A virtual reality system to analyze neural activity and behavior in adult zebrafish

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Virtual realities are powerful tools to analyze and manipulate interactions between animals and their environment and to enable measurements of neuronal activity during behavior. In many species, however, optical access to the brain and/or the behavioral repertoire are limited. We developed a high-resolution virtual reality for head-restrained adult zebrafish, which exhibit cognitive behaviors not shown by larvae. We noninvasively measured activity throughout the dorsal telencephalon by multiphoton calcium imaging. Fish in the virtual reality showed regular swimming patterns and were attracted to animations of conspecifics. Manipulations of visuo-motor feedback revealed neurons that responded selectively to the mismatch between the expected and the actual visual consequences of motor output. Such error signals were prominent in multiple telencephalic areas, consistent with models of predictive processing. A virtual reality system for adult zebrafish therefore provides opportunities to analyze neuronal processing mechanisms underlying higher brain functions including decision making, associative learning, and social interactions.
Head fixation of adult zebrafish and closed-loop VR. a, Attachment sites of L-shaped bars for head fixation. Tel, telencephalon; TeO, optic tectum; CB, cerebellum. b, Photograph of dissected set of stable skull bones. Red shading indicates attachment sites. Scale bar, 1mm. c, Top, schematic of VR projected by three projectors onto a panoramic screen with a 180° field of view. Arrow depicts head-fixed fish. Bottom, dorsal view of head-fixed fish. Scale bar, 5mm. d, Synchronization of closed-loop VR and two-photon imaging. The PMT and the LEDs of the projector are gated in a nonoverlapping manner using the line clock of the 8 kHz resonant scanner (RS) as a trigger. BF, back-projection film; BP, band-pass filter; CAM, camera; CL, collimating lens; DM, dichroic mirror; IR, infrared light source; LP, long pass filter; M, mirror; NF, notch filter; OL, objective lens; PJ, projector; SL, scan lens; SP, short-pass filter; Ti:Sa, titanium–sapphire laser; TL, tube lens. e, Back-projection of the VR onto a semihexagonal tank under the microscope (photograph of setup). Scale bar, 5cm. f, Three-dimensional model of the VR consisting of a cylindrical arena, a flat floor and two tunnels (top). Walls were textured with naturalistic rocks and plants (bottom). A set of three virtual cameras together capture a 180° field of view (green shading). An invisible collision wall (dashed line) kept virtual cameras at a distance from the VR boundaries to prevent pixelation of the texture. g, Algorithm to update the virtual cameras based on tail movements of head-fixed animals. Forward movement in the VR (green) is triggered by the zero crossings of the caudal tail curvature (black). Angular movement in the VR (red) is proportional to the caudal tail curvature after low-pass filtering. Numbers show total forward movement (10.2 and 2.8 mm) and rotation (0.01° and 41.1°) in the examples shown at default gain settings.

It is not possible to reproduce the exact kinematics of natural motor behavior in the VR because head fixation results in reactive forces on the body that do not occur during free swimming. Moreover, virtual swimming was restricted to a horizontal plane and fish received no vestibular feedback. Our goal was therefore to establish conditions in which fish swim spontaneously in the VR and show specific behavioral responses to sensory stimuli. When we decoupled the VR from motor output (open-loop), head-fixed fish often showed irregular, high-amplitude tail beats (struggling) and subsequently entered a prolonged period of inactivity. In closed-loop, we initialized gains to default values and fine-adjusted them subsequently entered a prolonged period of inactivity. In closed-loop, we initialized gains to default values and fine-adjusted them for each fish to minimize struggling and inactivity (Supplementary Fig. 3). Under these conditions, fish performed repetitive swims over long periods of time, resembling the discrete ‘bout-and-glide’ swimming pattern of unrestrained zebrafish (Supplementary Figs. 4 and 5).

Behavioral responses to naturalistic visual stimuli. We next examined behavioral responses to visual stimuli. Freely swimming adult zebrafish are visually attracted to conspecifics behind a transparent divider or to images and movies of other zebrafish. We tracked freely swimming adult fish in a rectangular tank while we presented visual stimuli behind the opposing small walls for 20s. One stimulus was a movie of a shoal of three adult zebrafish in a tank whereas the other stimulus was a static image of the empty tank (Supplementary Fig. 6a). During stimulus presentation, fish spent more time near the movie of the shoal, as visualized by probability maps (Supplementary Fig. 6b, n = 64 fish). Quantitative
Head-fixed fish in the VR in the absence of stimulus presentations frequently circled the environment, similar to the spontaneous behavior of unrestrained fish. When we presented stimuli in the two tunnels (Fig. 2a, duration 40–300 s), fish frequently stopped circling when they encountered the tunnel showing the shoal and performed persistent directional swims toward the shoal (Fig. 2b–d and Supplementary Video 1). We did not observe this behavior when fish encountered the tunnel showing the empty tank. The fraction of time spent close to the stimulus was higher when the shoal was present (Fig. 2c; 16.7% versus 10.4%; P = 0.038, Wilcoxon rank sum test) and the SPI was significantly biased toward the shoal (Fig. 2d; P = 0.0096, t-test, n = 46 fish). These results demonstrate that visual attraction to movies of conspecifics was retained in the VR.

