Blind cavefish retain functional connectivity in the tectum despite loss of retinal input

Graphical abstract

Highlights

- Cavefish retain functional connectivity in their visual center despite the loss of eyes
- Inhibitory connectivity is largely lost, whereas excitatory connectivity is retained
- Shared genetic architecture underlies multiple types of neurons in the tectum

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In brief

Lloyd et al. apply functional brain imaging in multiple populations of cavefish to define changes in visual processing that accompanies the evolution of eye loss.
Blind cavefish retain functional connectivity in the tectum despite loss of retinal input

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SUMMARY

Sensory systems display remarkable plasticity and are under strong evolutionary selection. The Mexican cavefish, Astyanax mexicanus, consists of eyed river-dwelling surface populations and multiple independent cave populations that have converged on eye loss, providing the opportunity to examine the evolution of sensory circuits in response to environmental perturbation. Functional analysis across multiple transgenic populations expressing GCaMP6s showed that functional connectivity of the optic tectum largely did not differ between populations, except for the selective loss of negatively correlated activity within the cavefish tectum, suggesting positively correlated neural activity is resistant to an evolved loss of input from the retina. Furthermore, analysis of surface–cave hybrid fish reveals that changes in the tectum are genetically distinct from those encoding eye loss. Together, these findings uncover the independent evolution of multiple components of the visual system and establish the use of functional imaging in A. mexicanus to study neural circuit evolution.

INTRODUCTION

Sensory systems are highly tuned through evolution to an animal’s environment and under stringent evolutionary pressure.1,2 Adaptation to novel environments is frequently associated with modification of sensory-processing governing vital behaviors, including foraging and avoidance of predation.3,4 The reliance on different sensory cues has been widely documented to be accompanied by changes in the anatomy of the primary sense organ, such as a tradeoff between expansion of the eye or olfactory epithelium or changes in cortical area devoted to processing sensory information.5,6 However, little is known about the evolution of neural circuitry in downstream sensory-processing centers.

The Mexican tetra, Astyanax mexicanus, provides a powerful biological system to study the genetic and neural basis of sensory evolution, consisting of eyed surface populations that inhabit rivers in Northeast Mexico and southern Texas, and at least 30 cavefish populations.5,10 Cavefish populations have evolved a series of morphological, physiological, and behavioral changes including eye loss, albinism, reduced sleep, loss of social behaviors, and expansion of non-visual sensory organs, including olfaction, taste, and mechanosensation.11,12 Many of these phenotypes and behaviors have evolved repeatedly in geographically isolated cavefish populations, positioning A. mexicanus as a model to investigate the genetic and ecological factors that shape the evolution of traits.13–15 One of the most striking traits is convergence on eye loss, presumably to conserve energy in a perpetually dark environment.16–18 This phenotype has been studied for over 80 years; however, the concomitant physiological changes in visual-processing centers have not been investigated.

In teleosts, the majority of retinal inputs project to the optic tectum, a brain region that is homologous to the mammalian superior colliculus.19 Although the tectum primarily receives sensory inputs from the retina, minor inputs from auditory and lateral line modalities have been reported.20 In sighted species, visual processing within the tectum is essential for generating appropriate visuomotor behaviors, including prey capture and predator avoidance.21–23 In comparison to surface fish, the size of the tectum is reduced in cavefish from multiple populations.6 This may be indicative of co-evolution of reduced inputs of the retina and its target centers within the brain. Alternatively, this may be an epiphenomena in which degradation of the retina and the optic tectum occurred independently of one another. Recently, we generated a quantitative neuroanatomical atlas of surface fish and multiple cavefish populations, revealing that the optic tectum is reduced by ~20% across populations.5,24 Although this represents a significant reduction in neural tissue, the presence of the remaining 80% raises the possibility that this system still contributes to brain computations, given the energetic cost of neural tissue.25,26 Therefore, the maintenance of the tectum despite the apparent loss of visual input provides a system to examine how brain structures are impacted by evolved differences in sensory input.
The optic tectum of the zebrafish, *Danio rerio*, is an established model to study development and function of visual sensory systems. Two-photon Ca²⁺ imaging has been used to identify cell types that respond to different properties of visual stimuli and infer the circuit’s functional connectivity. Recently, we have implemented transgenesis in *A. mexicanus*, permitting the application of approaches previously limited to zebrafish. The development of these tools has provided the first opportunity to investigate how neural responsiveness and functional connectivity of sensory circuits are shaped by evolutionarily derived changes in sensory function. Here, we used two-photon scanning microscopy to image surface and cavefish pan-neuronally expressing GCaMP to directly define differences in neuronal dynamics within the tectum across independently evolved populations of *A. mexicanus*.

