Published in final edited form as:

Nature. 2008 November 6; 456(7218): 102–106. doi:10.1038/nature07351.

Entrained rhythmic activities of neuronal ensembles as perceptual memory of time interval

Germán Sumbre^{1,†}, Akira Muto², Herwig Baier², and Mu-ming Poo¹

¹Division of Neurobiology, Department of Molecular and Cell Biology, Helen Wills Neuroscience Institute, University of California, Berkeley, California 94720, USA.

²Department of Physiology, University of California, San Francisco, California 94158, USA.

Abstract

The ability to process temporal information is fundamental to sensory perception, cognitive processing and motor behaviour of all living organisms, from amoebae to humans^{1–4}. Neural circuit mechanisms based on neuronal and synaptic properties have been shown to process temporal information over the range of tens of microseconds to hundreds of milliseconds^{5–7}. How neural circuits process temporal information in the range of seconds to minutes is much less understood. Studies of working memory in monkeys and rats have shown that neurons in the prefrontal cortex^{8–10}, the parietal cortex^{9,11} and the thalamus¹² exhibit ramping activities that linearly correlate with the lapse of time until the end of a specific time interval of several seconds that the animal is trained to memorize. Many organisms can also memorize the time interval of rhythmic sensory stimuli in the timescale of seconds and can coordinate motor behaviour accordingly, for example, by keeping the rhythm after exposure to the beat of music. Here we report a form of rhythmic activity among specific neuronal ensembles in the zebrafish optic tectum, which retains the memory of the time interval (in the order of seconds) of repetitive sensory stimuli for a duration of up to ~20 s. After repetitive visual conditioning stimulation (CS) of zebrafish larvae, we observed rhythmic post-CS activities among specific tectal neuronal ensembles, with a regular interval that closely matched the CS. Visuomotor behaviour of the zebrafish larvae also showed regular post-CS repetitions at the entrained time interval that correlated with rhythmic neuronal ensemble activities in the tectum. Thus, rhythmic activities among specific neuronal ensembles may act as an adjustable ‘metronome’ for time intervals in the order of seconds, and serve as a mechanism for the short-term perceptual memory of rhythmic sensory experience.

The zebrafish tectum processes visual information and integrates it with inputs from other sensory modalities^{13,14}. To investigate the ensemble neuronal activity triggered by visual stimulation, we used two-photon fluorescence imaging¹⁵ of Ca²⁺ dynamics¹⁶ to monitor the neuronal activities of a large population of cells (~200) simultaneously in intact, unanesthetized and unparalysed zebrafish larvae (5–14 days post-fertilization (d.p.f.), Fig. 1a, ref. 17). The amplitude of Ca²⁺ transients increases in individual neurons correlated with the number of spikes (Supplementary Fig. 1; see refs 17–20). Repetitive visual stimulation of the contralateral eye with a moving light bar across the visual field induced reliable responses in some tectal

© 2008 Macmillan Publishers Limited. All rights reserved

Correspondence and requests for materials should be addressed to M.-m.P. (mpoo@berkeley.edu).

[†]Present address: Laboratoire de Neurobiologie, UMR 8544, École Normale Supérieure, 46 rue d’Ulm, 75005 Paris, France.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Author Information Reprints and permissions information is available at www.nature.com/reprints.

cells, but caused sporadic or habituating responses in others (Fig. 1b). Moreover, moving the bar stimuli in opposite directions activated different, but partially overlapping, neuronal ensembles (Fig. 1c and Supplementary Fig. 2a). The mean amplitudes of Ca^{2+} transients evoked in each neuron by consecutively moving bars in the same direction were highly correlated, whereas those induced by bars moving in opposite directions showed much lower correlation, suggesting that the tectal-ensemble-evoked responses are stimulus-patternspecific (Supplementary Fig. 2b, c).

