



## Review

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# Zebrafish forebrain and temporal conditioning

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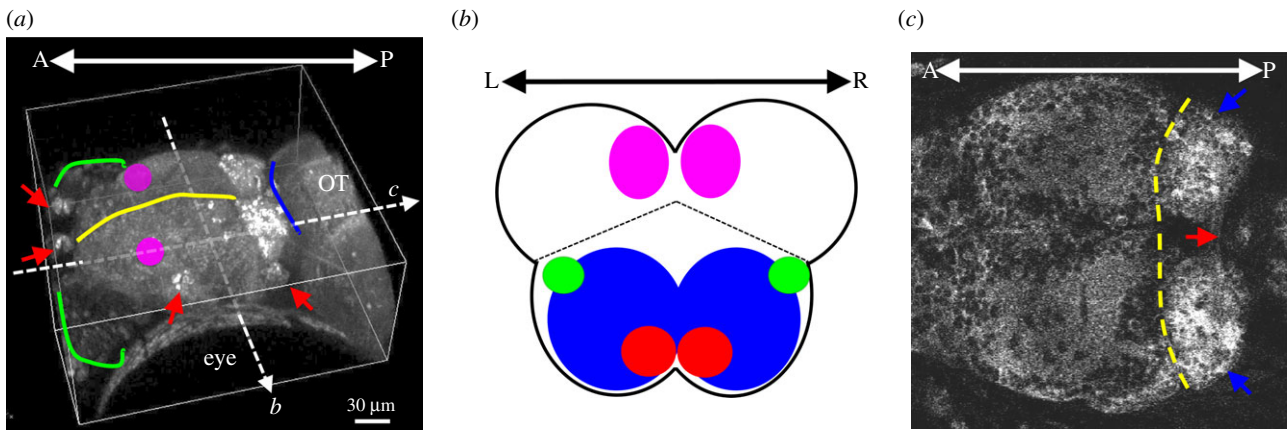
The rise of zebrafish as a neuroscience research model organism, in conjunction with recent progress in single-cell resolution whole-brain imaging of larval zebrafish, opens a new window of opportunity for research on interval timing. In this article, we review zebrafish neuroanatomy and neuromodulatory systems, with particular focus on identifying homologies between the zebrafish forebrain and the mammalian forebrain. The neuroanatomical and neurochemical basis of interval timing is summarized with emphasis on the potential of using zebrafish to reveal the neural circuits for interval timing. The behavioural repertoire of larval zebrafish is reviewed and we demonstrate that larval zebrafish are capable of expecting a stimulus at a precise time point with minimal training. In conclusion, we propose that interval timing research using zebrafish and whole-brain calcium imaging at single-cell resolution will contribute to our understanding of how timing and time perception originate in the vertebrate brain from the level of single cells to circuits.

## 1. Introduction

The ability to gauge the passage of time in the seconds-to-minutes range, defined as interval timing, is fundamental to vertebrate behaviour and cognition, but the underlying neural mechanisms at the circuit level remain elusive. Zebrafish (*Danio rerio*) offer great potential for studies of timing because of recent progress in neuroimaging techniques. Specifically, it is now possible to monitor neural activity in the entire brain at single-cell resolution by using calcium imaging in larval zebrafish [1,2]. This provides an unprecedented opportunity to investigate the relationship between brain, behaviour and cognition. In particular, whole-brain calcium imaging is especially suitable for studying functions that require cooperation among multiple brain regions. Interval timing is one such cognitive function because it involves cortico-striatal circuits [3]. To facilitate future interval-timing studies from molecular to cellular and circuit levels, we review zebrafish forebrain anatomy, neuromodulatory systems and behavioural repertoire. Calcium imaging data showing stimulus expectation that arises from presentation of temporally patterned stimuli are also presented to demonstrate that imaging the entire brain of a larval zebrafish opens a new window on how time perception is instantiated in the vertebrate brain.

