schematic showing the data processing pipeline. Control and CRFHy-ablated Tg(HuCH2B-GcamP6s, crf:Gal4FF;UAS:GcamP6s,UAS:NTR-mCherry) larvae were subjected to brain-wide calcium imaging as described in Fig. 5A. The data processing yields the information of each neuron’s activity (df), coordinates and anatomical regions (mask) it belongs to. Neurons were classified based on their max activity with respect to light and dark phases. For each anatomical region, the percent of cells in each photic class were scored and compared between control and CRFHy-ablated subjects. Anatomical regions showing significant difference in at least one of the photic classes were selected. **B** Relationship between perception, brain state, and behavior. Perception is the sensory experience of the world. Our experimental data show that it is possible to perceive (as evidenced by altered brain state) without altering the limited behavioral output measurable under the head-restrained condition. According to the evidence accumulation model [87, 88], animals need to integrate diverse sensory stimuli, evaluate, and decide on whether and when a behavioral response is warranted. The process of choosing whether a behavior is produced and when it is produced, is an active area of investigation in various organisms [31, 89–92]. In head-restrained/tail-free larval zebrafish, where both brain-wide neuronal activity and tail movement behavior can be recorded upon delivery of light/dark stimuli, we observed robust photic perception, but the probability of tail movement behavior was not different in light or in dark. Similarly, in CRFHy-ablated subjects, we observed altered neural activity and functional connectivity in discrete brain areas, but the probability of tail movement behavior was unaffected. Vigorous motion is accompanied by widespread neuronal activation. Extinction of vigorous motion (or giving up) involves astroglial activity and noradrenaline [30], but what generates vigorous motion is unclear. It has been observed that hypothalamic outputs of glutamatergic signals from multiple overlapping peptidergic neurons converge on brainstem neurons to drive vigorous tail turns [36]. We found that the top 1,000 neurons activated during vigorous motion were distributed across brain areas, suggesting a distributed property of the underlying circuitry.

**Evolutionary perspective of light, CRFHy, and mood disorders**

Several lines of evidence suggest that the effect of photic stimuli on brain state and behavior is evolutionarily conserved across vertebrates. Previous findings of the ipRGC-EmT-L-dHb involvement in light preference in zebrafish [52] and the ipRGC-PHb in light-mediated mood alterations in mice [5] highlight the importance of retinal melanopsin neurons and habenula. Our work further implicated the IPN-DTA pathway downstream of Hb as well as the pre-motor-motor areas (nMLF-Reticulospinal MNS). IPN is a phylogenetically conserved brain area that integrates information for the limbic system [93]. In mammals, the medial habenula (homologous to the dorsal habenula in zebrafish)-IPN circuitry is critical for addiction, anxiety, and mood regulation [94]. In zebrafish, IPN is involved in sensorimotor decision-making [92]. Downstream of the IPN is the DTA, which contains the mesencephalic pdf1a cluster, whose FC with seven brain areas was not different in light or in dark. Similarly, in CRFHy-ablated subjects, we observed altered neural activity and functional connectivity in discrete brain areas, but the probability of tail movement behavior was unaffected. Vigorous motion is accompanied by widespread neuronal activation. Extinction of vigorous motion (or giving up) involves astroglial activity and noradrenaline [30], but what generates vigorous motion is unclear. It has been observed that hypothalamic outputs of glutamatergic signals from multiple overlapping peptidergic neurons converge on brainstem neurons to drive vigorous tail turns [36]. We found that the top 1,000 neurons activated during vigorous motion were distributed across brain areas, suggesting a distributed property of the underlying circuitry.

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Last but not the least, a recent study in rodents showed that CRF<sub>Hy</sub> neurons in the preoptic area receive ipRGC input [95], suggesting that the role of CRF<sub>Hy</sub> neurons in regulating the perception and valence of light may also be conserved across species. In conjunction with the observed effectiveness of light therapy for treating mood disorders in humans [2–4], we propose that suppression of CRF<sub>Hy</sub> neuronal activity is a plausible mechanism by which light elevates mood.
CRF<sup>Hy</sup> neurons regulate functional connectivity of selective and distributed brain areas. A Flow chart showing steps in functional connectivity analysis to identify pairs of anatomical regions showing significant difference between control and CRF<sup>Hy</sup>-ablated subjects. The data carrying activity (df/F) and anatomical region information for each neuron was used to derive cell to cell correlation matrix with correlation value 0.5 as threshold and the correlation matrix between anatomical regions referred as mask-to-mask correlation matrix. For each anatomical region pair (mask to mask pair) average correlation value and percent cells pairs in correlation were calculated. Comparison of control and CRF<sup>Hy</sup>-ablated (n = 10 for each group) was carried out. Mask-to-mask pairs that showed significant increase or decrease in connectivity (both correlation value and percent of significantly correlated cell pairs) were selected to create functional connectivity matrix and to draw the functional connectivity map showing the effect of CRF<sup>Hy</sup> ablation. B, C Schematic diagram showing the brain regions with significantly increased (B1) or decreased (C1) functional connectivity in CRF<sup>Hy</sup>-ablated subjects. The color of connecting line represents the rescaled difference in correlation value and the thickness of line represents the rescaled difference in percent of correlated cell pairs. A matrix showing difference in correlation value (right- top) and difference in percent of cells correlated (left -bottom) (B2,C2). The table showing the description for anatomical region ID numbers in B1-B2 and C1-C2. D Schematic model describing the circuit controlling the dark avoidance behavior based on the anatomical analysis, comparison of photic response and functional connectivity between control and CRF<sup>Hy</sup>-ablated subjects. E Schematic diagram showing dorsal and lateral views of brain regions that showed significantly increased (red) or decreased (cyan) functional connectivity with at least two other brain regions. F Schematic model showing CRF acts as a modulator of the brain state primed for exploration vs anti-predation.

DATA AVAILABILITY
All primary data are stored on a secure server at the University of California, San Francisco and are available from the corresponding author.

CODE AVAILABILITY
Full coding implementation of all analysis tools are available at https://github.com/Mahdizairei/Brai-wide-perception-of-the-emotional-valence-of-light-is-regulated-by-distinct-hypothalamic-neuron.

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Acknowledgements

We thank our colleagues for sharing transgenic zebrafish lines: Misha Ahrens, Herwig Baier, Josh Bonkowsky, and Adam Douglass, as well as the Zebrafish International Resource Center (ZIRC). We thank Michael Munchua and Vivian Yuan for excellent fish care, and Guo lab members for helpful discussions. This work was supported by NIH R01 GM132500 (S.G.), NIH R35 NS122172 (D.A.P.), Gladstone Institutes (K.S.P.), NIH K99MH112840 (M.L.B.).

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AUTHOR CONTRIBUTIONS
MW and SG designed experiments. MW and KTP performed experiments. MW, MZ, and KTP analyzed data. MLB and KD contributed the electrophysiological data. JX and DP contributed the crhb CRISPR KO line. VR and KP performed bioinformatics analysis to identify cfr enhancers. JS advised on the CRF system. MW, MZ, and SG wrote the paper with input from all authors. SG supervised all aspects of the work.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41380-022-01567-x.

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