**RESULTS**

**Development of transgenic cavefish for brain imaging**

Multiple populations of cavefish have converged on reduced eye size and volume of the optic tectum, however, little is known about the underlying impacts on neuronal function. To investigate the changes in function of the optic tectum associated with eye loss, we sought to compare the functional connectivity and the responsiveness to light in the tectum of river-dwelling surface fish and two independently evolved populations of cavefish (Figure 1A). The Pachón population originates from old surface stock and is located in the Sierra del Abra region, whereas the Molino population evolved from new surface stock and is located in the Sierra de Guatemala range. Geological and genomic evidence suggest that these two populations of cavefish have independently evolved loss of eyes. To study changes in the function of the optic tectum in *A. mexicanus*, we generated surface and cave populations harboring the genetically encoded Ca²⁺ indicator (GCaMP6s) of the pan-neuronal promoter elavl3/HuC. The surface and Molino populations have previously been described; here, we also utilized a HuC:GCaMP6s Pachón line, allowing for direct comparison of brain function in independently evolved cavefish populations. Fish from the surface, Pachón, and Molino populations expressed the reporter as early as 1.5 days post-fertilization (dpf) through 8.5 dpf (Figures 1B and S1A–S1C), confirming stable, brain-wide expression throughout early development across all three *A. mexicanus* populations. Therefore, these lines recapitulate expression in zebrafish and can be applied to measure neuronal activity throughout the brain.

**Ongoing spontaneous activity in the optic tectum**

Correlation in the spontaneous activity between neurons has been used as a proxy of the circuit’s functional connectivity. In zebrafish, the ongoing spontaneous activity of the optic tectum is organized in assemblies composed of highly correlated neurons with similar spatial tuning curves. These neuronal assemblies show attractor-like dynamics and may improve the detection of prey-like visual stimuli. To quantify changes in the tectal functional circuitry that co-evolve with eye degeneration,
we compared spontaneous activity in the tectum between surface and cave populations. For this purpose, we performed two-photon Ca²⁺ imaging of surface and cave transgenic GCaMP6s larvae (6 dpf) for a period of 30 min while limiting as much as possible the presence of sensory stimuli (Figures S1D–S1G). Quantification of the number of neurons in the imaged region of the tectum by automated segmentation showed a reduction of ~20% in the cavefish population, consistent with previous reports of reduced cavefish tectum size (Figure 1C). We then analyzed the resulting fluorescence traces to detect significant Ca²⁺ transients as previously described (Figures 2A–3C). We first quantified the average frequency and duration of individual Ca²⁺ events across the tectal circuit. No differences were detected between surface fish and Pachón or Molino cavefish (Figure S2). Therefore, the overall levels of spontaneous activity within the tectum do not show evolutionary changes in two independently evolved cave populations. These findings reveal that the overall levels of spontaneous neuronal activity in the tectum are unaffected by evolutionary eye loss.

