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Circuit Neuroscience in Zebrafish

Review

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A central goal of modern neuroscience is to obtain a mechanistic understanding of higher brain functions under healthy and diseased conditions. Addressing this challenge requires rigorous experimental and theoretical analysis of neuronal circuits. Recent advances in optogenetics, high-resolution *in vivo* imaging, and reconstructions of synaptic wiring diagrams have created new opportunities to achieve this goal. To fully harness these methods, model organisms should allow for a combination of genetic and neurophysiological approaches *in vivo*. Moreover, the brain should be small in terms of neuron numbers and physical size. A promising vertebrate organism is the zebrafish because it is small, it is transparent at larval stages and it offers a wide range of genetic tools and advantages for neurophysiological approaches. Recent studies have highlighted the potential of zebrafish for exhaustive measurements of neuronal activity patterns, for manipulations of defined cell types *in vivo* and for studies of causal relationships between circuit function and behavior. In this article, we summarize background information on the zebrafish as a model in modern systems neuroscience and discuss recent results.

Introduction

Quantitative insights into the structure and function of neuronal circuits are essential to understand how higher brain functions arise from the physiological properties of individual neurons and their connections. This central question in neuroscience is addressed by a rapidly growing community of researchers and encompasses neuroanatomy, cellular neurobiology and systems neuroscience, as well as computational, behavioral and theoretical biology. The recent interest in this interdisciplinary field, which might be termed 'circuit neuroscience', has been triggered in part by the development of novel technologies. First, high-resolution imaging methods such as multiphoton microscopy [1] can visualize the structure and dynamics of small neuronal processes over extended time periods *in vivo* [2]. Second, multiphoton calcium imaging enables quantitative measurements of activity patterns across large populations of individual neurons in the intact brain [3]. Third, novel genetic tools provide the opportunity to stimulate or inhibit defined subsets of neurons by light and other means [4,5]. Such manipulations are important for dissecting circuit function and for establishing causal relationships between neuronal activity patterns and behavior. Fourth, new methods have been developed for visualizing neurons within a circuit, for determining their connectivity patterns and even for reconstructing entire circuits [6]. The morphology and projection patterns of up to approximately 100 neurons in complex circuits have been visualized by stochastic expression of multicolor fluorescent proteins in different ratios [7]. Viral vectors or other tracers have been engineered to label connected neurons across one or more synapses [4]. Finally, information on wiring diagrams can be obtained by 3D imaging of tissue ultrastructure using novel electron microscopy methods [8–10]. Automated data analysis methods are now required to apply this approach to large circuits [6]. Considering these developments, the rigorous quantitative analysis of neuronal circuits has become a realistic long-term goal.

The progress made by new methods is intimately related to the choice of appropriate animal models. A model system for circuit neuroscience should obviously be amenable to neurophysiology, imaging and behavioral analyses, as well as to advanced molecular manipulations. In addition, the brain should be small in terms of physical size and the number of neurons. This is an important and often underappreciated criterion because exhaustive analyses of activity and connectivity patterns currently have size constraints. Multiphoton microscopy can penetrate up to around 700 μm into the brain under most conditions — but signal-to-noise ratio decreases with depth. For 3D electron microscopy, a volume of approximately 300 x 300 x 300 μm appears to be a reasonable goal given the complexity of the data analysis task, although larger volumes are technically possible [6]. The number of neurons is important because detailed analyses of activity patterns require measurements of activity patterns across a substantial fraction of all neurons in the circuit. Currently, the number of neurons that can be recorded in a single animal is limited by experimental parameters such as the field of view in optical approaches, the density of cell bodies, and the need to present multiple stimuli. Hence, a small brain size is critical for exhaustive measurements of activity and connectivity patterns, which will eventually be important for extracting fundamental principles of circuit function by computational modeling and theoretical approaches.

Among vertebrates, the zebrafish (*Danio rerio*) is a promising model system. It has been selected as a genetic model system about 30 years ago by George Streisinger and colleagues [11] and subsequently became popular in developmental biology. Since then, many important techniques have been established [12–14], publicly available resources have been systematically accumulated (e.g., www.zfin.org), and the zebrafish has been adopted by other disciplines, including neurophysiology, medical research [15] and ethology [16]. Zebrafish are easy to breed and transparent at embryonic and early larval stages. The adult zebrafish brain (Figure 1A) is only about 4.5 mm long and between 0.4 and 2 mm thick [17,18]. The larval brain at five days post fertilization (dpf) is less than 500 μm thick and 1.5 mm long, making virtually all neurons accessible to multiphoton microscopy *in vivo* (Figure 1B). In this article, we will briefly discuss selected brain areas with an emphasis on the telencephalon, summarize important methods and discuss zebrafish behavior. We then review selected experimental studies and discuss the potential of zebrafish as a model system for circuit neuroscience.

Basic Organization of the Zebrafish Brain

Zebrafish are advanced freshwater teleosts that inhabit vegetation-rich, still or slow waters such as rice fields in India

