Supplemental Information

The Emergence of the Spatial Structure of Tectal Spontaneous Activity Is Independent of Visual Inputs

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Figure S1: Pair-wise correlations between tectal neurons for all developmental stages in intact and enucleated larvae, related to Figure 1. 
(A) Probability density of the distributions of the Pearson’s correlation coefficients between neuronal pairs, per developmental stage, in intact and enucleated larvae. The dashed curves depict the respective random surrogate versions. The distributions were calculated using correlation value bins of 0.001 ranging from -1 to 1. (B) Developmental dynamics of the average correlation coefficients for all neurons, in intact and enucleated larvae. (C) Average correlation coefficients for all pairs of neurons belonging to the same tectal hemisphere ('intra', solid curve) and all pairs of neurons belonging to different tectal hemispheres ('inter', dashed curve), in intact and enucleated larvae. (D) Average JSD for each stage in enucleated and intact conditions, for the pair-wise correlations of neurons belonging to the same tectal hemisphere ('intra', solid curve) and to both hemispheres ('inter', dashed curve). Comparison between stages are indicated on the curves (blue and red) and between intact and enucleated groups, at the bottom of the graph in black. Blue and red: intact and enucleated larvae respectively; p < 0.001: ***; p < 0.01: **; p < 0.05: *; Error bars: SEM.
Figure S2: Characterization of the neuronal assemblies, related to Figure 2.

(A) Average number of assemblies during development in intact and enucleated larvae. Although the average number of neuronal assemblies in the enucleated larvae are statistically smaller than the aged-match intact larvae (but at 5 dpf: p = 0.27), the trend over development is similar when comparing intact and enucleated larvae, with an increase in the number of extracted assemblies up to 5 dpf, followed by a decrease at 8 dpf. This trend is in line with the variation of the level of correlations observed for the whole neuronal population. 

(B) Average number of neurons per assembly during development in intact and enucleated larvae. In intact larvae, the average number of neurons per assembly increased up to 5 dpf, before sharply falling at 8 dpf (5 vs 8 dpf, p < 5.10^{-4}). In enucleated larvae, the average number of neurons per assembly show similar, although delayed, developmental dynamics and by 8 dpf this number is similar in intact and enucleated larvae (p = 0.18). 

(C) Raster plots of the significant Ca^{2+} events of the neurons composing two representative neuronal assemblies of a 5 dpf intact larva (top panels), their matching index (MI, in red and blue respectively, middle panel) and the matching indexes of the full population of assemblies (n = 64) of the same 5 dpf larva (bottom panel); peaks level with p values < 0.01 (i.e > 99% confidence) are considered significant. Scale bar: ΔF/F. 

(D) Average MI frequency during development in intact and enucleated larvae. No statistical difference are noticeable between intact and enucleated larvae, but at 3 dpf (p = 0.03). At this age the average frequency of MIs in enucleated larvae is however not different from the one of the intact 2.5 dpf larvae (p = 0.08). Blue and red in A, B and D: intact and enucleated larvae respectively; p < 0.001: ***; p < 0.01: **; p < 0.05: *; Error bars: SEM.
**Figure S3**: Spontaneous neuronal assemblies regroup functionally related neurons, related to Figure 2.