In the absence of visual stimulation, synchronized Ca^{2+} transients among different tectal cells were rarely observed and showed no apparent regularity. However, after repetitive stimulation of the contralateral eye with a moving bar stimulus 20 times, and with an interstimulus interval (ISI) of 6 s (CS), we observed post-CS synchronized Ca^{2+} transients in a subpopulation of tectal neurons, at a time corresponding to the multiples of the ISI of the CS (Fig. 2a). This was also shown by the average profile (Fig. 2b) and onset time (Fig. 2c, d) of the Ca^{2+} transients for all cells. In general, 'rhythmic' Ca^{2+} transients occurred for up to three cycles (18 s) and were found in a subpopulation of neurons that were responsive to the CS, spatially dispersed across the ensemble (Supplementary Fig. 3a). Altogether, post-CS rhythmic activities were observed in 53 out of 110 experiments (48%, 23 larvae, 20 moving bars, 6 s ISI). The average amplitude of the integrated Ca^{2+} signals associated with rhythmic activities and their probability of occurrence were lower than that of the CS-evoked responses ($P < 0.001$, *t*-test, Fig. 3b), and they decayed with time (Supplementary Fig. 3b). Such rhythmic activities were not observed in the 3-d.p.f. larvae ($n = 8$) and seemed to emerge in the 4-d.p.f. larvae (one out of seven). Furthermore, the minimal number of stimuli for inducing post-CS rhythmic activities was ~10, and the robustness of these activities (the mean number of rhythmic cycles) increased with the number of stimuli until it reached a plateau at ~50–100 cycles (Supplementary Fig. 4a, b). Uninterrupted rhythmic stimulation was required for inducing post-CS rhythmic activities, as shown by the lack of an apparent cumulative effect for two to four CS episodes (each consisting of ten stimuli) that were presented with 3-min spacing (Supplementary Fig. 4c). Similar post-CS rhythmic activities were also induced by CS other than moving light bars, including moving dark bars, looming light circles and wide-field light flashes (Supplementary Fig. 5).

Further experiments showed that the moving-bar CS of different ISIs (4, 6 and 10 s) induced post-CS rhythmic activities at the conditioning ISI (Fig. 2e). The temporal precision of these entrained rhythmic activities were analysed for all experiments by plotting the histogram of the onset time of Ca^{2+} transients relative to that of the visual stimulus during the CS, or relative to the expected onset time should the CS started earlier or continued for further cycles. The distribution of the onset times was largely uniform before CS, but became clustered during the CS. Post-CS Ca^{2+} transients showed non-uniform onset time distributions for up to three cycles, with clear peaks in the first cycle for all three ISIs, and peaks up to the second and third cycle for the ISIs of 6 and 4 s, respectively (Supplementary Fig. 6). Because rhythmic activities were observed for up to ~20 s for all ISIs, these activities may be limited by the time lapsed rather than by the number of entrained rhythmic cycles. The analysis of spontaneous ensemble Ca^{2+} transients showed that synchronous Ca^{2+} transients among an ensemble of tectal neurons occurred with a very low frequency (~0.02 Hz), and were rather uniformly distributed (Fig. 2f, 'spontaneous'). However, during the first 30 s after CS, the temporal distribution of synchronous Ca^{2+} events showed clear peaks at the entrained interval (Fig. 2f, '4, 6 or 10 s').

The post-CS rhythmic activities in the tectum are specific to the CS. Consecutive presentations (5 min apart) of two different types of CS—a light bar moving either caudo-rostrally or rostro-caudally, for 20 repetitions (ISI 6 s)—induced post-CS rhythmic Ca^{2+} transients in two partially overlapping tectal cell populations, which were essentially all within the ensemble that responded to the respective CS. In the experiment shown in Fig. 3, 54 out of the 67 neurons

that responded to both CSs had post-CS rhythmic activities, and among these neurons, many (36 out of 54) showed rhythmic activities induced only by one CS (Supplementary Fig. 7a). Data from four other experiments showed similar results. Furthermore, rhythmic activities occurred in neurons that were not necessarily among those that were highly responsive to the stimulus, because there was no clear correlation between the mean amplitude of stimulus-evoked and the post-CS rhythmic Ca^{2+} transients of these neurons ($R^2 = 0.09$, Supplementary Fig. 7b). Thus, rhythmic Ca^{2+} transients probably reflect the activity of a specific neuronal ensemble entrained by the CS, rather than the activation of neurons with higher excitability. Post-CS rhythmic activity is unlikely to originate from the retina, because it was absent in the axon terminals of retinal ganglion cells expressing the genetically encoded Ca^{2+} indicator G-CaMP²¹ (32 trials in 5 larvae, Supplementary Fig. 8 and Supplementary Methods).