## 2. Zebrafish forebrain neuroanatomy

Most cognitive functions rely on the forebrain, which comprises the telencephalon (cortical and sub-cortical regions in mammals; pallium and subpallium in fish) and the diencephalon (including the thalamus, pineal body and habenula). While this basic forebrain organization is conserved across vertebrates (figure 1a) [8], it is still uncertain whether the fish telencephalon contains a region that is functionally equivalent to the mammalian neocortex. One recent study implicated the dorsal part of the pallium as a functional homologue [9], but additional studies are required. Hence, here we only consider non-neocortical regions in the forebrain that have defined cognitive functions in mammals, interval timing in particular, and compare each region with its counterpart(s) in fish.



**Figure 1.** Anatomical structures of zebrafish forebrain. (a) Lateral view of a zebrafish forebrain that includes the telencephalon, in which the yellow line indicates the extent and divides this structure into two hemispheres, and the diencephalon, in which the blue line indicates the boundary between this structure and the optic tectum (OT) in the midbrain. The green line indicates the olfactory epithelium. The red arrows indicate the lateral line organ. The ADP (magenta area) is proposed to be the homologue of mammalian hippocampus [4]. (b) Coronal section (from the white dashed arrow *b* in *a*) of the telencephalon. The dashed line separates the dorsal telencephalon (pallium) and ventral telencephalon (subpallium, blue). Subpallium includes the potential homologue of mammalian BG based on *GAD67*-mRNA labelling [5]. The EN (green) is the homologue of the globus pallidus *internal segment* [4]. The dorsal medial pallium (magenta) is the homologue of mammalian basolateral/lateral amygdala, whereas the ventral subpallium (red) is presumably the homologue of mammalian central amygdala [6]. (c) Transverse section of the forebrain region (from the white dashed arrow *c* in *a*), in which the telencephalon and the habenula (blue arrows) are separated by the yellow dashed line. The red arrow indicates the habenula commissure that connects the two habenula. This 8-day old fish is a triple transgenic Nacre zebrafish, carrying the *HuC:Gal4* pan-neural driver, *GAL4<sup>S1011t</sup>* driver and the fluorescent calcium indicator *GCaMP3* under the UAS promoter [7]. Scale bar, 30  $\mu\text{m}$ .

### (a) Zebrafish homologues of basal ganglia: the subpallium

The mammalian basal ganglia (BG) include the striatum, globus pallidus, subthalamic nucleus and substantia nigra pars reticulata. A major characteristic of the BG is its dense populations of GABAergic medium spiny neurons (MSNs), which contain glutamate decarboxylase (GAD), an enzyme that helps to convert glutamate into GABA. Thus, one method for identifying the BG homologues in zebrafish, in addition to determining connectivity or immunohistochemical labelling and *in situ* hybridization [9,10], is to locate *GAD67*-mRNA-rich regions in the brain. Indeed, *GAD67* is expressed in all subpallial regions in zebrafish (figure 1*b*) [5]. In particular, the entopeduncular nucleus (EN) has been identified as equivalent to the mammalian globus pallidus *internal segment* (GPI), which gives rise to the BG output to the thalamus and habenula [4,11]. In rodents, primates and humans, the dorsal striatum is essential for time keeping [12–14]. One current biological model of interval timing—the striatal beat frequency model—posits that cortico-striatal circuits are critical for time perception [15,16]. In this model, the striatal MSNs serve as coincidence detectors that discriminate different cortical input patterns to quantify the passage of time [17]. Given that thousands of cortical inputs project to individual striatal MSNs [18], how multi-region connections between such large numbers of participating neurons give rise to timing is currently unobservable in mammals owing to the limited number of neurons that can be recorded simultaneously using traditional electrophysiological techniques. In zebrafish, it is possible to observe all brain neurons simultaneously [1,2], and therefore we can monitor both the pallium and the subpallium concurrently to reveal the functional microcircuits for interval timing.