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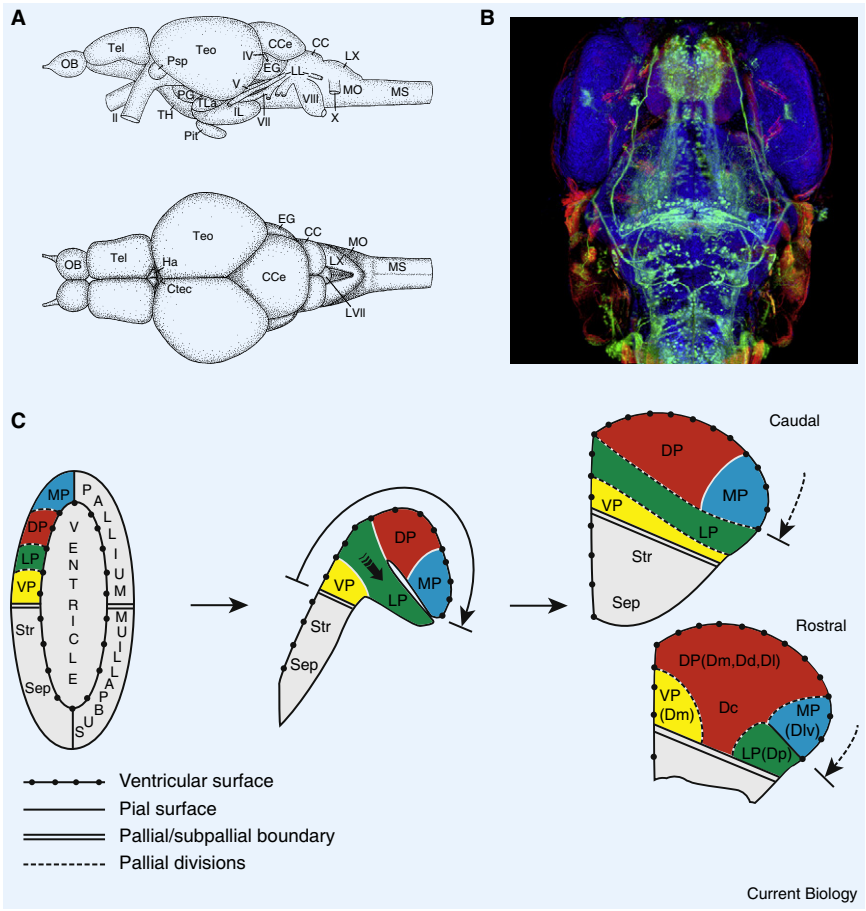

Figure 1. The zebrafish brain.

(A) Lateral and dorsal views of the adult zebrafish brain. Reprinted with permission from [18]. CC, cerebellar crest; CCe, cerebellar corpus; Ctec, tectal commissure; EG, granular eminence; Ha, habenula; IL, inferior lobe of hypothalamus; LL, lateral line nerves; LX, vagal lobe; MO, medulla oblongata; MS, spinal cord; OB, olfactory bulb; PG, preglomerular area; Pit, pituitary; PSp, parvocellular superficial pretectal nucleus; Tel, telencephalon; Teo, optic tectum; TH, tuberal hypothalamus; TLa, lateral torus. Roman numbers label cranial nerves. (B) Fluorescence imaging of the larval zebrafish brain (5 dpf; whole mount). Projection of a confocal stack, rendered using FluoRender (http://www.fluorender.com) [153]. Green, isl1–GFP expression; red, actin; blue, nuclear staining (TOPRO3). Courtesy of H. Otsuna and C.-B. Chien. Scale bar, 100 μm. (C) Partial eversion of the telencephalon during ontogeny of the zebrafish brain. The translocation of pallial regions (colored) is typical for teleosts and changes the topological relationship between compartments of the neural tube. Modified with permission from [24]. Dc, central zone of dorsal telencephalic area; Dd, dorsal zone of dorsal telencephalic area; Dl, lateral zone of dorsal telencephalic area; Dlv, ventral part of Dl; Dm, medial zone of dorsal telencephalic area; DP, dorsal pallium; Dp, posterior zone of dorsal telencephalic area; LP, lateral pallium; MP, medial pallium; VP, ventral pallium; Sep, septum; Str, striatum.

and Bangladesh [16,19]. Zebrafish are small compared to related species but specialized adaptations to tight ecological niches are not obvious. Because the teleost brain conforms to the basic vertebrate brain organization, the gross architecture of many brain areas, e.g., retina, olfactory bulb, cerebellum and spinal cord, is similar to that of other vertebrate classes. Other brain areas, including the telencephalon and tectum, in contrast, show more pronounced differences and are briefly discussed below.

Retinal ganglion cells of zebrafish project to at least ten target areas, the largest being the optic tectum [20]. Compared to its mammalian homologue, the superior colliculus, the architecture of the optic tectum appears to be more complex, comprising seven layers with unique cell types [21,22]. One possible reason for this complexity is that the optic tectum performs vision-related functions that have been taken over by the neocortex in mammals.

The gross morphology of the teleost telencephalon differs substantially from that of amniotes because the telencephalon undergoes a morphogenetic process (eversion) that relocates dorsal zones of the neural tube to lateral positions during ontogeny [23–25] (Figure 1C). Understanding telencephalic anatomy is, therefore, important to compare cortical and striatal brain areas to other vertebrates. The telencephalon of teleosts contains a pars ventralis and a pars dorsalis corresponding to the subpallium (striatum/pallidum and septum) and pallium, respectively. The pallium is homologous to cortical structures but no distinct layering is apparent except that neural cell bodies are denser near the surface in some regions [18]. Analyses of gene expression and projections between brain regions have established tentative homologies between a variety of forebrain areas in teleosts and mammals [24]. Among pallial areas, homologies have been proposed between the mammalian paleocortex (olfactory cortex) and the posterior zone of the dorsal telencephalic area (Dp in lateral pallium) in fish, as well as between mammalian archicortex (hippocampus) and the ventrolateral zone of the dorsal telencephalic area (Dlv in medial pallium) in fish and between the cortical amygdala in mammals and the medial zone of the dorsal telencephalic area (Dm in ventral pallium) in fish. For subpallial areas, it has been proposed that the ventral (Vv) and the lateral (Vl) nucleus of the ventral telencephalic area in fish correspond to the septal formation in mammals, while the commissural nucleus (Vc) and the dorsal nucleus (Vd) of the ventral telencephalic area correspond to the striatum/pallidum of mammals [24].