To investigate the physiological relevance of CS-induced rhythmic activities of tectal neurons, we examined the visuomotor behaviour of zebrafish larvae (7–14 d.p.f.). The heads of the larvae were immobilized in agarose and tail kinematics was analysed before, during and after the visual CS. Repetitive wide-field light flashes evoked ‘tail-flip’ behaviour (Fig. 4a, b) with a probability of ~0.6. Notably, after 20 flashes of CS at an ISI of 4, 6 or 10 s, 30% of the larvae (24 out of 81, 13 larvae) showed post-CS tail flips in the absence of sensory stimulation for at least one cycle at the entrained ISI for a period of up to ~20 s (Fig. 4c, d and Supplementary Movie 1). Spontaneous tail flips occurred at a low frequency (~0.015 Hz), without showing a preference for any specific interval (Fig. 4e, ‘spontaneous’). Nevertheless, during the first 30 s after the CS, the distribution of tail-flip events clustered around the entrained interval (Fig. 4e, ‘4, 6 or 10 s’).

By monitoring tectal ensemble Ca^{2+} transients and tail-flip behaviour simultaneously (Supplementary Fig. 9), we found a high correlation between the tail flip and the synchronous Ca^{2+} event in the tectal ensemble during the CS and the first 30 s after the CS, but a significantly lower correlation during the 60 s before, and the 31–60 s after, the CS (Fig. 4f and Supplementary Fig. 5). Moreover, normalized mean Ca^{2+} transients of the entire tectal ensemble during the first 30-s post-CS period were significantly higher for those associated with the rhythmic tail flip than those not associated with it (Fig. 4g and Supplementary Fig. 5).

The close resemblance and the correlation between entrained rhythmic tectal activities and post-CS tail flips suggest that entrained rhythmic activities contribute to short-term perceptual memory of visual experience. These rhythmic activities may raise the activity of specific neuronal ensembles at the entrained interval to a level closer to, and occasionally surpassing, that required for triggering the rhythmic activities, perhaps through the tectal outputs to the hind-brain^{22,23}. This idea was further supported by the observation that the subthreshold stimuli that were normally ineffective in eliciting a tail flip (probability 0.06) had a significantly facilitating effect on evoking tail flip after the CS (20 flashes, ISI of 6 s) when they were presented at the entrained interval time. For the first four cycles after the CS, the probability of rhythmic tail flips was increased from 0.30 (24 out of 81, 13 larvae) to 0.49 (18 out of 37 trials, 7 larvae, $P = 0.045$, chi-squared test), but only when the subthreshold stimuli were applied in phase with the entrained interval. In contrast, the same subthreshold stimuli applied in antiphase (at 3, 9, 15 and 21 s post-CS) rarely evoked a tail flip (1 out of 14 trials, 4 larvae), and the probability of rhythmic tail flips at the entrained interval (0.29, 4 out of 14 trials, 4 larvae) was similar to that which was observed with no subthreshold stimulation ($P = 0.99$, chi-squared test). Furthermore, during the late phase of the CS, tail-flip behaviour was sometimes initiated shortly before the light stimulus onset in an ‘anticipatory manner’ (Fig. 4d and Supplementary Movie 1). Thus, the rhythmic activity may increase the sensitivity of the neural circuit to a specific sensory stimulus occurring at the entrained time interval.