When we replaced each of the three fish in the movie by a vertical black ellipse of matching size that followed the same motion patterns, fish occasionally also started circling in the VR when they encountered the movie (Fig. 2e). However, this behavior was rare and circling usually resumed during movie presentation. The fraction of time spent close to the movie was not significantly different from the no-movie condition, and we did not observe a significant change in the SPI (Fig. 2f, fraction of time near movie = 7.8%, P = 0.36, Wilcoxon rank sum test; SPI: P = 0.81, t-test, n = 46 fish). Nevertheless, the heading direction was significantly biased toward the movie of ellipses (Fig. 2g, P < 0.001, Kuiper’s test, n = 3091 orientation measures) and ellipses (P < 0.001, Kuiper’s test, n = 1201 orientation measures). Body orientation was measured every 30 s. Radial unit represents 2.5% of total recording time.

Analysis of a side preference index (SPI) confirmed that fish spent significantly more time on the side where the movie was presented (Supplementary Fig. 6c, P = 10−10, paired t-test versus premovie period, n = 64 fish). Hence, freely swimming fish were attracted to movies of conspecifics, consistent with previous results.

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**Fig. 3** | Quantification of brain motion during motor output. **a.** Simultaneous recording of neuronal activity and behavior in Tg(neuroD:GCaMP6f) fish. Left, time-averaged raw fluorescence (top; t-avg) and maximum intensity projection of mean-subtracted image series, revealing active neurons (bottom). Right, traces of calcium signals (ΔF/F) from individual neurons (green, numbers indicate neurons in the maximal projection image) together with the simultaneously recorded tail movement intensity (black). Experiments with similar results were performed in 26 fish. Scale bar, 50 μm. **b.** Time-averaged fluorescent image of GFP-expressing interneurons acquired in an adult Tg(SAGFF212C:Gal4), Tg(UAS:ArchT-GFP) zebrafish during behavior (30 s, single focal plane). Scale bar, 20 μm. **c-g.** Measurements of brain motion and tail movement. **c.** Image motion in the field of view in red in x (medial-lateral direction) and y (rostro-caudal direction) during tail movements (black). Positive values of x and y correspond to leftward and forward motion, respectively. The recording was performed in the dark. An asterisk denotes an episode of U-shaped tail bends (at approximately 230 s). Image motion was measured by cross correlation of individual, raw images to the mean of a stable image series. The resolution of the measurement is 0.82 μm in x and 0.32 μm in y. **d-g.** Selected single frames of a two-photon imaging time series (33 ms per frame; top, raw data; scale bar, 20 μm, Supplementary Video 2) and the simultaneously recorded video frames of the tail (bottom; scale bar, 1 cm). Gray dashed lines indicate time in c of the respective frames. Similar results were obtained in two fish. **h.** Mean image displacement in x and y aligned on swim offset. Shading indicates s.e.m. (n = 40 swim events). **i.** Image displacement in x and y aligned on swim offset. **j.** Fraction of neurons that could be continuously identified as a function of recording time (manual tracking of 224 neurons in ten fields of view from eight fish). Box plots show median, 25th and 75th percentiles. Dots and error bars show mean ± s.d.

**Imaging neuronal population activity during behavior.** To explore in vivo imaging in adult zebrafish we measured point spread functions and imaged neurons using a two-photon microscope. Although the skull influenced optical resolution (Supplementary Fig. 7), we could resolve individual somata and neurites up to a depth of >200 μm below the brain surface (not including the thickness of the skull; Supplementary Figs. 8 and 9), which comprises roughly 30% of the pallium. To measure neuronal activity in the dorsal telencephalon we performed two-photon calcium imaging in Tg(neuroD:GCaMP6f) fish. Photons of the VR were temporally segregated from fluorescence measurements by exclusive gating of the photomultiplier tube (PMT) (Fig. 1d). Maximum projections of image series (8,000 frames at 30 Hz) along the time axis usually revealed no obvious blurring (Fig. 3a), indicating that motion artifacts were negligible. In addition, we analyzed image motion in Tg(SAGFF212C:Gal4), Tg(UAS:ArchT-GFP) fish, which express the fluorescent transmembrane protein ArchT-GFP in sparsely distributed telencephalic neurons (Supplementary Fig. 10). We acquired image series when the VR was off and fish showed episodes of struggling that were longer and more vigorous than regular swim events (Supplementary Fig. 11). Nonetheless, image shifts were small (usually <0.3 μm, rarely >1 μm) and reversible after cessation of tail movements (Fig. 3b–i and Supplementary Video 2). To assess long-term stability, we manually tracked individual somata and found that 67 ± 8% of somata could be identified throughout image series of 30 min (n = 224 neurons from eight fish, Fig. 3i). A linear fit yielded a median half-life of 49 min for individual neurons without adjustments of the focal plane.

We measured neuronal activity in different fields of view in the dorsal telencephalon of individual Tg(neuroD:GCaMP6f) fish (Fig. 4a,b and Supplementary Fig. 9; first 200 μm; seven image series with a total of 54,000 frames per field of view).
We distinguished four telencephalic areas: (1) Dm (medial zone of the dorsal telencephalon, likely to be homologous to the basolateral amygdala and related areas38), (2) the dorsorostral (rDc) and (3) dorsocaudal (cDc) parts of area Dc (central zone of the dorsal telencephalon, presumably related to the isocortex33,34) and (4) the dorsal part of DI (lateral zone of the dorsal telencephalon, potentially related to the hippocampus34). During spontaneous swimming in the VR, individual neurons in all brain areas showed sparse and discrete fluorescence transients. We first highlighted active neurons by a maximum projection of pixel intensities along the time axis of each image series (267 s, 8,000 frames) after subtracting the mean (Fig. 3a). This analysis revealed a region in DI with a high density of active neurons (Fig. 4b). Consistent with this observation, spontaneous neuronal activity was significantly higher in DI than in other regions (P < 0.001; two-sided t-test, n = 26 fish, Fig. 4d).