In goldfish, lesions in telencephalic brain areas produced behavioral effects comparable to those of lesions in the homologous mammalian brain areas [26]. Reflex conditioning using temporally overlapping conditioned (light) and unconditioned (shock) stimuli was not impaired after telencephalic lesions, whereas trace conditioning without temporal overlap between conditioned and unconditioned stimuli required the Dl region (medial pallium including Dlv, the presumed homolog of hippocampus) but not Dm (ventral pallium, the presumed homolog of cortical amygdala). In a form of instrumental fear conditioning with temporal overlap,

learning required Dm but not Dl, whereas instrumental fear learning with no temporal overlap was also impaired after lesioning Dl (predominantly Dlv) [27]. Lesions of Dl (predominantly Dlv, the presumed homolog of the mammalian hippocampus) produced substantial deficits in spatial learning but not in cue learning [28]. These findings suggest that telencephalic divisions homologous to hippocampus and amygdala serve similar functions as their mammalian counterparts. Further studies are now required to compare the underlying circuits and computations.

Zebrafish as an Experimental Model System

Genetic and Molecular Methods

In pioneering mutagenesis screens a large number of zebrafish mutants have been isolated with a wide range of phenotypes, including mutants defective in retino-tectal axon guidance [29,30]. However, only few of these mutants have been exploited for physiological or behavioral studies of neuronal circuit function [31–35], presumably because initial screens identified primarily developmental phenotypes. Indeed, additional mutants with neurobiological phenotypes have been discovered in dedicated behavioral screens [36–40].

Circuit neuroscience often takes the reverse approach to genetic screens and analyzes the function of defined genes or neuron types. In zebrafish embryos and early larvae, the function of specific genes can be disrupted conveniently by the injection of morpholinos, peptide nucleic acids or short interfering RNAs into one-cell embryos. These methods are fast but knockdown is incomplete and the efficiency decays as larvae become older (after around 3–6 dpf for morpholinos). Stable point mutations in defined genes can be generated by high throughput genotyping of mutagenized genomes (TILLING) [41]. Knockdown or overexpression of genes by light has been achieved by caged RNA, DNA or morpholinos [42,43]. These approaches allow, in principle, for precise spatial and temporal control of genetic manipulations but require optical technology that is not standard in most laboratories. Recently, it has been demonstrated that targeted knock-out and knock-in of genes can be achieved using zinc finger nucleases [44,45]. This approach has not yet been widely used but clearly has great potential.

Circuit neuroscience often relies on molecular approaches to visualize, probe or manipulate neurons with minimal perturbations of endogenous gene function. This approach requires sophisticated methods to express transgenes in specific subsets of neurons. In mammals, fast and easy gene delivery is achieved using viruses [4]. Initial studies indicate that viruses are promising tools also in zebrafish [46,47]. Viruses have, however, certain limitations such as the need for invasive injections and the resulting variability in gene expression among individuals. The specific and non-invasive expression of transgenes in defined cell types usually requires stable transgenic lines that are produced by injection of DNA constructs into one-cell embryos [12,14]. This procedure has become much more efficient thanks to the Tol2 transposon [48,49] but is still slow because the generation time of zebrafish is about three months. Classically, expression is targeted to selected cell types by promoter sequences in the DNA construct. However, the limited experimental control over the promoter sequences often results in incomplete or ectopic expression patterns. An alternative approach is to create pseudo-random libraries of enhancer or gene trap lines that can then be screened for desired expression patterns (Figure 2A). As in *Drosophila*, this approach is based on a minimal promoter that becomes regulated by enhancers near the integration site. Because this strategy requires the screening of large numbers of transgenic lines it is usually not practical in mice but feasible

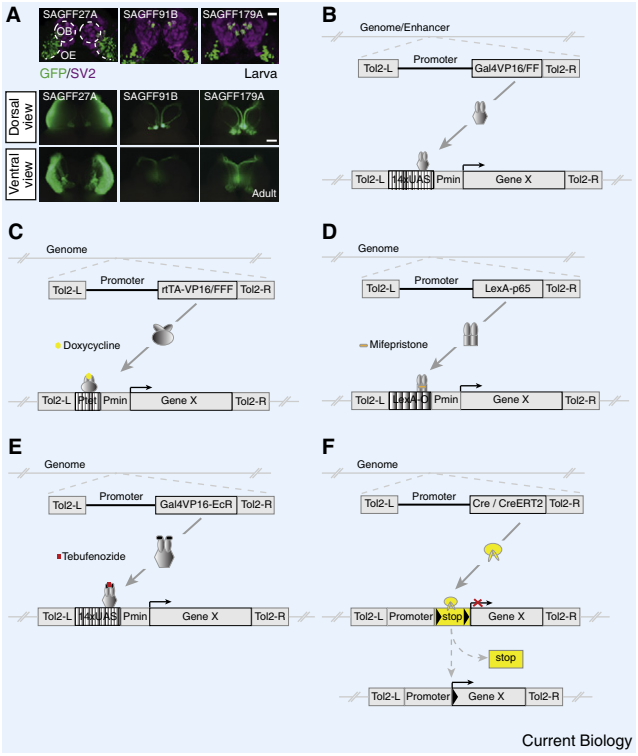

Figure 2. Advanced gene expression systems in zebrafish.