(A) Examples of neuronal assemblies induced by light-spots of 20° angular size, across the visual field (from -46° to +46°) at 3 and 8 dpf; the center of mass of each visually induced assembly is indicated by a red dot; these dots are color-coded in the bottom panels according to the position of the visual stimulus that induced the response. Note the match between the order of the colors along the caudo-rostral axis and the colors representing the different positions of stimulation, indicating that the tectal retinotopic map is organized along the tectal caudo-rostral axis. (B) Probability density distributions of the MIs of the significant visually induced neuronal groups (3 dpf: green; 8 dpf: red), during the periods of spontaneous activity. The dashed curves represent the distribution of the non-significant MI values (baselines). Note that distributions and magnitudes of induced assemblies were comparable at 3 and 8 dpf: the probability density peaks of the significant matching index were 0.226 ± 0.013 at 3 dpf and 0.254 ± 0.013 at 8 dpf, while the ones of their respective baseline were 0.047 ± 0.006 (p < 10^{-5} by comparing MIs and baselines) and 0.067 ± 0.004 (p < 10^{-4}). (C) Examples of 3 dpf (left panels) and 8 dpf (right panels) spontaneous assemblies and corresponding two topographic null assemblies (out of 50). Notice that the distributions of the distance between pair of neurons in the assembly and their respective null model assemblies are similar (bottom panels); positive and negative distance correspond to intra- and inter-hemisphere neuronal pairs, respectively. (D) Distribution of the normalized probability density of the pooled pair-wise correlation coefficients of the spatial tuning curves (azimuth tuning curves) of neurons belonging to spontaneous assemblies at 3 (green) and 8 dpf (red) and their respective null models (black). For the normalization, the distributions were divided by the null-model distributions. Note the large bias of the spontaneous assemblies toward grouping neurons with highly similar tuning curves, both at 3 and 8 dpf. The confidence intervals were calculated with a Jack-Knife procedure, across experiments. The significant bias range and median are indicated by the top line and the dot, respectively. The bias ranges became significant above 0.32 ± 0.01 and 0.26 ± 0.08, with medians of 0.633 ± 0.004 and 0.642 ± 0.003, for 3 and 8 dpf larvae respectively (the medians between stages were not statistically different: p = 0.116). (E) Same as D, but the normalization was performed using the distribution of the topographic null-models. Remarkably, the original assemblies still had a significant bias toward neurons with similar tunings, despite the preservation of the distribution of pair-wise distances in these topographic null-models. (F) Examples of topographic localization of the neurons responding to at least 25% of the auditory (top panels) or lateral line stimuli (bottom panels) in a ventral (left panels) and a dorsal (right panels) layer of the optic tectum. The scale bar indicates the probability of response (resp.) of the neurons to at least 25% of the stimuli. (G) To test whether the weak tectal response to lateral line stimuli is a direct consequence of lateral line stimulation failure, we monitored the response in the torus semicircularis (TS, its neuropil is outlined by a pale dashed blue line). Regions of interest (ROIs, delineated out of a homogeneous 10 x 10 pixels grid) in red show the TS regions responding to at least 25 % of the stimuli. The histogram of the significant Ca^{2+} events of the ROIs show a reliable and strong response to lateral line stimuli in the TS. (H) Proportion of neurons responding to at least 25 % of the auditory (left panel) and lateral line (right panel) stimuli, in intact (n = 3, blue) and enucleated larvae (n = 3, red). The proportion of responsive neurons to visual stimulation (vis., moving bar across the visual field) in the dorsal layer of the optic tectum (n = 13, black). Note the significant large differences of the visual responses with respect to the auditory and lateral line ones (p < 0.005). In the ventral layers (vent.) of the optic tectum, we observed no significant differences between intact and enucleated larvae for auditory and lateral line responses (p > 0.97). In the dorsal layers (dor.), the proportion of cells responding to the stimulation tends however to be smaller in the enucleated than in the intact larvae (p < 0.04). Error bars: SEM.
Figure S4: The neuronal assemblies are distributed along the caudo-rostral axis, related to Figure 3.

(A) Example of normalization of the neuronal coordinates in a common spatial reference map, where each dot represents a neuron in its original position (left) and after the normalization (right); the morphological medio-lateral (mla) and caudo-rostral axis (cra) are represented in green and purple, respectively. (B) Correlation coefficients between the neurons belonging to each assembly and their respective centroids along different axes of the common spatial reference map, rotated every 15° for a total of 180°, for each tectum and for all developmental stages, in intact (blue) and enucleated (red) larvae. Note that the highest correlations were obtained along the tectal caudo-rostral axis in all considered conditions. (C) Null-model density plots of the caudo-rostral normalized positions of each neuron against the normalized position of each neuronal assembly centroid, along the caudo-rostral axis of the tectum, in intact and enucleated larvae, at each developmental stage; 0 is the most rostral position and 1, the most caudal one (indicated in the enucleated 8 dpf larva density plot). (D) Distribution of the density of the neurons per assembly along the normalized caudo-rostral axis (the axis was normalized between -1 to 1, from lateral left to lateral right) at all developmental stages, in intact (n = 622, 800, 1101 and 864 assemblies for 2.5, 3, 5 and 8 dpf stage respectively) and enucleated larvae (n = 511, 768 and 688 assemblies for 3, 5 and 8 dpf stage, respectively) and their respective null models (built from 50 repetitions of each neuronal assembly). Each neuronal assemblies are represented on the y-axis.
Supplemental Experimental Procedures