To determine whether activity of individual neurons was modulated by swimming we first estimated the fluctuations in action potential probability underlying the observed somatic calcium transients using a convolutional neural network (Elephant)42 (Fig. 4c). We then calculated the difference in action potential probability ΔP in 0.5 s time windows before and after the onset of swim bouts for individual neurons. As a control, we triggered the same analysis on random time points. Neurons were considered swim-modulated when the distribution of ΔP was significantly different between swim-triggered and randomly triggered conditions (one-sided t-test with P < 0.05, Fig. 4e). The probability of observing swim-modulated activity was higher in cDc (9.4%, 158/1675 neurons) and rDc (7.9%, 233/2941) than in DI (5.5%, 438/7967) or Dm (3.7%, 275/7449, Fig. 4f).

**Analysis of predictive processing in the VR.** We exploited the VR to analyze predictive neural processing in zebrafish by manipulating sensory-motor feedback. The theoretical framework of predictive processing assumes that brains generate internal models of the world and suppress responses to predictable sensory inputs through top-down projections. As a consequence, bottom-up projections transmit error signals, rather than primary sensory information, and these error signals are used to update the internal model30,31. This process has been proposed to allow for experience-dependent learning of structures in the world and to drive not only sensory-motor behavior but also cognitive processes such as the inference of the mental states and actions of other individuals41. A key prediction of classical predictive processing models is that unexpected sensory inputs elicit neuronal responses that cannot be explained by sensory inputs or motor output alone but encode the mismatch between the expected and the actual sensory input. Error signals consistent with this assumption have been described in various vertebrate brain areas32,44–46. However, detailed characterizations of these error signals and the underlying circuitry are rare47.
We generated brief episodes of visuomotor mismatch by introducing left-right reversals to the angular update of the VR for 5–10 s without modifying forward swimming (Fig. 5a and Supplementary Video 3; mean duration 7.5 s and mean interevent interval 5 min). Hence, the VR rotated opposite to the expected direction during swim bouts. These perturbations frequently triggered a distinct sequence of motor outputs with a variable delay (Supplementary Video 4 and Supplementary Figs. 11 and 12). Behavioral responses typically started with 1–5 unilateral bends of the caudal tail with increasing frequency and amplitude that resembled J-turns, a motor motif that rotates the body axis. These movements were followed by vigorous and irregular movements of the entire tail. Using these motor features, we manually annotated behavioral responses to perturbation (BREPs) in each trial (Fig. 5b). We then divided the time after perturbation onset into two phases: the pre-BREP period, corresponding to the delay between perturbation onset and the BREP, and the BREP period, corresponding to the time between BREP onset and the end of the 10 s analysis time window.

The duration of the pre-BREP period varied substantially between trials (Supplementary Fig. 12; median = 2.1 s and 25th–75th percentile = 1.2–4.1 s). During this period, animals continued to perform short periodic swims (Fig. 5c) with an intensity and temporal structure that were not significantly different from swims during normal visuomotor coupling (Fig. 5d, pairwise t-test, P = 0.07). Tail movement intensity during the subsequent BREP period, in contrast, was significantly higher than the intensity during normal visuomotor coupling (Fig. 5d; pairwise t-test against normal coupling, P < 0.001, n = 26 animals; Supplementary Fig. 11). During the pre-BREP period, fish therefore received unexpected visual input without an obvious change in motor output.

We measured neuronal activity during brief VR perturbations in 2–8 fields of view per fish to sample activity in Dm, cDc, rDc and DI (1–7 perturbations per field of view). We detected a significant response to the perturbation in 882 out of 7,090 neuron-perturbation pairs (12.2%) from 26 animals (Dm 9.2%, 233/2520 neuron-perturbation pairs; cDc 15.0%, 121/805; rDc 19.4%, 171/882; DI 12.4%, 357/2883; see Methods for the definition of responses). To further characterize this activity, we first clustered the 882 responses based on their temporal activity profile and found that the average activity increased before the onset of the BREP in five out of 11 clusters (Supplementary Fig. 13). Hence, a substantial fraction of neuronal responses preceded the BREP.

Analyses of individual neuronal responses confirmed this observation (Fig. 6a). To compare these responses to activity related to spontaneous swims, we identified swim events similar to BREPs during normal visuomotor coupling (‘spontaneous strong swims’; swim duration >2 s). Across fish, the probability of detecting a response following the onset of a strong swim was 2.5 ± 0.6% (mean ± s.e.m., n = 26 fish; total of 64,600 neuron-swim pairs) and not significantly different from the probability of observing a significant response around randomly chosen time points (2.6 ± 0.2%, mean ± s.e.m., n = 26 fish; total of 35,450 time points; P = 0.13, two-sided t-test). Both of these probabilities were significantly lower than the probability of observing a response following a VR perturbation (13.4 ± 1.1%, mean ± s.e.m., n = 26 fish; total of 7,090 neuron-perturbation pairs; P = 10−14 and 10−14, two-sided t-test; Fig. 6b). VR perturbations therefore evoked neuronal responses that are unlikely to reflect only enhanced motor output.