(A) Top: Expression of GFP in the olfactory epithelium and olfactory bulb of zebrafish larvae (frontal view) in three enhancer/gene trap lines generated using the Gal4/UAS system. Scale bar: 50 μm. Bottom: GFP expression in the adult olfactory bulb of the same lines. Scale bar: ca. 120 μm. Modified, with permission, from [76]. (B) Gal4/UAS system. The transcription factor Gal4 activates transcription of the gene of interest (Gene X) by binding to the upstream activating sequence (UAS) in trans. Thus, the activator construct (top) determines the expression pattern while the responder construct (bottom) determines the gene of interest. Different genes of interest can therefore be expressed in different patterns by crossing appropriate activator and responder lines. When a minimal promoter is used to drive Gal4, the expression pattern will be mostly determined by enhancers in the vicinity of the integration site. This enhancer/gene trap approach is used to generate libraries of diverse expression patterns. (C) Tet system (Tet-ON configuration). In the presence of the small molecule Doxycycline (Dox), transcription of the gene of interest is activated by binding of the transactivator rtTA to the Ptet promoter [54]. In the Tet-OFF configuration (not shown), the transcriptional activator tTA activates transcription from the Ptet promoter in the absence of Dox, and Dox inhibits transcription. As the Gal4/UAS system, this two-component system allows for the easy exchange of transgenes. In addition, it provides temporal control of gene expression by Dox, high expression levels, and the possibility to create sparse expression libraries [47]. (D) LexPR system. This two-component system follows a similar logic and allows for temporal control of gene expression by the small molecule mifepristone [52]. (E) EcR system. This two-component system allows for temporal control of gene expression by the small molecule tebufenozide [53]. (F) Cre/CreER^{T2} system. In this two-component system, expression of Cre recombinase under the control of an appropriate promoter causes excision of a STOP cassette in trans and thereby irreversibly activates the transcription of the gene of interest.

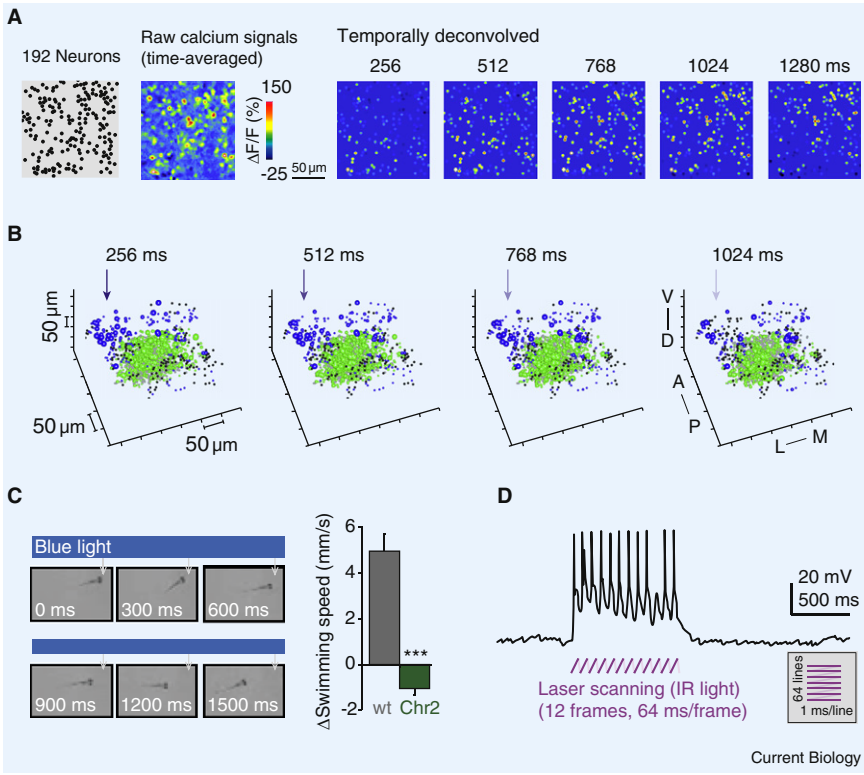

Figure 3. Optophysiology and optogenetics in zebrafish.

(A) Left: time-averaged calcium signals evoked by odor stimulation in an optical section through the granule cell layer of the adult olfactory bulb (multiphoton imaging). Positions of 192 somata are indicated. Right: time-resolved activity patterns reconstructed by temporal deconvolution. Modified with permission from [72]. (B) Time series of three-dimensional activity patterns across approximately 2000 neurons (blue: mitral cells identified by transgenic marker; green: interneurons) evoked by odor stimulation in the adult olfactory bulb. Activity patterns were measured by temporally deconvolved two-photon calcium imaging; time is given relative to the onset of the odor response. Size of spheres indicates magnitude of temporally deconvolved calcium signal. Reproduced with permission from [145]. (C) Backward swimming evoked by blue light stimulation with a remote LED in a zebrafish larva expressing Chr2 under Tet control. Right: mean change in swimming speed during two seconds after light onset in Chr2-expressing larvae and wild-type (wt) siblings. Wt fish show a transient increase in swimming speed (visual startle response). Chr2-expressing larvae, in contrast, swim backwards, which is slower than spontaneous forward swimming (***, $P < 0.001$; t-test). Modified from [47]. (D) Train of action potentials in an interneuron of the adult olfactory bulb expressing Chr2 under Tet control, evoked by frame scanning of a pulsed infrared (IR) laser at low intensity (two-photon activation of Chr2). Modified from [47].

in zebrafish [48,49]. Up to now, hundreds of enhancer/gene trap lines have been accumulated by the zebrafish community [5]. Combining this approach with the two-component Gal4/UAS system allows for the easy exchange of transgenes by crossing independent activator and responder lines (Figure 2B) [49–51]. An alternative method for generating expression pattern libraries is the Tet system (Figure 2C), which generates expression in neurons that appear to be subsets of the neurons targeted by the promoter controlling Tet activator expression [47]. Different transgenic lines exhibit different expression patterns that remain stable over generations. The Tet system, therefore, provides the opportunity to generate seemingly random collections of expression patterns within neuronal populations that can be preselected by choosing an appropriate promoter.

Two-component systems not only provide the opportunity to swap transgenes by simple crosses but some systems also allow for temporal control of gene expression by small molecules. These include the LexPR system [52], the EcR system [53] and the Tet system [47] (Figure 2C–E). The latter has been optimized extensively in mammals [54,55]. Other methods to control gene expression in zebrafish include electroporation, heat shock promoters and the Cre or CreER^{T2} system [56,57] (Figure 2F). Zebrafish, therefore, offer a wide range of opportunities for the expression of transgenes in specific sets of neurons at defined times.