Previous studies^{24–28} have shown that repetitive visual stimulation at a regular ISI can result in spontaneous neuronal activity roughly at the expected time interval during and after the end of stimulation—a phenomenon known as ‘omitted stimulus potential’. The omitted stimulus potential originates from the retina^{24,25,28} and occurs for repetitive stimuli with much shorter ISIs (15–500 ms). It was found in cells not necessarily responsive to the stimuli²⁸ and has not been observed beyond one cycle^{24–28}. The present results extend the phenomenon of the omitted stimulus potential by showing that the rhythmic activity of tectal circuits may act as an adjustable circuit ‘metronome’ that can be set to memorize stimulus time intervals in the order of seconds for a duration up to ~20 s, enabling the zebrafish larvae to estimate the time of a specific impending stimulus. Thus, short-term memory of rhythmic sensory experience may be represented by entrained rhythmic activities, the neural circuit basis of which remain to be determined.

METHODS SUMMARY

Zebrafish larvae (wild-type or nacre^{29,30}, 3–15 d.p.f.) were used for experiments. The optic tectum neurons were loaded with the fluorescent calcium indicator Oregon Green BAPTA-1 AM using methods previously described¹⁷. Visual-activity-induced Ca^{2+} transients in a large population (~200) of tectal cells were monitored at the periventricular layer by conventional confocal (488nm) or two-photon (790 nm) microscopy. Visual stimuli—for example, light bars moving in various directions or light flashes—were presented by an LCD screen positioned in front of the contra-lateral eye. Visuomotor behaviour of head-restrained larvae (7–14 d.p.f.) was elicited by whole-field brief light flashes, and the tail kinematics was measured from the images obtained by a video camera (at 60Hz). A custom-made mini-microscope was used for simultaneous recording of motor behaviour and tectal Ca^{2+} dynamics.

METHODS

Zebrafish preparation

Embryos from wild-type zebrafish and zebrafish nacre, with the latter lacking melanophores^{29,30}, were collected and raised at 28.5 °C in E3 embryo medium³¹. The larvae were kept under 14/10 h on/off light cycles and were fed after 6 d.p.f. All experiments were approved by the University of California Berkeley’s Animal Care and Use Committee.

Calcium imaging of ensemble neuronal activities

Calcium indicator dye Oregon Green 488 BAPTA-1 AM was dissolved in dimethylsulphoxide with 20% pluronic (10mM) and further diluted 10:1 in Evan’s solution³². For the dye loading into tectal neurons, larvae at 5–14 d.p.f. were embedded in 1.2% low-melting-point agarose and anaesthetized with 0.02% MS-222 or Evan’s solution at ~13 °C, similar to methods described previously^{17,32}. The larvae were bolus injected under a stereomicroscope, using pressure injection (2–3 pulses of 30 ms duration, at ~10 pounds per square inch) through a micropipette (tip opening 2–3 μm). Larvae were incubated in the dark (at ~24 °C) in E3 for 1 h before use. Ca^{2+} imaging was performed mainly in the periventricular layer of the optic tectum, using either a Zeiss confocal (at 488 nm) or a custom-built two-photon microscope system (at 790 nm), with $\times 40$ water-immersion objective (NA 0.8). Continuous scanning (1–2.7 Hz) was triggered by the visual stimulation software. Owing to pigmentation, wild-type larvae were imaged using the confocal system. Under low laser power (<2 mW), some tectal cells weakly responded to the onset of the 488nm laser light. We thus discarded data collected during the first 5-s period after the laser onset.

Visual stimulation

Custom-made software (written in Matlab and psychtool-box^{33,34}) were used to drive light bars to move in various directions (constant speed 65° s^{-1} , duration 1 s), and whole-field looming or flashing lights of various durations. Standard CS consisted of 20 repetitions with regular ISIs, defined as the time between the offset of the previous and the onset of the next stimulus. Visual stimuli were applied using a $14 \times 9 \text{ mm}$ LCD screen, with green light filtered (by Kodak-32) to avoid interference with the Ca^{2+} dye signal. The larvae were mounted dorsal-side up on the edge of a platform in an E3 solution-filled chamber, allowing an unobstructed view of the screen. The screen (covering $90^\circ \times 65^\circ$ of the visual field) was centred around the eye of the larvae, contra-lateral to the dye-loaded tectum and positioned 7mm from the eye—a distance that allows both the positioning of the objective above the tectum, and the proper focusing of the stimulus on the retina³⁵.