### (b) Hippocampus and amygdala: the pallium

The zebrafish homologue of the mammalian hippocampus appears to be the anterodorsolateral pallium (ADP, figure 1*a*),

because this region displays high spontaneous neural activity embedded with fast ripple oscillations (above 100 Hz) in the adult zebrafish brain [4]. The fish lateral pallium is also involved in trace conditioning, which is a hippocampal-dependent task in mammals [19]. Nevertheless, more anatomical pieces of evidence are needed to justify that the fish ADP is a hippocampus homologue, such as whether there are subfields (like CA1, CA3) in fish ADP with unique intrinsic connections, just to name one. The involvement of the hippocampus in interval timing is implied by rodent data showing that the remembered criterion time is persistently shorter (or earlier) than the actual criterion time following hippocampal lesions [20,21]. In addition, the hippocampus may also participate in temporal memory consolidation [22] and in bridging temporal gaps of discontinuous episodic events [23], leading to a recent proposal that there are time cells in the hippocampus for representing the flow of time in episodic-like memories [24].

For the amygdala, a recent review suggests that the dorsomedial portion of the pallium in *Actinopterygii* (the ray-finned fish class that includes zebrafish, goldfish and rainbow trout) is the homologue of the basolateral/lateral amygdala [6]. The homologue of the central amygdala seems to be located in the ventral subpallium (figure 1*b*). In mammals, the basolateral/lateral amygdala process fear-related information, while pain processing, expression of innate behaviours and anxiety are subserved by the central amygdala [25]. For timing, a recent study shows that amygdala neurons are sensitive to temporal reward structure and display expectation/anticipatory responses to reward [26]. In addition, emotion can modulate time perception, as demonstrated in rodent and human studies [27,28], but where and how this modulation occurs at the circuit level is currently unknown.

### (c) Habenula: the dorsal diencephalon

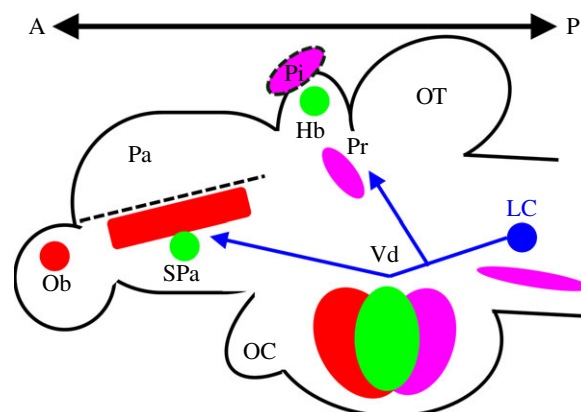
The habenula (figure 1*c*) is an evolutionarily conserved structure in vertebrates [29]. It comprises two sub-nuclei—medial and lateral in mammals and dorsal and ventral in zebrafish

[30], and each sub-nucleus has its own distinct input and output connections. The medial habenula (using mammalian nomenclature) receives inputs from the forebrain limbic circuits, such as the posterior septum, and projects to the interpeduncular nucleus (IPN), and the IPN further projects to the raphe nucleus and periaqueductal grey in the brainstem. The lateral habenula receives inputs from the BG (GPi) and projects to the midbrain ventral tegmental area, the locus coeruleus (LC), and the rostromedial tegmental nucleus [31–33]. Reflecting these complex connections, the proposed functions of the habenula are diverse and include value-based decision-making [34], anticipation of future events [35] and emotional regulation [36–38]. Although the habenula, especially the lateral habenula, is downstream of the BG and upstream of midbrain dopamine areas, both of which are critical for interval timing [3], it appears to be an overlooked brain region with respect to timing studies.