To determine whether correlated activity represents statistically significant interactions that are indicative of neural circuit connectivity in the optic tectum, we applied Pearson’s pairwise correlations between the spontaneous activity of the tectal neurons (Figures 2D–2F). The probability distribution of the correlations in spontaneous activity was compared with a null model that randomly shifted the time series of every neuron (the null model represents the correlations that can be explained by chance). To quantify the difference between the two distributions of the pairwise correlations (the correlations in the data that cannot be explained by chance), we used the Jensen-Shannon divergence (JSD). JSD measures the distance between two probability distributions (STAR Methods; Figures 2G–2I). Briefly, JSD allows assessing the similarity between two distributions: a low JSD value (close to 0) indicates that the distributions are very similar, whereas large JSD values indicate that the distributions are different. By comparing the JSD value of the dataset and the null model, we can assess the level of significant correlations (those that cannot be explained by chance). Thus, we computed for each larva of the Surface, Molino, and Pachón populations, the distribution of pairwise correlations as well as the distribution obtained from the null model, and computed the JSD value between these two. Using this approach, we first evaluated the functional excitatory connectivity by assessing only the “positive” pairwise correlations. We found no significant differences between the tectal neurons of surface fish, or Pachón and Molino cavefish (Figure 2J). These findings suggest that functional tectal connectivity was retained in cavefish despite eye loss. However, analysis of the “negative” correlations, which can reveal the functional inhibitory connectivity, showed a significant reduction in the Molino cavefish, with Pachón approaching significance (Figure 2K). Given the importance of the inhibitory/excitatory balance to visual processing, the reduction in negative correlations in cavefish may reflect a physiological correlate of relaxed selection on the visual circuit in blind cavefish that is driven by a lack of retinal input. Finally, analysis of the physical distance between correlated neurons revealed that both surface and cave populations exhibit spatially structured correlations, with an exponential decrease in correlations with the Euclidean distance between neurons (Figure 2L). Therefore, positive correlations are resistant to the evolved loss of visual function, suggesting that excitatory and inhibitory connectivity are under partially independent evolutionary selection.

**Visual responses in the optic tectum**

In zebrafish and other teleosts, the optic tectum is the main visual-processing center. Cavefish retain a diminished eye, and they behaviorally respond to some light cues; however, the eye is suggested to be non-functional as cavefish exhibit severely reduced retinotectal connections by adulthood. To examine whether cavefish retain a light-evoked response in the optic tectum, we used two-photon microscopy to image light-evoked activity in transgenic GCaMP6s surface and cavefish (6 dpf). Briefly, fish were immobilized in low-melting point agarose and imaged during visual stimulus presentation, which consisted of a white-light LED located in front of the larvae. The LED was turned on for 30 s and presented approximately ten times, separated by 60 s (STAR Methods; Figure S3). Using a multivariate linear regression model, we categorized the Ca²⁺ responses of individual tectal neurons during stimulus presentation and identified neurons with multiple distinct response profiles (Figure 3A). In
Figure 3. Partial loss of neuronal response to visual stimuli in cavefish populations
(A) Representative traces of the five types of light response.
(B–D) Left: raster plots of normalized neuronal activity over the whole population of surface (B, n = 6), Molino (C, n = 5), and Pachón (D, n = 3), averaged around the onset of whole-field visual stimulation. Visual stimuli were delivered by an array of white LEDs for a duration of 30 s (represented by horizontal light blue bar above the raster), with an inter-stimulus interval of 120 s, and neuronal activity was recorded with a frame rate of 1.96 Hz. Each experiment contains between 8 and 20 visual stimuli. Neurons responsive to the visual stimuli were detected using a linear regression model and classified into 5 categories: “On” cells (blue), “Off” cells
surface fish, the most common were cells that responded to either the onset ("On" cells) or offset ("Off" cells) of light, with another subset that responded to both the onset and offset of light ("On/Off" cells). In addition, we identified a small number of neurons that exhibited a sustained activation throughout the period of the light stimulus ("sustained" cells) and another that showed a sustained decrease in activity during light stimulus ("inhibited" cells). Therefore, the tectal light response in *A. mexicanus* surface fish contains cell types previously observed in zebrafish.44,45