Surgical procedures
Since the connection between retina and optic tectum becomes functional at ~ 73 hpf (first visually induced responses in the optic tectum, Niell et al., 2005), we chose to perform bi-lateral ablations of the eyes on 54 - 58 hpf embryos. Enucleations at this stage led the tectal circuit to develop under normal conditions, just before the onset of the functional retinal inputs, therefore, leaving the tectum in a naive state with respect to the visual inputs (retinal spontaneous activity and visual induced activity). Embryos were placed in 1.8 % low-melting agarose with 0.02% MS-222 (anesthetic; Sigma-Aldrich, France) in high-Ca\(^{2+}\) Ringer solution (116 mM NaCl, 2.9 mM KCl, 10 mM CaCl\(_2\), 5 mM HEPES, pH 7.2) for immobilization purposes, and then submerged in a high-Ca\(^{2+}\) Ringer solution. Custom-made micro-scalpel (fabricated from insect pins) were used the remove the eyes of the embryos. In order to assure the complete ablation of the retina and prevent any regeneration, we paid particular attention on the full removal of the photoreceptor layer. The enucleation procedure took just a few minutes and the embryos recovered from anesthesia a few seconds later after being released from the agarose. The embryos were then allowed to recover from the surgery in high-Ca\(^{2+}\) Ringer solution, at 28.5°C for two hours, before to be returned to 0.5x E3 embryo medium. In an attempt to feed enucleated larvae after 5 dpf, we added to their Petri dish large amounts of paramecia. This procedure showed limited success, precluding the use of enucleated larvae beyond 8 dpf.

In addition to the enucleations at 54-58 hpf, we performed enucleations at 48 hpf (the time at which the very first RGC axons reach the optic tectum, although these axons have not yet generated functional synapses; n = 3). To control for the effects of the surgical procedure, we also performed sham experiments (n = 3) in which the spinal cord were incised at 55-57 hpf at the level of the cloaca. These two sets of experiments were then compared with the 54-58 hpf enucleated and intact larvae, respectively. For the comparison, we measured two key parameters assessing the temporal and spatial components of the spontaneous activity, namely the Jenson-Shannon distance (JSD, Figure 1F), and the r coefficient of the linear regression of the normalized neuron’s positions against the neuronal assembly centroid’s positions along the caudo-rostal axis (Figure 3B), at 8 dpf for the 48 hpf enucleations and 5 dpf for the sham experiments. No statistical differences could be observed between the larvae enucleated at 54-58 hpf and those enucleated at 48 hpf (JSD = 0.46 ± 0.02 vs. 0.52 ± 0.04; p > 0.34 (Kolmogorov-Smirnov test), and r = 0.615 ± 0.012 vs. 0.618 ± 0.013; p = 0.70). Also, no statistical difference could be observed between the sham control experiments and the intact larvae (JSD = 0.27 ± 0.04 SEM vs. 0.29 ± 0.02 SEM, p > 0.91 and r = 0.70 ± 0.04 SEM vs. 0.71 ± 0.02 SEM, p > 0.98). These control experiments suggest that the enucleations performed at 54-58 hpf efficiently precluded retinal activity influence on the early tectal circuit maturation, without adversary impacting the global development of the larvae due to the surgical procedure.

Choice of the tectal regions for imaging
Larvae were embedded in 2 % low-melting agarose in 0.5x E3 embryo medium, ventral side down on an elevated stage placed in a cylindrical chamber filled with 0.5x E3 embryo medium, and let to adapt for at least 20 min, at room temperature (~ 22 °C) in the dark, before the onset of the experiments.