### 3. Zebrafish neuromodulatory systems

#### (a) Dopamine

Dopamine neurons project from midbrain to forebrain regions, such as the cortex (mesocortical) and the striatum (mesostriatal) in mammals. Psychoactive drugs that alter dopamine neurotransmission can also change the perception of time [39] in a manner that can be characterized as a change in the speed of a hypothetical internal clock. That is, immediately following systemic injections of dopaminergic agonists, the speed of the internal clock is relatively faster, thus leading to the experience of a longer subjective time for a given objective time [40]. In contrast, when synaptic dopamine levels are low, the speed of the internal clock is relatively slower and time seems to drag [41]. The presence of tyrosine hydroxylase (TH), a rate-limiting enzyme, which catalyses the transformation of L-tyrosine to the dopamine precursor L-DOPA, can be used to identify catecholamine neurons in zebrafish. Because TH is expressed in both dopaminergic and noradrenergic neurons, it is not a conclusive marker for dopaminergic neurons. A secondary marker is required to clearly distinguish dopamine- and norepinephrine (NE)-releasing neurons in the zebrafish brain. That said, recent labelling studies revealed that the olfactory bulb, subpallium and ventral diencephalon express *TH* genes (figure 2, red areas) [42]. In addition, zebrafish have paralogous *TH* genes (*TH1* and *TH2*), two genes that code for similar proteins, while mammals only have one *TH* gene [43,44]. The functional differences, if any, between *TH1*- and *TH2*-expressing neurons remain to be determined as some neurons express one *TH* gene only while others express both. In larval zebrafish, the developing putative dopamine regions start functioning 3–4 days post fertilization (dpf), immediately after hatching [47]. Indeed, altering dopamine neurotransmission by exposure to D1 or D2 receptor agonists/antagonists affects locomotion in 5-dpf zebrafish [48]. In addition, it is interesting to note that the *TH1*-expressing region in the subpallium is near the border between the ventral pallium and dorsal subpallium that stretches from anterior to posterior into an elongated striped area (figure 2, the red area below the dashed line) [43]. Although the function of this striped area remains to be determined, it is possible that it is the equivalent of the mammalian dorsal striatum.



**Figure 2.** Schematic diagram of zebrafish neuromodulatory systems in a sagittal view of the brain. Dopamine: the red areas indicate potential dopaminergic regions in the olfactory bulb, subpallium and many regions in the ventral diencephalon based on expression of *TH* genes and secondary markers [42–44]. The dashed line, which is right above the *TH1*-expressing subpallial region, separates the pallium and subpallium. Acetylcholine: the green areas show the ChAT-positive regions in the habenula, subpallium and sparsely in the ventral diencephalon [45]. Norepinephrine: the blue area indicates the NE containing cell bodies of the LC. The blue lines indicate its projections to the forebrain regions, such as the subpallium, prectum and ventral diencephalon [42]. Serotonin: the magenta areas indicate the 5-HT cell body regions in the ventral diencephalon, raphe nucleus (below LC), prectum and the pineal (between and above Hb) [46]. Note that we have not labelled individual subregions in the ventral diencephalon for dopamine (red), ACh (green) and 5-HT (magenta), because this is beyond the scope of the current review. Thus, the colour ellipsoids in the ventral diencephalon overlap. Hb, habenula; LC, locus coeruleus; Ob, olfactory bulbs; OC, optic chiasm; OT, optic tectum; Pa, pallium; Pi, pineal body; Pr, prectum; SPa, subpallium; Vd, ventral diencephalon.

#### (b) Acetylcholine

The neuromodulator acetylcholine (ACh) is also involved in mammalian interval timing. The effects on interval timing of modifying ACh levels are quite different from the effects of dopamine manipulations. In rodent studies, increasing ACh activity by systemic injection of centrally active cholinergic agonists gradually changes timing performance, as if the remembered target duration is shorter, while decreasing brain ACh activity level leads to the opposite pattern [49]. Unlike the dopamine clock effect described above, the ACh memory effect develops across several sessions of chronic drug injection, suggesting that it is probably related to how temporal memory is consolidated after each training session and how the remembered temporal content is retrieved under the influence of chronic cholinergic drug injections [50]. In zebrafish, immunohistochemical labelling of choline acetyltransferase (ChAT, an ACh synthesis enzyme) indicates that there are several ChAT-positive areas in the adult forebrain [45]. These include the subpallium and medial habenula, as well as some distributed regions in the ventral diencephalon (figure 2, green areas). The ChAT-positive neurons in the subpallium may be equivalent to the septal cholinergic neurons in the mammalian basal forebrain, especially the nucleus basalis of Meynert (NBM) [45]. In mammals, this region is rich in ACh and projects to the neocortex [51]. Lesioning the NBM in rats reduces brain ACh activity and leads to longer remembered durations [52]. In the timing literature, systemic injection of nicotine has been