For further analyses we divided responses into two subsets depending on whether their onset occurred before the transition in motor behavior (pre-BREP responses, n = 382) or thereafter (BREP responses, n = 500; Supplementary Fig. 13). During normal visuomotor coupling, activity was modulated by swimming in 16.5% (63/382) of the pre-BREP responses but only in 2.8% (14/500) of the BREP responses. Hence, the probability of swim modulation was substantially higher in pre-BREP responses than across all responses to VR perturbations (5.6%, P < 0.001, χ² test). Consistent with this observation, the mean activity increased sharply at the onset of swim bouts in pre-BREP responses but not in BREP responses (Supplementary Fig. 13).
The modulation of pre-BREP responses by swimming may reflect sensory responses to visual input or motor-related activity. Alternatively, pre-BREP responses may represent an error signal that arises from a mismatch between the expectation of the animal and the actual sensory feedback. To distinguish between these possibilities, we identified pre-BREP responses that were swim-modulated (n=63) and compared the swim-triggered average action potential probability during two conditions: (1) spontaneous swims during normal visuomotor coupling, and (2) swims during the pre-BREP period. The swim-modulated activity was substantially higher during the pre-BREP period (Fig. 6c) while the intensity and temporal structure of motor output were indistinguishable from spontaneous swims during normal visuomotor coupling (Fig. 5d). Hence, enhanced activity during the pre-BREP period cannot be explained by changes in motor output or visual flow amplitude, indicating that it was caused by the unexpected direction of visual flow during active swimming.

We next identified neurons that responded during the pre-BREP phase when the visuomotor perturbation evoked a BREP (positive trials) and examined their activity during trials when the perturbation failed to evoke a BREP (negative trials; n=65 neurons). Neurons showed a strong increase in activity in positive trials but not in negative trials (Fig. 6d,e; pairwise t-test, P<0.001). The activity in positive trials increased before the onset of a BREP (Fig. 6d) and predicted the occurrence of the BREP (Supplementary Fig. 13f), consistent with the hypothesis that responses during the pre-BREP phase represent a behaviorally relevant prediction error.

In all telencephalic areas examined, changes in neuronal activity occurred more frequently during the pre-BREP or BREP phase (Fig. 6f) than at randomly chosen time points (Supplementary Fig. 14). Pre-BREP responses were more abundant in rDc than in other brain areas, while BREP responses were more abundant in both rDc and cDc (Fig. 6f). These results demonstrate that a substantial fraction of telencephalic neurons respond to a mismatch between expectation and sensory feedback, thus representing a sensory-motor prediction error.
Discussion
We established a closed-loop VR system for optical measurements of neuronal activity during behavior in head-fixed adult zebrafish. Traditionally, higher brain functions of vertebrates are studied in rodents and primates but the large size of the brain severely constrains the fraction of optically accessible neurons in these species. Large-scale imaging of neuronal activity is possible in larval zebrafish but their potential to study associative learning, social interactions or cognitive behaviors is limited. Other small vertebrates such as *D. translucida* are not well established as animal models. We therefore focused on adult zebrafish, which exhibit a rich repertoire of behaviors and allow for imaging of neuronal population activity throughout the dorsal pallium with single-neuron resolution. We measured activity across brain areas that are difficult to target simultaneously in mammals, including the proposed homologs of the basolateral amygdala (Dm), of isocortex (Dc), and possibly of part of the hippocampus (Dl). Conceivably, noninvasive optical access to deeper structures could be achieved using adaptive optics or three-photon imaging. Hence, activity measurements in behaving adult zebrafish offer opportunities to analyze neuronal population activity during complex behaviors. Moreover, the zebrafish brain offers exceptional opportunities to study the ontogeny and maturation of neural circuit functions in a vertebrate.

Large-scale optical measurements of neuronal activity have recently been achieved in freely moving larval zebrafish using a specialized tracking technique but this approach cannot easily be scaled to adults. Juvenile and adult zebrafish can be examined in a VR designed for freely moving animals but this method does not permit simultaneous activity measurements. A VR for head-fixed zebrafish cannot fully emulate physical reality and principal constraints restrict the potential for kinematic analyses of motor output. Nonetheless, swimming behavior of head-fixed adult zebrafish in the closed-loop VR resembled free swimming in the home tank and fish showed differential and directional behavioral responses to visual stimuli. Our approach therefore enables measurements and manipulations of neuronal population activity in adult zebrafish during complex behaviors.

We briefly and specifically manipulated interactions of animals with their environment to test the hypothesis that telencephalic neurons represent sensory-motor prediction errors, which are key elements in models of predictive processing. Prominent error signals were observed in multiple areas of the dorsal telencephalon, indicating that error detection is a prominent computation in the telost brain. It is currently unknown how and where these error signals are computed, and how they are related to alertness or arousal. Our results provide a starting point to address these questions by dissecting the mechanisms of error detection at the level of individual neurons and circuits.