Ca^{2+} imaging analysis

A series of images obtained for each trial were aligned to compensate for drifts in the xy plane, by minimizing the mean square difference of intensities between the first frame and the rest of the data set, using spline processing (TurboReg³⁶). Data showing drifts in the z plane were discarded. Regions of interest (ROIs) corresponding to each of the imaged tectal neurons were manually marked on the average image calculated from the entire series, and the averaged pixel intensity within ROIs was calculated. The change in intensity of each ROI was calculated as

$\Delta I = \frac{I - I(t)_{\text{base}}}{I(t)_{\text{base}}}$, in which I is the average intensity of the ROI and I_{base} is a polynomial function of second or third degree fitted to the whole trace, demarking the trace's baseline. The polynomial function also served to correct for slight baseline changes due to photobleaching. A Ca^{2+} transient was considered an activity event when it surpassed a level of 1.5 s.d. above the baseline average and had a typical profile of fast rise and slow decay (as determined by visual inspection). Cells showing typical glial morphology (with triangular soma and long thick projections) or Ca^{2+} transients of prolonged decay time ($>5 \text{ s}$) were excluded from further analysis. A 'synchronous Ca^{2+} event' among neurons in the ensemble is considered to have occurred when the onset histograms of Ca^{2+} transients (Fig. 2d) showed a peak that surpassed the threshold of 1 s.d. above the average. A given cell (or ensemble) was considered as showing 'entrained rhythmic activities' when at least one Ca^{2+} transient (or synchronous Ca^{2+} event) fell within $\pm 0.5 \text{ s}$ around the multiples of the ISI of the CS.

Behavioural assay

Wild-type and nacre larvae (7–14 d.p.f.) were embedded in agarose and submerged in E3 medium in the recording chamber. The agarose around the tail was removed to allow escape/swimming behaviours with kinematics similar to those of free behaviour³⁷. Only larvae showing low frequency of spontaneous motor behaviours and reliable visuomotor behaviour ($>50\%$ success rate) without habituation were used (Supplementary Movie 1 and ref. 38). Experiments were performed under low ambient light and filmed at 60Hz. Whole-field light flashes (duration 200 ms) were used as the CS instead of moving-bar stimuli because they more reliably elicited visuomotor responses. To determine the onset and duration of the tail flip, we fitted a backbone curve along the midline of the larva's tail and calculated the absolute value of the average time derivative of its curvature (the derivative of the tangent angle with respect to arc length) for all time frames. Subthreshold stimulation was defined as the maximal light intensity that consistently failed to evoke motor responses. This intensity varied between larvae and was determined 5 min before the experiment.

Simultaneous recording of Ca²⁺ dynamics and motor behaviour

Zebrafish larvae (7–15 d.p.f.) were restrained in 2% agarose except for the last third of the tail. The larva was then placed under an upright confocal microscope and illuminated with infrared LEDs. Larva behaviour was monitored with a $\times 20$ custom-made mini-microscope (connected to a video camera and shielded with a 488 nm notch filter; Supplementary Fig. 7). Synchronized acquisition of both fluorescence and bright-field images was done by custom software (Matlab). Data showing movement artefacts were discarded. Synchronous Ca²⁺ events (see definition described previously) and tail-flip behaviours were considered correlated if the synchronous Ca²⁺ event fell within a 1-s time window (± 0.5 s around the onset time of the tail flip).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank A. Kampff, F. Engert, Y. Fu and S. Smith for their help with two-photon microscopy, C. Niell for advice on the zebrafish preparation, N. Farchi, Y. Loewenstein, B. Hochner, G. de Polavieja and A. Noe for comments on the manuscript, and A. Arrenberg, B. Barak, V. Yoon, R. Chen and J. Sumbre for their technical help. This work was supported by the US National Institutes of Health.