We imaged the optical section that best responded to visual stimuli. For this purpose, we presented light bars moving across the visual field, while focusing at different depths (z-planes) of the optic tectum. Stimuli were projected on a screen (#216 White Diffusion, Rosco Cinegel) placed around the circular imaging chamber via a pico-projector (Pocket Projector ADPP-305, Adapt). To avoid interference of the visual stimulus with the GCaMP3 emitted fluorescence (peaking at 547 nm and filtered using a 520/50 band-pass filter), only the red LED of the projector was used (620 nm). In addition, a 561 nm long-pass filter (BLP01-561, Semrock, USA) was placed in the front of the projector. Visual stimuli were created using the Psychtoolbox (Brainard, 1997) for Matlab (The MathWorks, Inc.) and designed to compensate for the curvature of the chamber.

The z-planes that best responded to the visual stimuli were observed in the superficial layers of the optic tectum, at depths of ~ 40 – 50 mm (with respect to the dorsal skin) for the 3 dpf larvae, and ~ 60 – 70 mm for the 5 and 8 dpf larvae. For the 2.5 dpf larvae, not responding to visual stimuli, we used the same depth as for the 3 dpf larvae. We used the same imaging depths for the enucleated larvae experiments and several anatomical landmarks, especially the distinctive morphology of the cerebellar region, immediately caudal to the tectum (for instance the lateral positioning of the parallel fibers crossing our optical plan of interest) and the anatomy of the rostral part of to the tectum (characteristic triangular shape), to assure similar positioning of optical planes between larvae at each developmental stage. This strategy enabled us to find similar optical planes of the optic tectum across the different intact and enucleated larvae, at each developmental stage.

To control for unwanted sensory stimulation, the larvae were placed in a complete dark environment, the medium in the large recording chamber was static (no medium flow), and the microscope was place on a 500 kg active air table to filter out vibrations from and outside the table. We also paid a particular attention to perform all experiments in an isolated room away from external noise sources. We thus considered that under these sensory-suppressed conditions, the zebrafish larvae did not experience any sensory stimulation.

Visual stimulation
To accurately assess the onset of the functional connection between the retina and the optic tectum, we continuously imaged
latter from 70 to 74 hpf at different depths of the optic tectum, while presenting moving light bars. We confirmed that the first response to visual stimuli was observed at ~ 73 hpf (as in Niell and Smith, 2005) with a variability of less than one hour. Accordingly, experiments were stopped before reaching 72 hpf for the 2.5 dpf stage, and started after 74 hpf for the 3 dpf stage.

For some experiments, we estimated the receptive field of the neurons (spatial tuning curves) by presenting the zebrafish larvae with light spots of 20° in size at different azimuthal angles of the visual field (between -46° to 46°). Light-spot stimuli were presented four times at each position in a random order. We then averaged the neuronal responses induced by stimuli at the same spatial position. Induced responses were calculated over a 2-s window after the onset of the stimulus. Neurons were considered responsive to visual stimuli if they had a significant Ca²⁺ event of an average amplitude response larger than one standard deviation of the average amplitude, and responded to at least 50 % of the stimuli. This procedure enabled us to group neurons in visually induced neuronal groups.

For some experiments, we simultaneously monitored the calcium dynamics in the tectum and the tail movements of the larva. For that purpose, we removed agarose around the tail and added a custom-made small microscope from the ventral side of the larva. This small microscope was connected to a high-speed camera (TXG02, Baumer), controlled by a custom-made script written in C++. Visual stimuli and tail images (acquired at 200 Hz) were both synchronized with the two-photon calcium imaging acquisition software, via a TTL pulse generated by an I/O board (ActiveWire or Arduino) and controlled by Matlab.

**Auditory and lateral line stimulation**

Auditory stimuli were delivered via a water-proof Visaton K28 miniature speaker placed in front of the larvae within a circular recording chamber. The chamber dimensions were 100 mm of diameter and 50 mm tall, and it was filled with 0.5X E3 medium. The larvae were placed on an elevated stage in the center of the chamber facing the speaker. Stimuli consisted of sinusoids of 1 s duration gated with a 150 ms cosine squared window to prevent spectral leakage. Recordings of the stimulation with a hydrophone (Bruel & Kjaer 8103) showed spectrograms with single pure tones without harmonics. For stimulation of the auditory system we used 600 Hz and for the lateral line, 30 Hz. We presented to each larva 10 stimuli repetitions with an inter-trial time interval of 6 s. Using two-photon Ca²⁺ imaging, we monitored the responses at two different layers of the tectum, namely the visual layer (superficial) where all other experiments were performed, and in a ventral tectal layer, known to receive multisensory information, approximately at the level of the intertectal commissure. Neurons were considered responsive to auditory or lateral line stimuli if they had a significant Ca²⁺ event of an average amplitude response larger than one standard deviation of the average amplitude, and responded to at least 25 % of the stimuli. This procedure enabled us to group neurons in auditory and somatosensory induced neuronal groups.