Although cavefish have previously been reported to be blind with the exception of response to looming stimulus and lack physiological response to light in the tectum,46,47 we found that both populations of cavefish showed significant, although severely reduced, Ca²⁺ responses to the presented visual stimuli (Figures 3B–3D). In comparison to surface fish, Pachón and Molino larvae showed a significantly smaller number of responsive neurons to the visual stimulus, representing a reduction of responding neurons of ~75% in Molino and ~90% in Pachón (Figure 3E). The amplitude of the Ca²⁺ responses of the cavefish to the visual stimulus was also reduced by ~60% (Figure 3F). The probability of the neurons to respond to visual stimuli was also significantly smaller in cavefish with respect to surface fish (Figure S3). Moreover, the "On/Off" neurons were absent in both Pachón and Molino larvae, and the inhibited neurons showed very weak responses that were almost absent in Pachón (Figures 3B–3D). The onset precision of the visual response with respect to the onset of the stimulus was also significantly more variable in the cavefish with respect to that of the surface fish (Figures S4C and S4D). The extent of the reduction in light response was severe enough that responses to light in cavefish tecta were undetectable by the naked eye, requiring rigorous statistical analyses to definitively determine whether neurons were truly responsive (STAR Methods). Together, these findings reveal that although cavefish exhibit severe reductions in visual tectal function, they maintain the genetic and cellular functions necessary to process visual stimuli.

The presence of light responsiveness in the cavefish tectum raises the possibility that the retina is still functional in larval cavefish. Alternatively, responses in the tectum may be downstream of extra-retinal light-responsive organs such as the pineal gland.48 To distinguish between these possibilities, we imaged the retinal response in GCaMP-expressing Molino and Pachón larvae while presenting a series of 30 s light stimuli. Due to pigmentation, recording of the intact retina in surface fish was not possible. In the Pachón population, we identified strong spontaneous retinal waves known to play a role in the development of retinotectal pathways (Video S1).49–51 However, no light-evoked activity was detected (Video S1; Figures 4A–4C). The Molino retina showed neither retinal waves nor visually induced responses. These findings suggest that although the retina is still alive and active, at least in the case of the Pachón population, it does not respond to light. Therefore, light responsiveness observed in the optic tectum of cavefish must stem from a non-visual source (e.g., the pineal gland or deep-brain internal photoreceptors).45,52

**Analysis of visual response in hybrid populations**

It is possible that the decrease in the evoked tectal light response in cavefish is due to the partial loss of eyes that, at 6 dpf, are still in the evolutionary process of degenerating.53 The ability to generate surface-cave hybrid fish has been widely used to examine the functional and genetic relationship between traits.54 To determine whether changes in the anatomy of the tectum are related to the loss of eyes, we examined the relationship between traits in genetically and morphologically variable hybrid fish. By crossing transgenic HuC:GCaMP6s surface fish to Pachón or Molino cavefish and re-crossing the siblings for one generation, we generated genetically variable F2 surface × cave hybrids harboring the HuC:GCaMP6s transgene (Figure 5A). To determine whether there is a developmental or genetic relationship between eye size and the number of neurons in the tectum, we examined the relationship between these two traits. We found an association between eye diameter and the number of neurons in the tectum in hybrids from the Molino, but not in Pachón, caves (Figure 5B). These findings suggest that the reductions in the size of the tectum and eyes of Molino cavefish is controlled by shared genetic architecture, whereas these factors are independent in Pachón cavefish, suggesting that the evolutionary mechanisms that confer eye loss and/or tectal size in these two populations differ. The finding that tectal development is linked to eye size in Molino hybrids led us to question whether the tectum’s functional response is also related to eye size. We measured eye size in F2 fish, performed Ca²⁺ imaging in response to changes in illumination, and examined correlations between the two. As the tectal response in cavefish is so slight as to be nearly undetectable relative to the surface responses, we presumed the majority of the tectal response to be visual in nature and derived from genetic contributions from the surface parent. We found no significant relationship between eye size and tectal response, in any of the cell types previously identified (Figure S5). Therefore, there is a functional relationship between eye and tectum size in Molino, but eye size does not impact light-induced tectum activity in either cavefish population.
To determine whether there is a shared genetic basis underlying the development of distinct neuronal functional populations in the tectum, we quantified different neuronal response types in surface-cave hybrids. In both Pachón and Molino cavefish, there was no relationship between the number of "On" cells and "Off" cells, suggesting that each population is independently regulated (Figure 5C). This finding suggests that distinct genetic pathways guide the development of these cell types within the tectum. However, a relationship was identified between each singly responsive cell type and dual-responsive "On/Off" cells, suggesting a shared genetic basis (Figures 5D and 5E). Additional correlation analyses between incidence of cell types, including the "sustained" and "inhibited" cells, yielded no significant correlations (data not shown). Taken together, these findings suggest that shared genetic architecture underlies the loss of "On/Off" cells and cells that exclusively respond to light onset or offset across both Molino and Pachón populations of cavefish. These findings are indicative of convergence on shared developmental processes contributing to changes in tectum function across independently evolved cavefish populations through different genetic mechanisms. Future studies building on these findings may provide a more in-depth understanding of the genetic factors underlying the independent evolution of the visual system across caves.