shown to increase the speed of the internal clock in rats [53], possibly through its interaction with dopamine systems, an interaction recently shown in zebrafish [54]. Notably, a recent study shows that cholinergic neurons in the rodent medial habenula display spontaneous pacemaking activity at a frequency of 2–10 Hz, a range that can be increased by exposure to nicotine *in vitro* [55]. Whether this spontaneous pacemaking activity is related to any aspect of interval timing remains to be determined.

### (c) Norepinephrine and serotonin

Two other major neuromodulators, NE and serotonin (5-HT), that might be related to timing, are also present in zebrafish [56]. In brief, zebrafish NE is synthesized in the LC, which lies in the metencephalon (part of the brain stem) and projects to the dorsal diencephalon (e.g. pretectum) and ventral telencephalon (subpallium) in its ascending pathway by 3 dpf (figure 2, blue area and its projections) [42]. In human studies, blocking NE reuptake with reboxetine (leading to more NE activity at the synapse) improved the subject's performance in a temporal discrimination task [57]. The effects of NE on timing behaviours are usually attributed to working memory and attention [58].

Regarding 5-HT, a recent study suggests that in zebrafish there are 5-HT-containing neurons in the pineal, pretectum, hypothalamus and raphe nucleus in the hindbrain (figure 2, magenta areas) [46]. In addition, numerous 5-HT projections are present in the dorsolateral and dorsomedial pallium as well as the ventral subpallium. These areas may correspond to the mammalian hippocampus and amygdala as shown in figure 1*b* [6]. Accordingly, we can infer that 5-HT in zebrafish may also participate in the functions of the hippocampus and amygdala, such as memory and emotion. A recent study using calcium imaging shows that neural activity in the dorsal raphe can modulate how visual information is processed when a 5–7-dpf zebrafish larva is in a heightened emotional state [59]. Different emotional states may affect information processing and therefore distort the perception of time [28].

## 4. Behaviour and cognition in larval zebrafish

Given the potential for using zebrafish to investigate the neural circuits underlying interval timing, it is important to develop appropriate behavioural assays. Furthermore, to directly observe brain activity change as a function of training, the behavioural training in zebrafish should be completed during the larval stage, a time window during which the skull is absent and the brain has relatively low lipid content—two ideal factors for non-invasive *in vivo* calcium imaging. The larval stage of zebrafish lasts from 3 dpf to approximately 30 dpf. During this period, the brain is still growing, but most of the critical brain regions and neuromodulatory systems are already functioning. At 5–6 dpf, the yolk of the embryo is completely consumed and the fish's brain must be mature enough to support a complex repertoire of foraging-related behaviour and cognition, possibly including interval timing. Taking this into consideration, the behavioural and cognitive abilities of larval zebrafish first need to be explored. A review of the full behavioural repertoire in larval zebrafish is available elsewhere [60], so here we focus on recent studies of conditioned behaviours because animal timing work

usually requires conditioning techniques. In addition, tasks that can be quickly acquired during the larval stage are of great interest for imaging purposes.