**Data analysis: Ca²⁺ dynamics data processing and detection of neuronal assemblies**

Image segmentation to obtain regions of interest corresponding to neurons, curation of movement artifacts and detection of the significant calcium transients were performed as described in Romano et al. (2015), using custom-written Matlab scripts (The MathWorks, Inc.). Frames with movement artifacts were not further considered and the non-significant portions of the ΔF/F traces were then set to 0 in all subsequent analysis. To extract the functional neuronal assemblies from the GCaMP3 fluorescence fluctuations recorded in zebrafish larvae, we use the analytical framework established by Romano et al. (2015).

Briefly, regions of interest corresponding to single neurons were semi-automatically detected using a watershed-based algorithm from an average across the entire time series images. Movement artifacts were automatically detected and discarded. We developed a method to infer the statistical significance of single-neuron calcium transients using a data-driven noise model and imposed biophysical constraints of the fluorescent calcium indicator. All further analysis was performed on significant calcium events. To extract functional neuronal assemblies, we started by representing the spontaneous activity of the entire neuronal population in a space of reduced dimensionality through principal component analysis (PCA), by only keeping principal components (PCs) with significant eigenvalues (Peyrache et al., 2010). To further partition the data into groups of co-varying neurons, we used factor analysis (promax) which broke the PCA orthogonality condition (Lopes-dos-Santos et al., 2013). Following this analysis, the PC loadings tended to sparsely concentrate along the non-orthogonal rotated PCs. We then defined the neuronal composition of the assemblies by setting a data-driven threshold on the PC loadings along these non-perpendicular rotated PCs.

**Matching index**

To analyze the temporal dynamics of the activation of the neuronal assemblies, we implemented a *Dice coefficient* index (Dc) previously used to evaluate the degree of similarity in the connection patterns of brain regions (Hilgetag et al., 2002; Sporns et al., 2007). This index first measures the proportion of active neurons at each time point of the recording period (each frame) in both the spontaneous neuronal assembly and the full neuronal population, creating two neuronal activation patterns. Then it quantifies the proportion of neuronal activation that is common to both patterns, with respect to the total number of neuronal activation in each pattern. The neuronal assembly *matching index* ranges between 1, where there is no overlap between the respective patterns and 0 where there is a complete overlap between the respective patterns, at each time frame. We used the hypergeometric distribution to evaluate the probability of observing a common activation pattern by chance (at p < 0.01), which estimate the significance of the assembly activation patterns. For this analysis we considered that the neuronal activations were independent.
from each other.

**Random surrogates and Jensen-Shannon distances**

To assess the statistical significance of the pair-wise correlations of neuronal activities across different developmental stages, we generated random surrogate data sets. This is particularly important when comparing experiments, with different neuronal activity dynamics (e.g. frequency of significant neuronal Ca\(^{2+}\) events). To this end, we repeatedly shuffled the time stamp of significant Ca\(^{2+}\) events of each neuron and observed that 50 repetitions of this procedure were sufficient to create a surrogate data set, as adding more repetitions did not change neither the mean nor the variance of different measured parameters of the surrogate data set.

To test whether the pair-wise Pearson's correlation coefficients can be indicative of a structure in the spontaneous activity (in contrast to correlations explained by chance), we measured the Jensen-Shannon distance (JSD) between the distribution of the spontaneous correlation coefficients and distribution of the respective random surrogate sets. The JSD is a metric measurement of the dissimilarity between two distributions. It is derived from the classical information-theory Kullback-Leibler divergence (Kld), with the advantage of being a dimensionless and symmetric measure (Lin, 1991).

\[
\text{JSD}(p_1 \parallel p_2) = \sqrt{\text{KLd}(p_1 \parallel q) + \text{KLd}(p_2 \parallel q)}
\]

where

\[
q = \frac{p_1 + p_2}{2}
\]

\(p_1\) is the probability distribution of the correlation coefficients of the data, and \(p_2\), of the random surrogates, at each discrete bin \(r\) along the distributions. We used a base 2 logarithm to calculate the \(\text{Kld}\) to bound the JDS between 0 and 1.