**DISCUSSION**

Cave animals have evolved numerous phenotypic changes that are likely adaptive to a cave environment, including eye loss and expansion of olfactory and mechanoreceptive organs.12,55,56 These changes occur in diverse cavefish species throughout the world, suggesting that the shared ecological features in cave environments, such as perpetual darkness and reduced food availability, underlie the evolved changes in sensory processing.57 The repeated evolution of eye loss in the different A. mexicanus cavefish populations has provided a model to investigate the genetic basis of evolution. Several studies have identified numerous factors underlying eye loss, including a critical role for the degeneration of the lens and retina.58-62 Furthermore, genetic complementation studies have revealed that different genetic mechanisms underlie eye loss across cave populations.39,63 Despite our detailed understanding of genetic and morphological changes that occur in sensory organs, strikingly little is known about evolutionary adaptations in sensory-processing centers of the brain. We utilized...
GCaMP-expressing transgenic *A. mexicanus* to examine the changes in the main visual-processing center in surface and cavefish, the optic tectum.

In teleost fish, the optic tectum is the main visual-processing region; it is involved in the detection and processing of visual stimuli to generate goal-directed motor behaviors such as prey capture.64 Similar to our findings in *A. mexicanus*, numerous cellular response types have been previously identified in the zebrafish tectum, including those which respond selectively to changes in light level.65 We identified shared cellular response types across surface and both cave populations of *A. mexicanus*, although the number and sensitivity of responsive cells was severely reduced across cave populations. Imaging from the cavefish retina did not identify responses to flashes of light, raising the possibility that the light-induced activity that we observed in the tectum derives from non-visual sources such as internal photoreceptors and/or the pineal body.66 These differences support the hypothesis that the light response in the tectum is vestigial in cavefish, suggesting a transformation in the function of the cavefish tectum. Also, in support of this hypothesis is the finding that fish from the evolutionarily older Pachón population exhibit more severe deficits in tectal light response relative to fish from the Molino caves.

In zebrafish, the tectum is primarily devoted to visual processing but also receives mechanosensory and auditory information. Previous studies have identified a laminar organization of the zebrafish tectum, with different layers corresponding to distinct functional specializations.29,67,68 Numerous cellular response types have been previously identified in zebrafish, including those which respond selectively to changes in light level.65 Their identification in *A. mexicanus* provides an opportunity to investigate the genetic basis of tectal wiring through hybridization experiments. Our findings show that the prevalence of “On” cells and “Off” cells is uncorrelated in the tectum of hybrid individuals, suggesting that separate genetic mechanisms regulate these traits, and that likely distinct axon guidance molecules regulate the wiring of the tectum.69,70 Conversely, the correlation of both of these cell types with dual-responsive “On/Off” cells suggests a shared developmental basis, e.g., overlapping expression of axon guidance molecules. Further experiments in surface-cave F2 hybrids, in combination with quantitative trait locus (QTL) mapping, could elucidate the genetic basis of these, and other, functional cell types in the tectum.

Our analysis of the spatiotemporal correlations in the spontaneous activity within the tectum suggests cavefish have maintained functional connectivity despite the complete loss of a light-responsive retina and the near-complete loss of tectal light response. In the zebrafish optic tectum, spontaneous activity reflects the functional connectivity of the circuit, which is adapted to improve visual detection of prey-like visual stimuli.31 These findings suggest that eye loss has not influenced the tectum’s
functional connectivity. However, this trend did not hold true for the negative correlations within the tectum, which tended to be reduced in both Molino and Pachón populations. This suggests that tectal inhibition is tightly linked to visual processing or that only positive correlations and not inhibition play a role in the potential repurposing of the optic tectum in cavefish.