### (a) Conditioned swimming behaviours

Timing studies using laboratory animals require the animals to be conditioned to perform a task. The conditioning task can be Pavlovian, i.e. pairing a conditioned stimulus (CS) with an unconditioned stimulus (US), or instrumental, such as requiring rodents to press a lever to obtain a reinforcer. The task can be appetitive or aversive, but most animal timing studies are based on appetitive conditioning. In fish, swimming can be conditioned, and variables such as swim speed, tail movement and location in a tank can be measured. For example, when adult goldfish were trained in a swimming task by pairing a 5 or 15 s light CS with an aversive US (an electrical shock), the fish displayed a response function (water displacement induced by fish activity) with a peak centred around the scheduled time of shock delivery following 200 training trials distributed across 20 sessions [61]. In a different CS–US aversive conditioning task, both adult [62] and larval [63] zebrafish acquired the CS–US contingency (less than 15 s) after about 10 conditioning trials. In this type of conditioning task, accurate timing is required to ensure that responses do not occur too early or too late, but are optimized.

In an escape/avoidance version of the CS–US conditioning paradigm, in which the fish could prevent US onset (avoidance) or terminate it (escape) by swimming to the non-CS side within 10 s (the CS duration), adult goldfish attained the performance criterion after 120–180 conditioning trials with 10 trials per daily session [19]. In the same study, when a 5-s temporal gap was introduced in between the 10-s CS and US (i.e. trace conditioning), adult goldfish could still attain the performance criterion after about the same amount of training. More importantly, fish that received an ablation of the dorsomedial pallium (equivalent to basolateral/lateral amygdala in mammals) after reaching the learning criterion failed to retain their performance post-operatively, in both trace and non-trace versions of the task. In contrast, fish that received an ablation of the lateral pallium, a region that may be functionally equivalent to the mammalian hippocampus, performed normally in the non-trace task, but not in the trace task. These findings suggest that the dorsomedial pallium is critical for maintaining avoidance responses in an aversive CS–US conditioning task, while the lateral pallium is responsible for keeping track of the temporal gap between the CS and US. In an appetitive CS–US trace conditioning paradigm, juvenile rainbow trout (another ray-finned fish species) learned the task with a 3.4-s trace interval after about three weeks of training [64]. In addition, in a recent appetitive Pavlovian conditioning study, adult zebrafish displayed timed conditioned responses at 4, 8 and 16-s CS–US delay conditions after one month of training (20 trials per day) [65].

Because adult zebrafish are not suitable for non-invasive whole-brain calcium imaging (other than imaging the dorsal surface of the brain after removal of the skull as shown by Aoki *et al.* [9]), a recent study examined the time course of conditioned learning in larval zebrafish [66]. The fish were trained in an aversive visuospatial classical conditioning task in a daily session (six trials per session) continuously from 7 to 42 dpf. Fish began showing improvement on the task when older than 21 dpf and reached the adult performance

level at around 42 dpf. In an operant version of the task in the same study, five-week old fish learned the task within 36 trials (shocks), whereas a group of four-week old fish required 100 shock trials to achieve the same performance level. Fish younger than four weeks performed even worse. In conclusion, behaviourally conditioning larval zebrafish can be difficult compared with conditioning adult fish owing to developmental constraints. Thus, if one wants to conduct calcium imaging using a behavioural conditioning paradigm, one needs to optimize the task parameters to ensure that fish can acquire the task during the larval stage.

### (b) Virtual environment and conditioned tail movement

Another requirement of calcium imaging is that the fish's head must be immobilized. One solution is to provide a virtual reality environment such that the visual feedback the fish receives indicates it is moving (i.e. fictive movement) [67,68]. Another approach that allows the fish to slightly 'move' in a way that does not disturb brain imaging is to permit tail movement, while the remaining body, especially the head, is embedded in agarose. Using this approach, one study showed that a 4-s light CS and a tactile US (that elicits unconditioned tail movement) activated different populations of neurons in the cerebellum, while only a few cerebellar neurons (2.12%) responded to both CS and US [69]. This learning can be observed in 6–8-dpf larvae within seven trials of CS–US pairing. In a similar study, Sumbre *et al.* [70] observed that tail movement was evoked and entrained by a strong rhythmic visual pattern, which included at least 10 cycles of repetitive stimuli and a fixed inter-stimulus interval (ISI). In addition to tail movement, it also elicited synchronized calcium transients in the tectum, a midbrain structure critical for visual information processing [71]. This rhythmic responding pattern extended beyond the offset of the visual stimulus presentation for both the tail movement and the tectal neuron response, as if the fish was following the visual stimulus rhythm after it stopped. The finding suggests that the temporal properties of external stimuli are encoded at the neuronal level and followed by the fish at the behavioural level.