Electrophysiology and Optophysiology

Various preparations have been developed for extra- and intracellular recordings from neurons in the intact zebrafish brain and spinal cord. Larvae are usually immobilized pharmacologically or embedded in agarose, while recordings in adult fish can be performed conveniently in an explant preparation of the entire brain, including sensory organs on the head [58]. This preparation can be maintained *ex vivo* for many hours and does not require the use of anesthetics. In most preparations, neurons can be visualized by transmitted light optics for targeted patch clamp recordings.

Optical recording methods used in zebrafish include voltage-sensitive dye imaging [59,60] and calcium imaging with synthetic or genetically encoded indicators [61–73]. Using confocal or multiphoton microscopy, calcium signals can be measured from hundreds or, in sequential trials, thousands of individual neurons in the intact zebrafish brain (Figure 3A,B). Changes in neuronal firing rate have been reconstructed from calcium signals by a simple temporal deconvolution technique [62]. These methods permit optical measurements of activity across large fractions of neurons in many areas of the zebrafish brain (Figure 3B). Neuronal activity can also be detected using GFP-aequorin, a calcium-sensitive bioluminescent protein. In combination with non-spatial detectors, this approach allows for remote activity measurements from genetically defined neuronal populations in freely behaving fish [74].

Circuit Manipulation Approaches

Dissecting the function of neuronal circuits and their relation to behavior requires specific physiological manipulations. While classical pharmacological manipulations act on defined molecules, it is often desired to manipulate circuits at the level of cell types or even individual neurons. To this end, the development of genetically encoded tools to control

neuronal physiology by light and other means has created a plethora of new opportunities [4].

In zebrafish, synaptic output has been suppressed by tetanus toxin light chain (TeTxLC), a permanent blocker of synaptic vesicle release. Expression of tetanus toxin light chain in different neurons using UAS/Gal4 enhancer/gene trap lines caused specific effects on mechanosensory and olfactory behaviors [75,76]. This approach can identify subsets of neurons involved in defined behaviors, even in large-scale screens, and help uncover their function.

Light-activated molecular tools include the monolithic light-gated cation channel, channelrhodopsin-2 (Chr2) [77,78], and a modified ionotropic glutamate receptor that covalently binds a photoswitchable agonist (LiGluR system) [79]. Activating these molecules by light depolarizes neurons and can cause action potential firing. In zebrafish embryos, optical stimulation of mechanosensory neurons using Chr2 has demonstrated that activation of an individual sensory neuron is sufficient for triggering an escape reflex [80]. In larvae, the stimulation of different neurons using the LiGluR system has led to the identification of neurons that modify responses to mechanosensory stimulation and control swimming behaviors [79,81]. In a transgenic line expressing Chr2 under Tet control, optical stimulation with a remote LED evoked backward swimming, a behavior that does not occur spontaneously [47] (Figure 3C).

Optical techniques can increase the specificity of purely genetic approaches and provide the opportunity to precisely control neuronal manipulations in space and time [5]. Scanning an optical fiber over the brain of zebrafish larvae expressing Chr2 or halorhodopsin (NpHR), a light-gated chloride pump that hyperpolarizes neurons [82], identified brain areas associated with motor behaviors [83]. The spatial extent of these optical manipulations can be determined elegantly using transgenic lines that express photoswitchable proteins [83]. Recently, it has also become possible to trigger action potentials by two-photon activation of Chr2 in zebrafish with a spatial precision that is likely to provide single-neuron resolution (Figure 3D) [47]. This is not trivial, because the spatial resolution of Chr2 activation degrades with increasing intensity of the excitation light due to the long lifetime of the activated state [84]. Precise two-photon activation of Chr2, therefore, requires high expression levels that have been achieved using the Tet system [47].

Zebrafish Behavior

Quantitative behavioral experiments are essential to study end points of neuronal computations. Up to approximately 6 dpf, zebrafish larvae are transparent and require no feeding, which facilitates the combination of behavioral studies with genetic screening and optophysiological analyses. Motor behaviors, including spontaneous contractions, directed escape movements and simple swimming, develop between approximately 18 hours post fertilization and 3 dpf [85,86], followed by the emergence of free swimming and visually guided prey capture between 4 and 9 dpf [87–89]. Robust visual behaviors of zebrafish larvae include the opto-kinetic reflex, which stabilizes retinal image motion by compensatory eye movements, and the optomotor response, which stabilizes retinal image motion by pursuit swimming [36,37,90,91]. After 3 dpf, zebrafish larvae also exhibit phototaxis [36,92] and startle responses to changes in light intensity [90,93]. Other visual behaviors such as the dorsal light reflex [94] emerge later during development. Mechanosensory stimuli evoke startle or escape responses in larvae [95]. Changes in ambient temperature and chemical irritants evoke increased locomotor activity, which may also reflect escape behavior [96]. Some olfactory stimuli cause increased swimming behavior [97], and a few odors have been identified that are either attractive or repulsive at larval stages [40]. Spontaneous locomotor activity comprises epochs of swimming activity and rest and exhibits a circadian, light cycle-dependent pattern. Already at larval stages, this pattern shows the hallmarks of sleep–wake cycles and is modulated by hypocretin [98]. Many behaviors have been studied quantitatively in zebrafish larvae, making them an excellent model system to study neuronal circuits in the context of defined, relatively basic behaviors.

Adult zebrafish offer the opportunity to study more complex behaviors. In juveniles and adults, an innate olfactory avoidance response is triggered by an alarm substance ('*Schreckstoff*') that is released from the skin of injured fish [16]. Fish can learn to associate neutral stimuli with the alarm substance [99], and naïve fish can even learn these associations by observing the behavior of experienced fish [100,101].