Online content
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References
1. Holscher, C. Rats are able to navigate in virtual environments. *J. Exp. Biol.* 208, 561–569 (2005).
2. Harvey, C. D., Collman, F., Dombreck, D. A. & Tank, D. W. Intracellulardynamics of hippocampal place cells during virtual navigation. *Nature* 461, 941–946 (2009).
3. Minderer, M., Harvey, C. D., Donato, F. & Moser, E. I. Virtual reality explored. *Nature* 533, 324–325 (2016).
4. Reiser, M. B. & Dickinson, M. H. A modular display system for insect behavioral neuroscience. *J. Neurosci. Methods* 167, 127–139 (2008).
5. Kim, S. S., Rouault, H., Druckmann, S. & Jayaraman, V. Ring attractor dynamics in the *Drosophila* central brain. *Science* 356, 849–853 (2017).
6. Dombreck, D. A. & Reiser, M. B. Real neuroscience in virtual worlds. *Curr. Opin. Neurobiol.* 22, 3–10 (2012).
7. Larsch, J. & Baier, H. Biological motion as an innate perceptual mechanism driving social affiliation. *Curr. Biol.* 28, 3532–3534.e4 (2018).
8. Stowers, J. R. et al. Virtual reality for freely moving animals. *Nat. Methods* 14, 995–1002 (2017).
9. Keller, G. B., Bonhoeffer, T. & Hubener, M. Sensorimotor mismatch signals in primary visual cortex of the behaving mouse. *Neuron* 74, 809–815 (2012).
10. Dreosti, E., Lopes, G., Kampff, A. R. & Wilson, S. W. Development of social behavior in young zebrafish. *J. Exp. Biol.* 208, 35–44 (2005).
11. Portugues, R., Severi, K. E., Wyart, C. & Ahrens, M. B. Optogenetics in a transparent animal: circuit function in the larval zebrafish. *Curr. Opin. Neurobiol.* 23, 119–126 (2013).
12. Friedrich, R. W., Jacobson, G. A. & Zha, P. Circuit neuroscience in zebrafish. *Curr. Biol.* 20, R371–R381 (2010).
13. Muto, A., Ohkura, M., Abe, G., Nakai, J. & Kawakami, K. Real-time visualization of neuronal activity during perception. *Curr. Biol.* 23, 307–311 (2013).
14. Kim, D. H. et al. Pan-neuronal calcium imaging with cellular resolution in freely swimming zebrafish. *Nat. Methods* 14, 1107–1114 (2017).
15. Buske, C. & Gerlai, R. Shaping develops with age in Zebrafish (*Danio rerio*). *Prog. Neuropsychopharmacol. Biol. Psychiatry* 35, 1409–1415 (2011).
16. Frank, T., Mônig, N. R., Satou, C., Higashijima, S. & Friedrich, R. W. Associative conditioning remaps odor representations and modifies inhibition in a higher olfactory brain area. *Nat. Neurosci.* 22, 1844–1856 (2019).
17. Gerlach, G. & Lysiak, N. Kin recognition and inbreeding avoidance in zebrafish, *Danio rerio*, is based on phenotype matching. *Anim. Behav.* 71, 1371–1377 (2006).
18. Valente, A., Huang, K.-H., Rodrigues, R. & Engert, F. Ontogeny of classical and operant learning behaviors in zebrafish. *Learn. Mem.* 19, 170–177 (2012).
19. Minderer, M. A., Genoud, C., Masudi, T., Sikszö, L. & Friedrich, R. W. Dense EM-based reconstruction of the interglomerular projectome in the zebrafish olfactory bulb. *Nat. Neurosci.* 19, 816–825 (2016).
20. Friedrich, R. W., Genoud, C. & Wanner, A. A. Analyzing the strength and function of neuronal circuits in zebrafish. *Front. Neural Circuits* 7, 7 (2013).
21. Zhu, P., Fajardo, O., Shum, J., Zhang Schäfer, Y.-P. & Friedrich, R. W. High-resolution optical control of spatiotemporal neuronal activity patterns in zebrafish using a digital micromirror device. *Nat. Protoc.* 7, 1410–1425 (2012).
22. Collman, F., Dombreck, D. A., Khabbou, J., A. E., Adelman, T. L. & Tank, D. W. Imaging large-scale neural activity with cellular resolution in awake, mobile mice. *Neurosci. 56*, 43–57 (2007).
23. Dreosti, E., Lopes, G., Kampff, A. R. & Wilson, S. W. Development of social behavior in young zebrafish. *J. Exp. Biol.* 208, 35–44 (2005).
24. Rupprecht, P., Prendergast, A., Wyart, C. & Friedrich, R. W. Remote z-scanning with a macroscopic voice coil motor for fast 3D multiphoton laser scanning microscopy. *Biomed. Opt. Express* 7, 1656 (2016).
37. Koide, T. et al. Olfactory neural circuitry for attraction to amino acids revealed by transposon-mediated gene trap approach in zebrafish. Proc. Natl Acad. Sci. USA 106, 9884–9889 (2009).
38. Lal, P. et al. Identification of a neuronal population in the telencephalon essential for fear conditioning in zebrafish. BMC Biol. 16, 45 (2018).
39. Mueller, T., Dong, Z., Berberoglu, M. A. & Guo, S. The dorsal pallium in zebrafish, Danio rerio (Cyprinididae, Teleostei). Brain Res. 1381, 95–105 (2011).
40. Aoki, T. et al. Imaging of neural ensemble for the retrieval of a learned behavioral program. Neuron 78, 881–894 (2013).
41. Rodriguez, F. et al. Conservation of spatial memory function in the pallial forebrain of reptiles and ray-finned fishes. J. Neurosci. 22, 2894–2903 (2002).
42. Berens, P. et al. Community-based benchmarking improves spike rate inference from two-photon calcium imaging data. PLoS Comput. Biol. 14, e1006157 (2018).
43. Koster-Hale, J. & Saxe, R. Theory of mind: a neural prediction problem. Neuron 79, 836–848 (2013).
44. Blakemore, S.-J., Wolpert, D. M. & Frith, C. D. Central cancellation of self-produced tickle sensation. Nat. Neurosci. 1, 635–640 (1998).
45. Cullen, K. E. Vestibular processing during natural self-motion: implications for perception and action. Nat. Rev. Neurosci. 20, 346–363 (2019).
46. Schultz, W., Dayan, P. & Montague, P. R. A neural substrate of prediction and reward. Science 275, 1593–1599 (1997).
47. Attinger, A., Wang, B. & Keller, G. B. Visuomotor coupling shapes the functional development of mouse visual cortex. Cell 169, 1291–1302.e14 (2017).
48. McElligott, M. B. & O’Malley, D. M. Prey tracking by larval Zebrafish: axial kinematics and visual control. Brain. Behav. Evol. 66, 177–196 (2005).
49. Wang, T. et al. Three-photon imaging of mouse brain structure and function through the intact skull. Nat. Methods 15, 789–792 (2018).
50. Ji, N. Adaptive optical fluorescence microscopy. Nat. Methods 14, 374–380 (2017).