It is possible that the visual circuitry of the sighted teleost has been repurposed for processing a different sensory modality in cavefish. This hypothesis is supported by the fact that in contrast to the significant reduction in visual response, which may represent a vestige of visual degeneration evolution, the functional connectivity of surface and cavefish did not show significant differences. Systematic characterization of the response of the tectum to a battery of sensory stimuli can determine whether this brain region has been repurposed to respond predominantly to non-visual sensory stimuli.

The functional imaging described here represents the first use of genetically encoded Ca$^{2+}$ indicators to examine evolved differences in brain function in a vertebrate model. In addition to loss of vision, cavefish are widely used to study many different behaviors including enhanced lateral line function, sleep loss, prey capture, olfactory processing, and stress.$^{11,54,71}$ The application of whole-brain imaging approaches that have been developed in zebrafish to evolutionary models including $A.\text{mexicanus}$ has the potential to identify neural mechanisms associated with evolved differences in these behaviors. Therefore, this manuscript provides a framework from applying comparative functional imaging to identify variability in brain function.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2022.07.015.

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**AUTHOR CONTRIBUTIONS**

A.C.K., G.S., and E.L. designed the experiments. E.L. and B.M. performed the experiments. E.L., M.P., and B.M. analyzed the data. E.L., B.M., and J.B.J. generated the transgenic lines used in all experiments. A.C.K., G.S., E.R.D., E.L., and M.P. wrote the manuscript. E.R.D. and J.B.J. provided additional help with the experimental design and data analysis.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | | |
| Mivacurium chloride | Sigma-Aldrich | CAS: 106861-44-3 |
| | | |
| Deposited data | | |
| Raw data | This paper | https://doi.org/10.17632/y9j999yk25.1 |
| | | |
| Experimental models: Organisms/strains | | |
| Surface A. mexicanus Tg(Huc:H2B-GCaMP6s) | This paper | N/A |
| Molino A. mexicanus Tg(Huc:H2B-GCaMP6s) | This paper | N/A |
| Pachón A. mexicanus Tg(Huc:H2B-GCaMP6s) | This paper | N/A |
| Software and algorithms | | |
| MATLAB 2020a | Mathworks | https://mathworks.com/ |
| Octave 6.2.0 | GNU | https://www.gnu.org/software/octave/ |
| Other | | |
| Low-melting point agarose | Thermo-Fisher | CAT#17856 |
| Nunc glass bottom dish | Thermo-Fisher | CAT#150680 |
| Arduino Uno | Arduino | https://www.arduino.cc/ |

RESOURCE AVAILABILITY

Lead contact
Further information and requests should be directed to and will be fulfilled by the lead contact, Alex Keene (akeene@bio.tamu.edu).

Materials availability
A. mexicanus lines used in this study can be made available by request to the lead contact.

Data and code availability
All data used for analysis has been uploaded to Mendeley Data and is publicly available as of the date of publication. The DOI is listed in the key resources table. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fish
Animal husbandry was carried out as previously described and all protocols were approved by the IACUC Florida Atlantic University. Fish were housed in the Florida Atlantic University core facilities at 23 °C ± 1 °C constant water temperature throughout rearing for behavior experiments. Lights were kept on a 14:10 h light-dark cycle that remained constant throughout the animal’s lifetime. Adult fish were fed a diet of blood worms to satiation 2-3x daily (Aquatic Foods, Fresno, CA), and standard flake fish food during periods when fish were not being used for breeding (Tetramin Pro).

Larval fish used were collected as embryos from adult tanks following breeding, and raised in a temperature-controlled incubator at 23 °C ± 1 °C until 6 dpf, when they were used in imaging experiments.