**Null models of the neuronal assemblies for the significance assessment of the different features**

To assess the significance of the topographic and dynamic features of the neuronal assemblies, we compared them against surrogate assembly null models. We shuffled the indexing (neuronal identity) of the entire neuronal population for each experiment and then built null models by grouping neurons according to the original assemblies, but using the shuffled neuronal indexes. We created 50 sets of randomly shuffled surrogate assemblies. This procedure randomized the topography of each assembly, while keeping intact the number of neurons per assembly and the topographical position and activation time series of each neuron. Since pair-wise correlations can be sensitive to the amount of neuronal activity, we selected the null-model assemblies according to their average level of activity. We kept those within mean ± standard deviation of the activity level of the considered assembly. In some cases, we also built random shuffle assembly null model, with the constraint of conserving the distribution of relative pair-wise physical distances between neurons found in the original assemblies. For a given spontaneous assembly, we randomly chose a first neuron in the contralateral tectum and iteratively added neurons to the topographic null model in a way that conserved the neuron pair-wise assembly physical distance as good as possible by minimizing the mean-squared-error of each new pair of neurons in the topographic null model. Furthermore, we also respected, for each topographic null assembly, the proportion of neurons in either tectal hemisphere as observed in the original assembly. When the last iteration was performed, we only kept the topographic null model if the physical distance off all its pairs of neurons was not significantly different (\(p > 0.05\)) from the original assembly. The preservation of these properties enabled testing the specificity of the neuronal associations present in the assemblies. Assessing the significance of the different properties of the neuronal assemblies with respect to their null models enabled the comparison between experiments, independently of the variable number of tectal neurons found at the different developmental stages.

**Compactness and Lateralization indexes**

To assess the topographic dispersion of each assembly, we implemented a compactness index (CI), defined as:

\[
CI = 1 - \frac{\sum_{i=1}^{n} d_i}{\left(\sum_{j=1}^{n} d_j\right)}
\]

The sum of the all distances of each neuron of an assembly to its centroid \(d_i\) was divided by the averaged sum of each neuron of the respective null-model assembly to their centroid \(d_j\). This allows taking into account the variable number of neuron in each
assembly, and compare within and between each condition.

We defined a lateralization index ranging from -1 to 1. One represents a neuronal assembly with all its neurons in a given hemisphere of the tectum, while the neurons of its corresponding null-model assemblies are equally present in both hemispheres. Reciprocally, -1 depicts a case where all the neurons of the null-model assemblies are in a given hemisphere, while neurons of the neuronal assembly are equally present in both hemispheres. Zero represents a neuronal assembly and its corresponding null model assemblies with the same proportion of neurons in a given hemisphere.

Normalization of the tectal anatomical coordinates
We transformed the position coordinates of the tectal neurons into a normalized reference space to compare topographical features across larvae of different developmental stages and in the intact and the enucleated conditions. The tectum possesses two clear morphological axes: the caudo-rostral axis and its perpendicular one, the medio-lateral axis (Figure S4A). We chose to project and normalize the coordinates of all imaged neurons with respect to these two axes. We first manually delineated the boundaries of the tectum, and used its midline and the border between the stratum periventricular and the neuropil to calculate the curve that best described the two axes. These axes were then normalized from -1 to 0, caudal to rostral for the left hemisphere, and 0 to 1, rostral to caudal, for the right one. Neurons from the left and right hemispheres of the tectum were projected into a [-1,0] x [-1,1] and [0,1]x[-1,1] normalized space respectively. Due to the curvature of the caudo-rostral axis, some neurons could be potentially equally mapped to two distinct normalized positions. In these rare cases, we chose to project the neuron over the axis position closest to its original position to solve this ambiguity.

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