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Methods

Animal models. All experiments were performed in adult (5–21 mo-old) zebrafish (danio rerio) of both sexes. Fish were raised and kept under standard laboratory conditions (26–27 °C, 13 h/11 h light/dark cycle). All experiments were approved by the Veterinary Department of the Canton Basel-Stadt (Switzerland).

Calcium imaging was performed using mGluR6-GCaMP6f fish in a nacre background. Tg(SAGFF212C:Gal4), Tg(UAS:ArchT-GFP) fish were created by multiple crossings. Tg(SAGFF212C:Gal4), Tg(UAS:ArchT-GFP) fish were created by the gene trap method described by Asakawa et al.51. ArchT-GFP encodes a fusion protein consisting of the light-sensitive proton-pump archaerhodopsin 3 (Arch3) and green fluorescent protein (GFP). Tg(UAS:ArchT-GFP) fish were generated using the Tol2Kit52, involving a multisite recombination reaction (Invitrogen Multisite Gateway manual Version D, 2007) between p5E–UAS (5xUAS and E1b minimal promoter53), pME–ArchT-GFP (first-generation archaerhodopsin-3 fused to GFP54) and pDest20(222) as a destination vector55.

Overview of the experimental procedure. Light-weight head bars were fabricated from stainless steel syringe needles and attached to the skull of the fish using tissue glue and dental cement under anesthesia. After gluing of head bars to mounting posts, the anesthetic was removed and fish were transferred to the VR environment under the two-photon microscope. During the subsequent 20–30 min, fish were allowed to habituate to the closed-loop VR and gains were adjusted manually until the animal exhibited quasi-periodic swimming behavior. Multiple calcium imaging sessions were performed in each fish that was prepared by 15 min. Within each session, series of 54,000 frames (30 min) were acquired and subsequently divided into shorter series (usually 8,000 frames, 267 s) for analysis. The field of view was constant in each session and covered one or more forebrain regions. VR perturbations (left-right reversal) were introduced either automatically every 7 min, each lasting for 10 s, or manually every 2–7 min, each lasting 5–10 s. Between imaging sessions, a new field of view was chosen while animals continued to behave in a closed-loop VR.

Head fixation of adult zebrafish for in vivo imaging. Fish were anesthetized in 0.03% tricine methanesulfonate (MS-222), wrapped in moist tissue and placed under a dissection microscope. The head was then cut apart by 15 min. Within each session, series of 54,000 frames (30 min) were acquired and subsequently divided into shorter series (usually 8,000 frames, 267 s) for analysis. The field of view was constant in each session and covered one or more forebrain regions. VR perturbations (left-right reversal) were introduced either automatically every 7 min, each lasting for 10 s, or manually every 2–7 min, each lasting 5–10 s. Between imaging sessions, a new field of view was chosen while animals continued to behave in a closed-loop VR.

VR combined with two-photon microscopy. Adult zebrafish were head-fixed and positioned at the rear center of the water-filled VR chamber (water depth 7 cm), around 3 cm below the water surface. A x16 water-immersion objective with a working distance of 3 mm (Nikon, CF175 LWD x16 W) was used for two-photon imaging. A custom-built water-proof sleeve was allowed for immersion of objectives >3 cm below the water surface. A custom-built multichannel microscope with resonant scanners was used to acquire series of images with 512 × 512 pixels at a rate of 30 Hz using custom-written software based on Scanimage56,57. Because the PMT was gated on alternating lines, the effective resolution frame was 512 × 256 pixels at 30 Hz. Calcium imaging through the intact skull was performed using an excitation wavelength of 920 nm with a power of 50 mW at the sample. The microscope did not include compensation for group velocity dispersion or other optical components designed to optimize imaging depth.