METHOD DETAILS

Molecular cloning and transgenesis
The A. mexicanus Tg(Huc:H2B-GCaMP6s) was generated using the previously published zebrafish Tg(elavl3;H2B-GCaMP6s). Briefly, the zebrafish elavl3 promoter was cloned to a Tol2 vector with the subcloned H2B, which restricts expression to the nucleus. The transgene plasmid and transposase RNA were injected into one to four cell-stage embryos at 25 ng/µl to a 1-µl volume, as described previously. Injected (F0) founders were grown to sexual maturity and bred, and transgenic lines were isolated by identification of high expression of fluorescence in the F1 and F2 generations. All transgenic animals were raised under standard conditions.
**Functional Imaging**

Prior to imaging experiments, larvae were paralyzed by 60 s of immersion in 0.5mg/ml mivacurium chloride, rinsed thoroughly with system water, and embedded in 2% low-melting point agarose (Thermo-Fisher, 17856), in clear plastic 35mm dishes (Nunc, Thermo-Fisher, 150680), with a strip of white LED lights (Lepro) affixed to the mounting dish rostral to the larvae. The microscope was shielded to prevent unintended light stimulus. Following a minimum of 1 minute of recording to capture basal activity levels, a 30 second visual stimulus was applied. In follow-up experiments, multiple 30 second stimuli were applied, separated by additional 1 minute intervals. Visual stimulus was controlled by custom code running on Octave 6.2.0, utilizing the Arduino package. All visual stimulus experiments were acquired on a Nikon A1 confocal microscope, at 20X magnification, 2X digital zoom, at a frame rate of 1.96 Hz.

**Two-photon Ca$$^{2+}$$ imaging**

To capture spontaneous activity in the tectum, larvae were prepared as described above, and Ca$$^{2+}$$ activity in the tectum was monitored for a period of 30 minutes, at a frame rate of 3.96 Hz on a Nikon A1R Multiphoton microscope, with a Chameleon Vision II Ti:Sapphire tunable laser at an excitation wavelength of 940 nm. Images were acquired with a 25x objective, and 1.5X digital zoom.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Ca$$^{2+}$$ activity analysis**

Ca$$^{2+}$$ activity of individual cells was extracted using a MATLAB toolbox developed by Romano et al. Briefly, an average image was generated from acquired time-lapse images, and a watershed algorithm was applied to generate ROIs outlining individual cells. These were used to extract raw fluorescence data from the time-lapse images, and calculate the ΔF/F values for individual cells (Figure S1). Cell counts (for Figure 1C) were derived from the automated segmentation.

**Correlation analysis**

In order to compute the average probability density function (pdf) for correlations in surface, Molino and Pachón, we estimated the correlation pdf for each larva by counting the number of correlations in regularly spaced (0.05) correlation bins between -1 and 1. We then averaged those pdf together within each population. In order to quantify the amount of correlation that emerged from the spontaneous rate of activity, we built a null model by shifting circularly the neuron’s activity time series by a random lag between 0 and 30min. The latter corresponds to the total duration of the experiments. This keeps the time series untouched while destroying the temporal correlations between time series. We performed 1000 repetitions of this procedure to obtain the null model.

To compare the distributions of correlations between the surface, Molino and Pachón populations, we quantified the distance between the null-model distribution and the distribution of correlations in our dataset using the Jensen-Shannon divergence for each larva. The discrete Kullback-Leibler divergence $D_{KL}$, or relative entropy, quantifies the similarity between probability distributions $P$ and $Q$ defined on some probability space $X$.

$$D_{KL}(P \parallel Q) = \sum_{x \in X} P(x) \log \frac{P(x)}{Q(x)}$$

The Jensen-Shannon divergence $D_{JS}$ is based on the Kullback-Leibler divergence, but is symmetric, such that $D_{JS}(P \parallel Q) = D_{JS}(Q \parallel P)$. It can be defined as:

$$D_{JS}(P \parallel Q) = \frac{1}{2}D_{KL}(P \parallel M) + \frac{1}{2}D_{KL}(Q \parallel M)$$

where

$$M = \frac{1}{2}(P + Q)$$

Finally, to investigate the spatial structure of correlations in the optic tectum of surface, Molino and Pachón, we averaged the correlation between pairs of neurons falling into regularly spaced (20 µm) distance bins between 0 and 200 µm to obtain the distribution of correlations against distance.