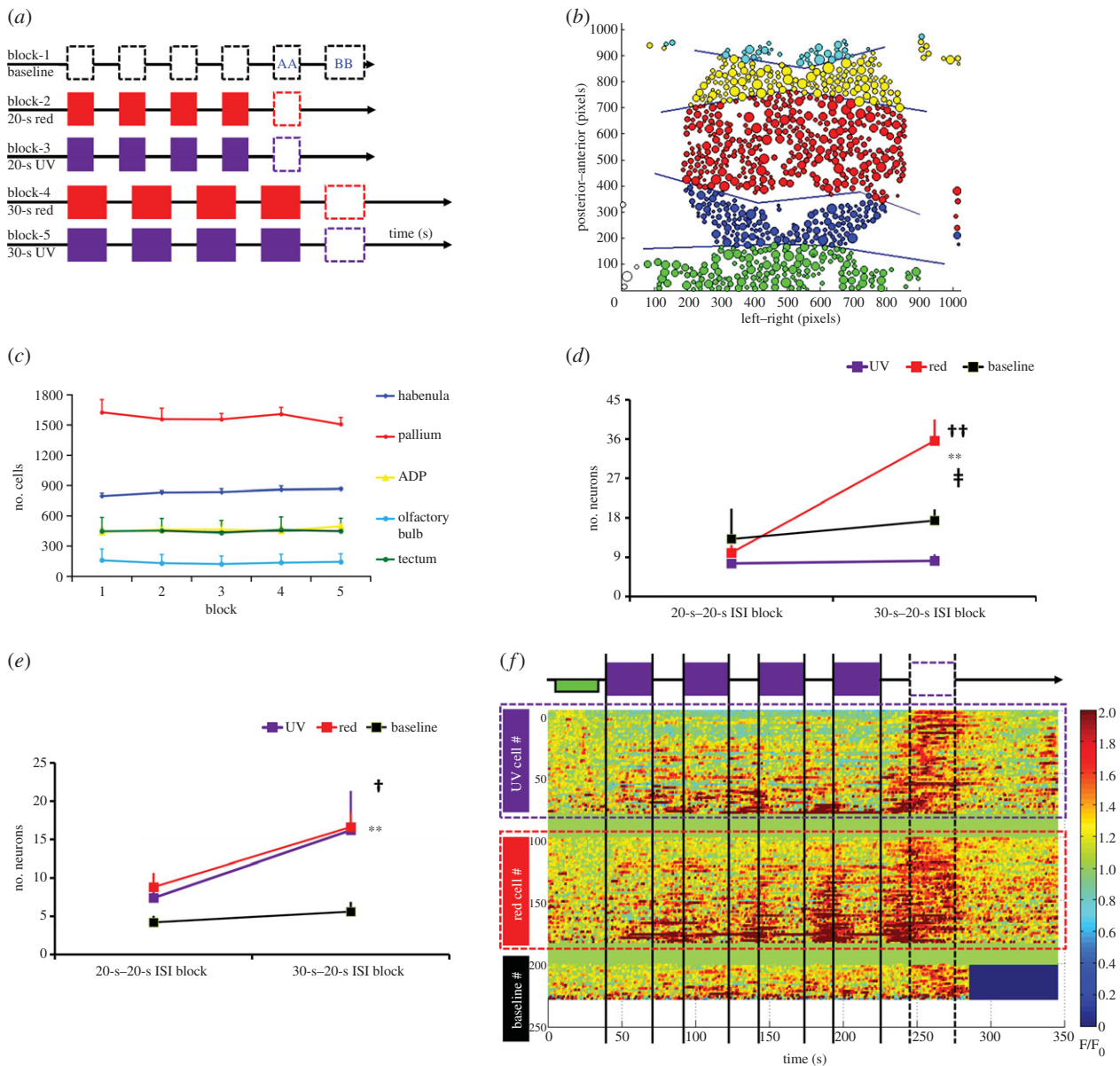
## 5. Time-based expectation in the larval zebrafish brain