References

- Gibbon J, Malapani C, Dale CL, Gallistel CR. Toward a neurobiology of temporal cognition: Advances and challenges. *Curr. Opin. Neurobiol* 1997;7:170–184. [PubMed: 9142762]
- Buhusi CV, Meck WH. What makes us tick? Functional and neural mechanisms of interval timing. *Nature Rev. Neurosci* 2005;6:755–765. [PubMed: 16163383]
- Buonomano DV. The biology of time across different scales. *Nature Chem. Biol* 2007;3:594–597. [PubMed: 17876310]
- Saigusa T, Tero A, Nakagaki T, Kuramoto Y. Amoebae anticipate periodic events. *Phys. Rev. Lett* 2008;100:018101. [PubMed: 18232821]
- Carr CE. Processing of temporal information in the brain. *Annu. Rev. Neurosci* 1993;16:223–243. [PubMed: 8460892]
- Buonomano DV. Timing of neural responses in cortical organotypic slices. *Proc. Natl Acad. Sci. USA* 2003;100:4897–4902. [PubMed: 12668762]
- Buonomano DV, Karmarkar UR. How do we tell time? *Neuroscientist* 2002;8:42–51. [PubMed: 11843098]
- Rainer G, Rao SC, Miller EK. Prospective coding for objects in primate prefrontal cortex. *J. Neurosci* 1999;19:5493–5505. [PubMed: 10377358]
- Quintana J, Fuster JM. From perception to action: temporal integrative functions of prefrontal and parietal neurons. *Cereb. Cortex* 1999;9:213–221. [PubMed: 10355901]
- Brody CD, Hernandez A, Zainos A, Romo R. Timing and neural encoding of somatosensory parametric working memory in macaque prefrontal cortex. *Cereb. Cortex* 2003;13:1196–1207. [PubMed: 14576211]
- Leon MI, Shadlen MN. Representation of time by neurons in the posterior parietal cortex of the macaque. *Neuron* 2003;38:317–327. [PubMed: 12718864]
- Komura Y, et al. Retrospective and prospective coding for predicted reward in the sensory thalamus. *Nature* 2001;412:546–549. [PubMed: 11484055]
- Meek J. Functional anatomy of the tectum mesencephali of the goldfish. An explorative analysis of the functional implications of the laminar structural organization of the tectum. *Brain Res* 1983;287:247–297. [PubMed: 6362772]
- Vanegas H, Ito H. Morphological aspects of the teleostean visual system: a review. *Brain Res* 1983;287:117–137. [PubMed: 6315186]

15. Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. *Science* 1990;248:73–76. [PubMed: 2321027]
16. Stosiek C, Garaschuk O, Holthoff K, Konnerth A. *In vivo* two-photon calcium imaging of neuronal networks. *Proc. Natl Acad. Sci. USA* 2003;100:7319–7324. [PubMed: 12777621]
17. Niell CM, Smith SJ. Functional imaging reveals rapid development of visual response properties in the zebrafish tectum. *Neuron* 2005;45:941–951. [PubMed: 15797554]
18. McLean DL, Fan J, Higashijima S, Hale ME, Fetcho JR. Atopographic map of recruitment in spinal cord. *Nature* 2007;446:71–75. [PubMed: 17330042]
19. Ramdya P, Reiter B, Engert F. Reverse correlation of rapid calcium signals in the zebrafish optic tectum *in vivo*. *J. Neurosci. Methods* 2006;157:230–237. [PubMed: 16765450]
20. Yaksi E, Friedrich RW. Reconstruction of firing rate changes across neuronal populations by temporally deconvolved Ca^{2+} imaging. *Nature Methods* 2006;3:377–383. [PubMed: 16628208]
21. Nakai J, Ohkura M, Imoto K. A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nature Biotechnol* 2001;19:137–141. [PubMed: 11175727]
22. Gahtan E, Tanger P, Baier H. Visual prey capture in larval zebrafish is controlled by identified reticulospinal neurons downstream of the tectum. *J. Neurosci* 2005;25:9294–9303. [PubMed: 16207889]
23. Sato T, Hamaoka T, Aizawa H, Hosoya T, Okamoto H. Genetic single-cell mosaic analysis implicates ephrinB2 reverse signaling in projections from the posterior tectum to the hindbrain in zebrafish. *J. Neurosci* 2007;27:5271–5279. [PubMed: 17507550]
24. Bullock TH, Hofmann MH, New JG, Nahm FK. Dynamic properties of visual evoked potentials in the tectum of cartilaginous and bony fishes, with neuroethological implications. *J. Exp. Zool* 1990;256:142–155.
25. Bullock TH, Hofmann MH, Nahm FK, New JG, Precht JC. Event-related potentials in the retina and optic tectum of fish. *J. Neurophysiol* 1990;64:903–914. [PubMed: 2230933]
26. Bullock TH, Karamursel S, Hofmann MH. Interval-specific event related potentials to omitted stimuli in the electrosensory pathway in elasmobranchs: an elementary form of expectation. *J. Comp. Physiol. [A]* 1993;172:501–510.
27. Bullock TH, Karamursel S, Achimowicz JZ, McClune MC, Basar-Eroglu C. Dynamic properties of human visual evoked and omitted stimulus potentials. *Electroencephalogr. Clin. Neurophysiol* 1994;91:42–53. [PubMed: 7517843]
28. Schwartz G, Harris R, Shrom D, Berry MJ II. Detection and prediction of periodic patterns by the retina. *Nature Neurosci* 2007;10:552–554. [PubMed: 17450138]
29. Lister JA, Robertson CP, Lepage T, Johnson SL, Raible DW. nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* 1999;126:3757–3767. [PubMed: 10433906]
30. O'Malley DM, et al. Optical physiology and locomotor behaviors of wild-type and nacre zebrafish. *Methods Cell Biol* 2004;76:261–284. [PubMed: 15602880]
31. Westerfield, M. *The Zebrafish Book; A Guide for the Laboratory Use of Zebrafish (Danio rerio)*. Eugene, Univ. Oregon Press; 2000.
32. Brustein E, Marandi N, Kovalchuk Y, Drapeau P, Konnerth A. “*In vivo*” monitoring of neuronal network activity in zebrafish by two-photon Ca^{2+} imaging. *Pflugers Arch* 2003;446:766–773. [PubMed: 12883893]
33. Brainard DH. The psychophysics toolbox. *Spat. Vis* 1997;10:433–436. [PubMed: 9176952]
34. Pelli DG. The Video Toolbox software for visual psychophysics: transforming numbers into movies. *Spat. Vis* 1997;10:437–442. [PubMed: 9176953]
35. Easter SS Jr, Nicola GN. The development of vision in the zebrafish (*Danio rerio*). *Dev. Biol* 1996;180:646–663. [PubMed: 8954734]
36. Thévenaz P, Ruttimann UE, Unser M. A pyramid approach to subpixel registration based on intensity. *IEEE Trans. Image Process* 1998;7:27–41. [PubMed: 18267377]
37. Ritter DA, Bhatt DH, Fetcho JR. *In vivo* imaging of zebrafish reveals differences in the spinal networks for escape and swimming movements. *J. Neurosci* 2001;21:8956–8965. [PubMed: 11698606]

38. Burgess HA, Granato M. Modulation of locomotor activity in larval zebrafish during light adaptation. *J. Exp. Biol* 2007;210:2526–2539. [PubMed: 17601957]

Figure 1. Visual-stimulation-evoked Ca^{2+} transients in an ensemble of tectal neurons

a, An optical section of zebrafish larva tectum (6 d.p.f.), labelled with Ca^{2+} indicator and imaged using a two-photon microscope. np, neuropil; pvl, periventricular layer; scale bar, 20 μm . **b**, Five example neurons showing evoked Ca^{2+} transients in response to 20 repetitions of the moving bar (vertical grey bars) at an ISI of 6 s. Scale bar, 0.5 (change in baseline fluorescence, $\Delta F/F_0$). **c**, Raster plot of Ca^{2+} transients observed in 186 tectal neurons evoked by the moving bar at an ISI of 6 s. Grey, caudo-rostral (C-R); black, rostro-caudal (R-C). Neurons are sorted and numbered by their average evoked responses to the rostro-caudal stimulation. Grey scale represents the amplitude of Ca^{2+} transients in $\Delta F/F_0$. Scale bar, 20 s.