**Identification and categorization of cells responsive to whole-field visual stimuli using linear regression**

To identify and categorize neurons that are responsive to visual stimuli, we used a multivariate linear regression model. To search for neurons with specific responding profiles, we used a regressor-based analysis. We used 3 binary regressors based on the timings of the onset and offset the visual stimuli ($R_1$) or the offset ($R_2$) of the visual stimulation, or a sustained activation or inhibition during the whole duration of the stimulus ($R_3$). To model the slow dynamics of the H2B-GCaMP6s Ca$$^{2+}$$ sensor, we convolved those regressors with a decaying exponential kernel with characteristic time constant $\tau = 3.5s$. More specifically, if we denote $y_n$ the time series of the relative change of fluorescence over time for neuron $n$, we have:

$$y_n = \beta_0 + \beta_1 \times R_1 + \beta_2 \times R_2 + \beta_3 \times R_3 + \epsilon_n$$
Where $\beta_0^n$ is the intercept, $\beta_i^n$ are the regression coefficients associated to each regressor $R_i$, and $\epsilon_n$ represent the residuals for neuron $n$.

We compared the fraction of variance explained by the linear model $R^2$, which is a measure of the goodness of fit and compared this to a null model. We also compared the regression coefficient $\hat{\beta}_i$ obtained for each regressor, which captured the intensity of the response of a neuron, against the distribution of regression coefficients under the null model.

A neuron was classified as responsive if its $R^2$ value exceeded the 95th percentile of the distribution of $R^2$ over the whole population of recorded neurons in the null model ($R^2_{95}$), or lower than the 95th percentile in the case of inhibition ($\beta_i^{95}$). This procedure ensures that silent neurons, which may present a very good fit (high $R^2$ values) but low $\beta$ value are excluded from the population of responsive neurons, and also allows to categorize the neurons into 5 response profiles:

- **ON cells**: $R^2_n \geq R^2_{95} \land \beta_1 \geq \beta_1^{95} \land \beta_2 < \beta_2^{95} \land \beta_3 < \beta_3^{95} \land \beta_3 > \beta_3^{95}$
- **OFF cells**: $R^2_n \geq R^2_{95} \land \beta_1 < \beta_1^{95} \land \beta_2 \geq \beta_2^{95} \land \beta_3 < \beta_3^{95} \land \beta_3 > \beta_3^{95}$
- **ON/OFF cells**: $R^2_n \geq R^2_{95} \land \beta_1 \geq \beta_1^{95} \land \beta_2 \geq \beta_2^{95} \land \beta_3 < \beta_3^{95} \land \beta_3 > \beta_3^{95}$
- **Sustained cells**: $R^2_n \geq R^2_{95} \land \beta_1 < \beta_1^{95} \land \beta_2 < \beta_2^{95} \land \beta_3 \geq \beta_3^{95}$
- **Inhibited cells**: $R^2_n \geq R^2_{95} \land \beta_1 < \beta_1^{95} \land \beta_2 < \beta_2^{95} \land \beta_3 > \beta_3^{95}$

**Detection of significant Ca$^{2+}$ events**

In order to detect significant Ca$^{2+}$ events in the fluorescence time series, we used the method described in Romano et al. This enables us to compute the fraction of neurons active at any time point during the recording.

**Quantification of the temporal precision of the neural response to the onset of the visual stimulus**

Due to the level of noise in the data, especially in the Molino and Pachón populations, we could not reliably detect the onset of the visually induced Ca$^{2+}$ response for each neuron. Therefore, we chose to estimate the variability of the onset of the response by averaging together the across-trial probability of response (Figures S3G–S3I) for all cells (ON, ON/OFF and Sustained cells). Then, we performed a linear regression of the obtained average in a small time window (-2s to +2s) around the onset of the stimulus. As a proof of principle of this method, we computed the slope of the linear regression from synthetic data with different jittering levels (S6A,B).

**Statistical inference and data analysis**

Non parametric statistical tests were performed when the normality assumption could not be upheld. Data analysis was performed using custom scripts written in MATLAB (R2020a).