A prominent social behavior of zebrafish is shoaling [16]. Shoaling preferences are determined by early olfactory and visual experience. Olfactory imprinting to kin-related olfactory cues at 6 dpf results in a preference for kin at juvenile stages that turns into kin avoidance at adulthood, presumably to avoid inbreeding [102,103]. In the visual modality, juvenile and adult zebrafish prefer to shoal with fish that display the pigment patterns they encountered as larvae [104–107]. Zebrafish can also depart from shoals and exhibit territorial behavior. When adult males are kept in pairs they develop dominant-subordinate relationships that are correlated to differential patterns of vasotocin expression [108].

Mating behavior of zebrafish involves a sequence of signals between males and females. First, female egg production depends on the presence of male olfactory signals [109–111]. Second, spawning requires male courtship, which is triggered by female pheromonal cues and involves visual and mechanical stimuli to elicit female egg laying [110,112]. Male courtship tactics can vary from active pursuit to territorial defense, depending on the density of males [113], and intra-sexual competition exists also in spawning females [114,115].

Learning behavior of zebrafish has been studied in laboratory assays for conditioned place preference with a positive reinforcement such as food or cocaine, or with a negative reinforcer such as a mild electric shock [38,116,117]. Other studies established learning paradigms for aversive or appetitive conditioning to visual and olfactory stimuli [118–121]. In summary, zebrafish offer an opportunity to study complex phenomena such as social behavior, associative conditioning and observational learning.

Analysis of Neuronal Circuit Function in Zebrafish

Motor Systems in the Brainstem and Spinal Cord

Pioneering studies by Fetcho and colleagues [69,122] examined the control of escape swimming by the Mauthner cell and its serial homologs which are central parts of reflex circuits that control rapid startle behaviors [123,124]. Escape swimming is directed away from a mechanical stimulus by adjusting the amplitude of the initial body bend (C-bend) as a function of stimulus position or direction. Experiments in goldfish have demonstrated that the Mauthner cell alone

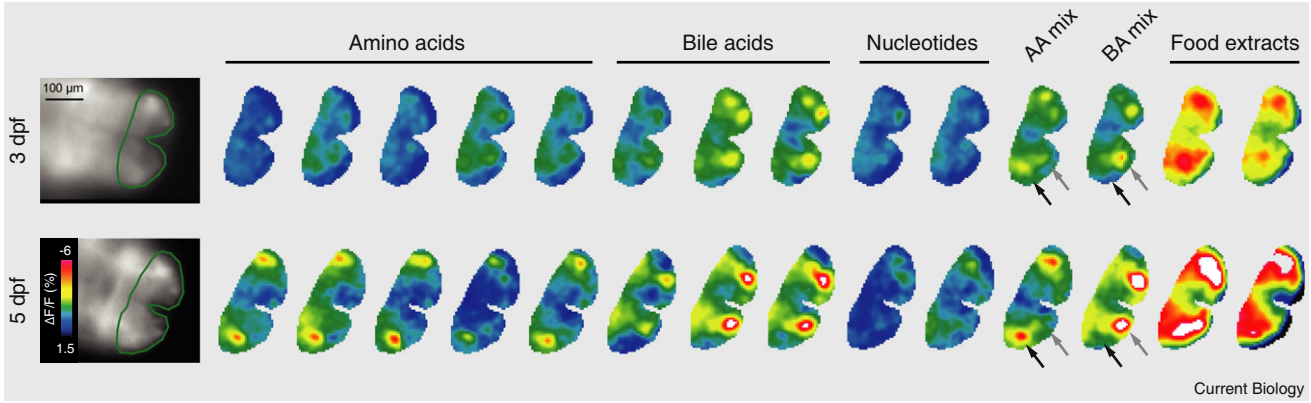

Figure 4. Emergence of chemotopic activity maps during development, visualized by a genetically encoded calcium sensor.

Odor-evoked calcium signals in the developing olfactory bulb were measured at successive developmental stages (3 and 5 dpf) in the same zebrafish larva expressing the genetically encoded calcium sensor, inverse pericam [154]. Left: raw fluorescence *in vivo*. Inverse pericam is expressed throughout the olfactory bulb and telencephalon; olfactory bulbs are outlined. Decreasing fluorescence intensity corresponds to increasing calcium concentration. Right: fluorescence signals evoked in the developing olfactory bulb by different odors. Responses to some odors were not yet detectable at 3 dpf but emerged at 5 dpf in the same fish. Even at 3 dpf, however, responses to amino acids and bile acids are already spatially segregated to the lateral and anterior-medial olfactory bulb, respectively. This is most obvious by comparing responses to mixtures of amino acids (AA mix) and bile acids (BA mix; arrows). Hence, a coarse chemotopic map is already established at early developmental stages when the olfactory bulb starts to respond to odors. Modified with permission from [71].

cannot account for the graded amplitudes of C-bends because it fires only a single action potential during escape. A model developed by Foreman and Eaton [125] predicted that graded C-bend amplitudes are produced by the recruitment of reticulo-spinal neurons in adjacent hindbrain segments. Indeed, calcium imaging in zebrafish larvae revealed that during small C-bends only the Mauthner cell was activated, while its serial homologs were recruited during larger C-bends [69]. Laser ablations then established causal relationships between the activity of hindbrain neurons and fast escape behavior [122]. Work in zebrafish, therefore, revealed a basic principle underlying the control of escape movements that cannot easily be examined in other vertebrates.

In a more recent study [65], different swimming behaviors in zebrafish larvae were evoked visually by gratings moving in different directions. Calcium imaging identified small groups of descending hindbrain neurons whose response properties were correlated with the observed behaviors. Laser ablations indicated that these neurons are necessary for the expression of visually evoked swimming patterns. These results provide insights into the command codes controlling various motor behaviors.