Figure 2. Repetitive conditioning stimulation induces post-CS rhythmic Ca^{2+} transients

a, Raster plot of Ca^{2+} transients for 126 tectal neurons before, during and after CS (20 moving bar stimuli, 6-s ISI). Note that after the CS, synchronous activity was observed at a regular interval corresponding to the ISI of the CS (arrows) for a subset of neurons (arranged as 1 to 89). **b**, The average of all Ca^{2+} transients in **a**. The green bar denotes the moving bar stimulus; the blue bar represents the expected time corresponding to the ISI (6 s) of the CS should the CS start earlier or continue for further cycles. **c**, The onset time of Ca^{2+} transients for the data shown in **a**. **d**, Onset time histograms for data shown in **c**. The shaded area represents the CS period. **e**, Examples of Ca^{2+} transients evoked by the last five conditioning stimuli (ISIs of 4, 6 or 10 s; three neurons each), and observed during the post-CS period. Vertical scales ($\Delta F/F_0$)

$F_0 = 0.3$. **f**, Histograms of inter-event intervals for synchronous Ca^{2+} events (see Methods for definition) in the absence of any sensory stimulation ('spontaneous', total time 130.5 min, from 35 trials), and for synchronous Ca^{2+} events during the first 30 s after CS at 4-, 6- or 10-s ISIs ($n = 16, 38$ or 17 , respectively). Arrows represent the CS ISI.

Figure 3. Rhythmic activities of neuronal ensembles are stimulus-specific

The onset time of Ca^{2+} transients in an ensemble of 105 tectal neurons in response to a CS of 20 caudo-rostral (grey) and 20 rostro-caudal (black) moving bars, with neurons showing rhythmic activities entrained by the caudo-rostral CS (sorted as 1–65) and those un-responsive to the caudo-rostral CS (as 91–105). The two CS trials were separated by 5 min (marked by '//'). The bottom histograms represent the onset times of the Ca^{2+} transients shown at the top. Arrows depict the post-CS rhythmic activities.

Figure 4. Repetitive visual CS induces post-CS rhythmic motor behaviour

a, Images depict the tail-flip behaviour of a head-immobilized zebrafish larva, evoked by a light flash (duration 0.2 s). Red dashed line represents the tail backbone curve; blue dashed line represents the contour of the restraining agarose. Scale bar, 1 mm. **b**, The absolute value of the average time (t) derivative of the tail backbone curvature, calculated from the images in **a**. Green bar represents the stimulus duration. **c**, Data from one example experiment before, during and after visual stimulation (20 flashes, 6-s ISI). Shaded area shows the CS period; arrows represent onset of the post-CS tail flips. Green lines show the CS ISI; blue lines denote the entrained interval time. **d**, Tail flips triggered by the last four CS stimuli of 4-, 6- or 10-s ISIs, and post-CS tail flips in the absence of sensory stimulation. Scale, 4 s. Arrowheads show

tail flips initiated shortly before the stimulus. **e**, Histograms of inter-tail-flip event intervals in the absence of sensory stimulation ('spontaneous', total time 30 min, from 15 trials), and for tail-flip events during the first 30 s after CS at 4-, 6- or 10-s ISIs ($n = 7, 8$ and 5 , respectively). Arrowheads represent the CS ISI and multiples. **f**, The percentage of tail flips that correlated with synchronous Ca^{2+} events (yellow) and the percentage of synchronous Ca^{2+} events that correlated with tail flips (magenta) during the period corresponding to 60-s pre-CS, CS, and 0–30-s (or 31–60-s) post-CS ($n = 10, 8$ larvae). Percentages for the post-CS 0–30-s group were significantly different from those of the corresponding pre-CS and post-CS 31–60-s groups ($P < 10^{-3}$, non-parametric analysis of variance (ANOVA); $**P < 0.01$, Kolmogorov–Smirnov tests), but not from those of the CS group. **g**, The mean Ca^{2+} transients during the first 30-s post-CS period (normalized by the mean CS value), for all cases associated or not associated with post-CS rhythmic tail flips ($**P = 0.02$, Kolmogorov–Smirnov test, $n = 10$ trials, 8 larvae). Error bars, s.d.