In contrast to a head-fixed rodent, where the objective front lens can be shielded from ambient light by the skull and head plate58, a substantial number of photons from the VR entered the objective lens. We therefore temporally separated calcium imaging from the VR by gating the LEDs of the projector and the fluorescence-detecting PMT (H11706P-40, Hamamatsu) in a nonoverlapping manner. The TTL line clock of the resonant scanner (Cambridge Technology, CR8) was used as a gating signal to switch between VR illumination and fluorescence detection on alternating lines using a data acquisition board with a retriggering function (National Instruments, PCIe-6321). The projector LEDs were turned on for 24 μs during the turn-on part of the resonant scanner while the shutter circuit of the PMT was switched to low gain. To further reduce optical contaminations a notch filter (514.5/25 nm, optical density 4, Edmund Optics) was positioned in front of each projector and an additional band-pass filter (510/50 nm, Chroma) was positioned in front of the PMT. Between imaging sessions, the resonant scanner was not scanning and the projector LEDs were triggered by an 8 kHz TTL signal generated by a data acquisition board (National Instruments) to maintain the VR. The frame count of the two-photon recording (30 Hz) was stored in each video frame (30 frames per second) to ensure precise frame-to-frame mapping between behavioral recordings and activity measurements.

Behavioral analysis in head-restrained adult zebrafish. To analyze the probability of animal position in the VR, the virtual position of the animal was labeled by a...
The resulting residence probability was convolved with a normalized Gaussian mask ($\sigma = 2$ cm). The SPI of each animal was calculated from the mean of the normalized position traces along the long axis of the VR during the analysis time window (90 s). $\Delta SPI = SPI (movie versus empty tank) − SPI (empty tank versus empty tank).$ Movies of conspecifics and ellipses were presented for 40–300 s.

Intensity of tail movements was quantified by the mean of the absolute difference in pixel values between adjacent video frames. To detect swim events, tail movement intensity trace was binarized using a threshold of three standard deviations calculated from the lower half of the distribution. Binarized events separated by a duration <100 ms were fused. The onset of a swim event was defined as the onset of these binarized events. The duration of a swim event was defined as the interval between adjacent swim onsets. Each swim event thus consisted of an active period (with tail movement) followed by an inactive period (without tail movement).

The onset of BREP was determined by visual inspection of the behavioral video. Usually, a transition in motor behavior was detected because fish started to perform unilateral bends of the caudal tail followed by vigorous and often bilateral movements of the whole tail, including the rostral trunk of the body (Supplementary Video 2). In some cases, clearly we could not identify the onset or the end of a swimming event because the response was not clearly detected. In such cases, BREP onset was set to the third tail flick after the VR perturbation.

Behavioral analysis in freely swimming adult zebrafish. Responses to movies of conspecifics and abstract shapes were analyzed in 64 freely swimming adult zebrafish. The behavior of individual freely swimming fish was recorded in a rectangular tank ($L \times W \times H = 20 \times 10 \times 15$ cm$^3$, water depth 10 cm) for 1 h at 10 Hz. The long walls of the tank were made of white PVC with matt surfaces. The short walls were made of antireflection glass (LUXAR) to prevent interactions of fish with their mirror reflection. The bottom of the tank was covered with a pixel value of one on a background of zeros and the average of all recording frames was convoluted by a normalized Gaussian mask ($\sigma = 2$ cm) to generate the residence probability map.

To measure the intensity of tail movements, an image patch with the fish at the center ($100 \times 100$ pixels) was extracted from each video frame and the mean of the absolute difference in pixel values between adjacent frames was calculated. The SPI, swim event onset and swim event period were defined by the procedure described above.

Definition of brain regions in the dorsal pallium. Anatomical definitions of canonical subdivisions of the dorsal pallium in adult zebrafish vary between previous studies36–39. We adopted the definitions of Aoki et al.34 with minor modifications because this definition best matched landmarks in the fluorescence images of the dorsal telencephalon from Tg(neuroD:GCaMP6f) fish. At the end of the experiment, two-photon image stacks of the dorsal pallium were acquired in each fish and forebrain regions were delineated manually based on anatomical features. This was separated from other forebrain regions by the sulcus ypsoliformis. cDC was separated from rDC by a boundary that was visible in the NeuroD:GCaMP6f expression (Supplementary Fig. 9). This boundary appears to correspond to a boundary in parvalbumin expression40. DI was lateral to rDC at the rostral end of the forebrain but covered rDC at a more caudal position (Supplementary Fig. 9).

Postprocessing of calcium imaging data and extraction of neuronal regions of interest (ROIs). In each session, a single field of view was recorded for 30 min at 30 Hz. The 54,000 frames were then separated into six files with 8,000 frames per file (267 s) and one file with 6,000 frames. For visualization, images were smoothed by a median filter with a $150 \text{ ms}$ time span. The lower 70% of the activity trace before and after perturbation were extracted to extract relative action potential probabilities using the Elephant algorithm42 (https://git.io/vNhbsz).

Analysis of neuronal activity modulated by VR perturbation. To determine whether a neuron was responsive to the VR perturbation, the difference in $\Delta F/F$ between 10 s windows before and after perturbation onset was calculated. The observed response was compared to the response distribution triggered by 500 random time points on the same activity trace. The neuron was considered responsive in this perturbation trial when the response exceeded the mean of the randomly sampled distribution by 2 s.d. Each combination of a ROI and a VR perturbation is defined as neuron-perturbation pair. Each neuron therefore contributed $n$ neuron-perturbation pairs, where $n$ is the number of VR perturbations that were applied while imaging the neuron.