In the spinal cord of zebrafish embryos and larvae, patch clamp studies have shown that spontaneous tail contractions (coiling) at early developmental stages are generated by a network of electrically coupled neurons [126], while swimming and escape movements at later stages require chemical synaptic transmission [86,127]. Some interneurons appear to be recruited only in specific motor patterns, whereas a subset of glycinergic interneurons is active in multiple patterns [68,128]. Further experiments demonstrated that motor neurons and pre-motor interneurons are topographically organized in a dorso-ventral map that reflects their excitability [129]. Presumably as a consequence of this topographic organization, motor neurons are recruited in a ventral-to-dorsal fashion as swimming frequency increases. Interestingly, inhibitory interneurons are also topographically organized but recruited in a dorsal-to-ventral fashion. Moreover, motorneurons activated at low swimming frequencies remained active at higher frequencies, consistent with the classical size-principle that increasing muscular force is generated by a recruitment of additional motor neurons. Pre-motor interneurons, in contrast, were active within a specific window of swimming frequencies, resulting in a gradual shift of activity across the population with increasing swimming frequency [130]. These results mark a breakthrough because they provided direct evidence for a functional topographic map in the spinal cord. Moreover, these and other studies, e.g. [81], associate identified types of neurons with defined functions and are important steps towards a functional dissection of spinal cord circuits. The simple layout of spinal cord circuits in zebrafish larvae and the potential for molecular manipulations, therefore, provide a powerful experimental framework that complements other model systems [131,132].

Visual Processing

Quantitative behavioral studies in combination with laser ablations have started to map visual behaviors to different target areas of retinal ganglion cells. Ablation of the optic tectum had only mild effects on optokinetic and optomotor responses but abolished orienting movements during prey capture [88,133]. Different behaviors depend on different color channels, and zebrafish can detect second-order motion [92,134], a computation that in mammals is usually attributed to visual cortex.

Tectal neurons have relatively large receptive fields that are shaped by excitatory and inhibitory inputs [135,136]. Two-photon calcium imaging has demonstrated that motion- and direction-selectivity in a subset of tectal neurons emerges soon after the retinotectal projection has formed [66]. After unilateral ablation of one tectum, axons from both retinas grow into the remaining tectum and create a zone of binocular innervation. Tectal neurons in this zone were not only responsive to input from both eyes but their receptive fields and direction selectivity were clearly correlated, suggesting that input from both eyes becomes

integrated into a common motion-detection circuit [64]. Indeed, tectal neurons responded to apparent motion, and direction selectivity was strongly reduced after blockade of $GABA_A$ receptors. These results indicate that direction selectivity is computed in the tectum and favor a model of asymmetric inhibition between retinotopically organized tectal neurons [64]. Another recent study [63] has demonstrated that rhythmic activity of tectal neurons and tail flicks evoked by a series of light flashes persist for a few cycles after termination of the stimulus. This striking result indicates that neuronal circuits form short-term memories of time intervals that are likely to be much longer than neuronal time constants. The use of optical measurements and manipulations of neuronal activity in the transparent zebrafish larvae now provides interesting opportunities to explore the cellular mechanisms underlying this peculiar form of temporal memory [137].

In the past, important quantitative studies on visual processing were performed in goldfish, a close relative of zebrafish. It may thus be fruitful to apply this knowledge in zebrafish. The goldfish is also being used to dissect the function of the hindbrain circuit that integrates eye velocity signals to produce motor commands controlling eye position [138]. Zebrafish would offer the opportunity to combine the sophisticated electrophysiological and behavioral approaches used in goldfish with calcium imaging and molecular manipulations.

Olfactory Processing

Molecular, electrophysiological and imaging methods have been used in zebrafish to study principles of neuronal coding and processing in the olfactory system. Odor-evoked patterns of activation across the input modules of the olfactory bulb, the glomeruli, were visualized in adult zebrafish by calcium- and voltage-sensitive dye imaging of convergent sensory axons [60,61]. These results allowed for a quantitative analysis of combinatorial odor representations and revealed fuzzy topographic maps of chemical odorant features that appear much less distinct than the topographic maps in other sensory systems. Subsequent imaging studies in other species showed that glomerular activation patterns are organized by similar principles throughout vertebrates [139] and probably also in insects [140–142]. Repeated imaging of odor-evoked activity patterns in zebrafish larvae demonstrated that coarse functional maps are established already during early development [71] (Figure 4). Silencing of sensory input to subsets of glomeruli by tetanus toxin light chain expression showed that behavioral responses to different odors require input to different subsets of glomeruli, linking the detection of topographically mapped odor classes to defined behaviors [76].

Patterns of glomerular activation are processed by networks of inhibitory interneurons and mitral cells, the output neurons of the olfactory bulb. Electro- and optophysiological measurements (Figure 3A,B) have demonstrated that odor-evoked activity of zebrafish mitral cells is temporally patterned on multiple time scales and rhythmically synchronized among odor-dependent ensembles [59,72,143,144], as observed in virtually all vertebrate and insect species examined. The relatively low number of output neurons in the zebrafish olfactory bulb enabled exhaustive measurements of dynamic activity patterns across the population of mitral cells. A direct comparison of these output activity patterns to glomerular input patterns

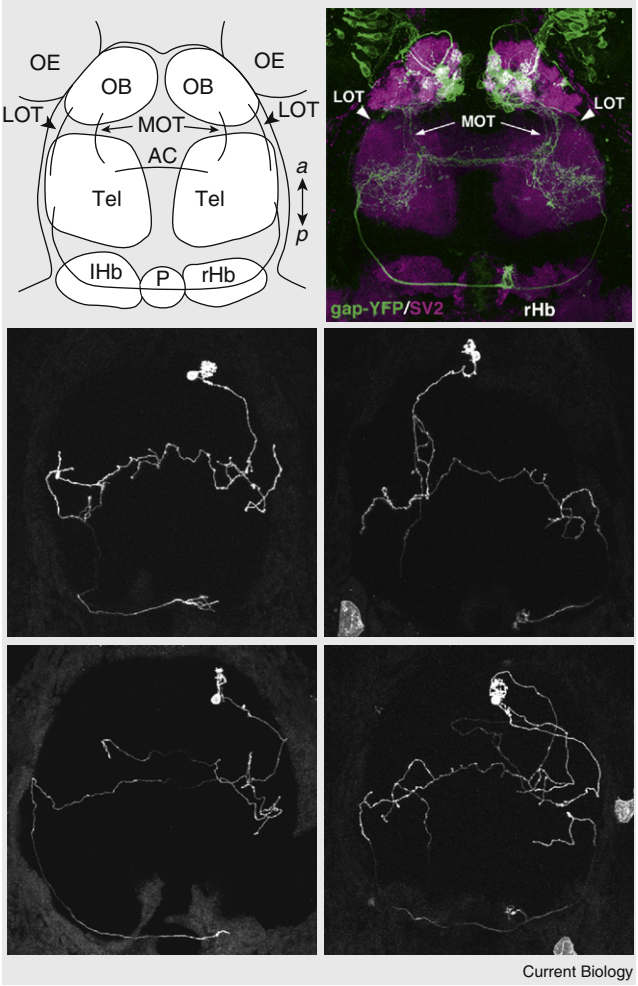

Figure 5. Genetic tracing of individual mitral cell projections from the olfactory bulb to higher brain areas.

Top left: schematic dorsal view of the larval forebrain. Top right: Expression of GFP in a subset of olfactory sensory neurons and mitral cells in a transgenic line at 7 dpf. Bottom: *In vivo* confocal imaging of individual GFP-labelled mitral cells at 7 dpf. Sparse stochastic GFP expression was achieved by injection of a DNA construct into one-cell embryos. Complex axonal projections to multiple telencephalic areas are observed. The soma with a small dendritic tuft is located at the top in each image. Mitral cells projecting to the habenula are located in a specific cluster in the olfactory bulb. Modified with permission from [147]. (AC, anterior commissure; lHb, left habenula; LOT, lateral olfactory tract; MOT, medial olfactory tract; OB, olfactory bulb; OE, olfactory epithelium; P, pineal organ; rHb, right habenula; Tel, telencephalon.)

uncovered multiple transformations of odor representations [145]. For example, neuronal circuits in the olfactory bulb reduce the overlap between activity patterns evoked by similar odors, resulting in a decorrelation of odor representations across mitral cells [59,143,144] that is associated with a local topological reorganization of activity patterns [72] (Figure 3B). Gradual changes in the molecular identity of a stimulus ('odor morphing') resulted in abrupt transitions between mitral cell activity patterns that were mediated by coordinated response changes among small mitral cell ensembles [146]. The olfactory bulb, therefore, performs a classification of inputs into discrete and defined output

patterns, a type of computation that may also be employed in other tasks such as decision making. Similar analyses of pattern processing have not yet been possible in mammals because activity can be recorded only from a small fraction of the neurons in the olfactory bulb.

Recently, zebrafish have also been used to analyze the transformation of odor representations in higher forebrain areas. Sparse genetic labeling in larvae revealed that individual mitral cells send widespread projections to multiple higher brain areas [147] (Figure 5). Consistent with this result, large-scale activity mapping by two-photon calcium imaging in adult zebrafish showed that the coarse topographic organization of glomerular activation patterns is not maintained in two target areas of the olfactory bulb, one of them being the posterior zone of the pallium (Dp) [73], the evolutionary homolog of mammalian olfactory cortex. Further results showed that neurons in Dp integrate synaptic input from functionally diverse mitral cells via excitatory and inhibitory synaptic pathways [73]. These results indicate that higher telencephalic circuits generate novel, synthetic representations of odor objects. In contrast to activity patterns in an associative olfactory brain area of insects, the mushroom body [148], activity in Dp was not ultra-sparse, suggesting that higher olfactory processing may differ to some extent between vertebrates and insects. The lack of chemotopy and the powerful inhibition observed in area Dp are consistent with recent observations in mammalian olfactory cortex [149,150]. The olfactory forebrain is evolutionarily old and may perform computations that are fundamental to other cortical circuits. Zebrafish are therefore a promising model to study such computations at the molecular, cellular and systems levels.

Conclusions and Outlook

As any other animal, the zebrafish has advantages and disadvantages as a model system in circuit neuroscience. A current weakness of zebrafish is that paradigms for studying complex behaviors are not as advanced as in other model organisms, but this situation will likely change in the future. Important advantages of zebrafish are its small size, the transparency at larval stages and the possibility to combine a broad range of experimental methods. Zebrafish permit not only forward genetic screens but also high-throughput behavioral and pharmacological profiling, which opens new perspectives for neuronal circuit research and drug discovery [151,152]. By capitalizing on experience in invertebrates and mammals, a spectrum of advanced genetic methods has been established that opens exciting perspectives for optogenetic and other approaches in zebrafish. Recent studies demonstrate how these methods can be combined with physiological and behavioral approaches to dissect the function of defined cell types and even individual neurons within a circuit. In the future, the zebrafish's small brain size may be exploited also for the reconstruction of neuronal wiring diagrams by 3D electron microscopy [6]. The exhaustive and quantitative analysis of neuronal activity and connectivity will eventually be important to derive principles governing neuronal circuit function by theoretical approaches. In zebrafish, detailed circuit analysis is not limited to simple systems but can also be applied to higher-order brain areas. The zebrafish thus offers unique opportunities for mechanistic analyses of neuronal circuits underlying complex computations and higher brain functions in vertebrates.

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