To investigate how many neurons responded during behavioral transitions in the absence of VR perturbations we identified ‘spontaneous strong swims’ (swim duration >2 s) during normal visuomotor coupling. A time interval was randomly sampled from the pre-BREP period and paired to the spontaneous strong swim to define an artificial onset. This artificial onset was used to identify responsive neurons by the procedure described above. Each combination of a ROI and a swim event is defined as neuron-swim pair. Each neuron therefore contributed $n$ neuron-swim pairs, where $n$ is the number of swims that were performed while imaging the neuron.

To determine the response onset of individual neurons (Fig. 6a) the activity trace from −10 s to $+10$ s around the perturbation onset was smoothed using a median filter with a $150 \text{ ms}$ time span. The lower 70% of the activity trace before the perturbation were used to calculate the baseline and s.d. Rising edges were detected from the post-perturbation activity trace using a threshold of 5 s.d. The first rising edge with a mean activity in the following 30 s higher than 3 s.d. was defined as the first calcium transient. The combination of criteria for height (5 s.d.) and duration (3 s.d. for 1 s) reliably separated responses from noise. From the rising edge of the response event, a retrograde search was applied for the first time point when the activity was lower than 2 s.d. This time point was set as the response onset of the neuron to this respective perturbation.

Clustering of activity traces was performed by affinity propagation11.

Statistical analysis. We tested for significant differences in the mean using a one- or two-sided $t$-test, for differences in the median using a two-sided Wilcoxon rank sum test, for differences in fractions using a $z^2$ test and for differences between angular distributions using a Kuiper’s test.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Original image series and VR presentations that support the findings of this study are too large to be included in the publication. These data are available from the corresponding author upon reasonable request.

Code availability. Software for controlling VR and coordinating VR with two-photon imaging can be downloaded at https://github.com/HUANGKUOHUA/Zebrafish-in-virtual-reality.git.

References.
51. Asakawa, K. et al. Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. Proc. Natl Acad. Sci. USA 105, 1255–1260 (2008).
52. Han, X. et al. A high-light sensitivity optical neural silencer: development and application to optogenetic control of non-human primate cortex. Front. Syst. Neurosci. 5, 18 (2011).
53. Kwan, K. M. et al. The Tol2kit: a multisite gateway-based construction kit forTol2 transposon transgenesis constructs. Dev. Dyn. 236, 3088–3099 (2007).
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Author contributions
K.H.H. developed the methodology, designed and performed experiments, analyzed data and wrote the manuscript. P.R. developed methodology and wrote the manuscript. T.F. and K.K. created and analyzed transgenic fish. T.B. supervised the project. R.W.F. supervised the project, analyzed data and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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Software and code

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**Data collection**

| Software and Tools | Details |
|--------------------|---------|
| Wings3D (v.1.5.3), Panda3D (v.1.8.1), LabVIEW (v.8.2), ScanImage (v.4.2 with modifications: Rupprecht et al., 2016), MATLAB (R2013a) | Software for controlling VR and coordinating VR with two-photon imaging can be downloaded at [https://github.com/HUANGKUOHUA/Zebrafish-in-virtual-reality.git](https://github.com/HUANGKUOHUA/Zebrafish-in-virtual-reality.git). |

**Data analysis**

| Software and Tools | Details |
|--------------------|---------|
| FIJI (Image), v.1.51, MATLAB (R2013a) | Analysis software for calcium imaging data is described in Berens et al. (2018) and can be downloaded at [https://git.io/vNBsz](https://git.io/vNBsz). |

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The data that support the findings of this study are available from the Lead Contact upon request (rainer.friedrich@fmi.ch).
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
The behavior of 46 head-restrained animals was examined in the closed-loop VR. Simultaneous calcium imaging was performed in 26 of these animals. Behavioral responses of freely swimming animals to movies of conspecifics were analyzed in 64 adult animals. Spontaneous behavior of freely swimming animals in the absence of movies was performed with 18 different animals.

Data exclusions
No data were excluded from the analyses.

Replication
Each animal was tested independently. The number of animals for each experiment are reported for each experiment. The means and standard errors or standard deviations of measurements are also reported.

Randomization
Animals were randomly selected from their home tanks. No animal was used twice in an experiment.

Blinding
Blinding was not relevant to our study because statistical measures were based on automated data extraction and did not require subjective judgment.

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Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | Antibodies           |
| ☑   | Eukaryotic cell lines|
| ☑   | Palaeontology        |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |
| ☑   | Clinical data        |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☑   | ChiP-seq              |
| ☑   | Flow cytometry        |
| ☑   | MRI-based neuroimaging|

Animals and other organisms

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Laboratory animals
All experiments were performed in adult (5 – 21 months old) zebrafish (Danio rerio). For two-photon imaging experiments on head-fixed animals, stable transgenic lines Tg[NeuroD:GcaMP6]lclm05 (Rupprecht et al., 2016), Tg[Gad1b:GFP] (Satou et al., 2013), Tg(212C:ArchT-GFP) (Frank et al., 2019) were used. For freely behavioral experiment, wild type adult zebrafish were used. Genders were equally represented.

Wild animals
The study did not involve wild animals.

Field-collected samples
The study did not involve samples collected from field.

Ethics oversight
All experiments were approved by the Veterinary Department of the Canton Basel-Stadt (Switzerland).

Note that full information on the approval of the study protocol must also be provided in the manuscript.