Given that a larval zebrafish as young as 4 dpf can repeat the temporal pattern of a series of visual stimuli [70], we assumed that imaging the entire forebrain under a similar condition may reveal the neural circuits involved in this type of timing. We developed a triple transgenic line carrying HuC:Gal4, a pan-neural driver, GAL4<sup>s1011t</sup> and the fluorescent calcium indicator GCaMP3 under the UAS promoter [7] in pigmentless Nacre zebrafish. Five ( $n = 5$ ) larval zebrafish (6–8 dpf) from the F<sub>2</sub> in-crossed generation were imaged under a two-photon microscope. Fish were anaesthetized by a muscle relaxant, mivacurium, 20 min before the experiment and mounted in 2% low-melting agarose in a glass-bottom dish, surrounded by two pairs of LEDs (red and UV) outside the dish and on each side anterior to the fish. Each fish was presented with a stimulus sequence that comprised four repetitions of the same-colour, same-duration stimulus with a fixed 20-s ISI as illustrated in figure 3a. This

visual sequence design was intended to evoke a rhythmic neural response in the brain as shown in the tectum by Sumbre *et al.* [70]. Because fish were imaged under conditions of otherwise complete darkness, each LED illumination was a long flash of light to the whole recording chamber. All five fish completed five blocks of testing: Block 1 was the baseline recording without any stimulus, Blocks 2 and 3 comprised 20-s stimulus (red in Block 2 and UV in Block 3), whereas Blocks 4 and 5 comprised 30-s stimulus presentations with red and UV, respectively. Each block took about 5 min to complete and the inter-block interval was about another 5 min. During light exposure, the brain was imaged at five focal planes, at an interval of 1 s per z-stack.

We noted that a number of neurons fired strongly when a fifth stimulus would have occurred had one been scheduled, although no stimulus was actually delivered as indicated by the fifth rectangular box in figure 3a. We term these cells omitted stimulus response (OSR) neurons, consistent with prior usage [72]. As shown in figure 3b, we divided the brain image into five subregions. The average number of recorded neurons from each subregion across blocks is plotted in figure 3c. A two-way ANOVA treating block (five blocks) and brain regions (five areas) as factors confirmed that there was no significant change ( $p = 0.91$ ) of the number of neurons across five blocks, suggesting that our automated cell segmentation routine using an IMAGEJ algorithm across recording blocks was reliable (see the electronic supplementary material for details). The OSR neurons were defined as those showing lower than a mean of 1.2  $F/F_0$  (a ratio change over baseline fluorescence level) value during the presentation of the stimulus sequence in conjunction with showing higher than 1.7  $F/F_0$  value in at least 3 consecutive seconds during the 20-s window in which a fifth stimulus would have occurred had the stimulus presentation pattern been continued. These thresholding criteria (1.2 and 1.7) ensure that the identified OSR neurons were responding the strongest during the omitted stimulus time period, but were less active during the presentation of the stimulus sequence.

For statistical purposes, we grouped data from Blocks 2 and 3, in which the stimulus duration was 20 s into the first level of the block factor. Data from Blocks 4 and 5 were grouped as the second level of the block factor. The colour factor comprised red, UV and baseline. For the number of identified OSR neurons among the five subregions, a two-way ANOVA on each subregion confirmed a significant colour effect in the habenula ( $p < 0.05$ ) and the pallium ( $p < 0.01$ ). A significant block effect was also observed in the habenula ( $p < 0.01$ ), pallium ( $p < 0.01$ ) and tectum ( $p < 0.001$ ). The interaction between colour and block was significant only in the pallium ( $p < 0.05$ ) region. No OSR neurons were identified in the olfactory bulb or the ADP region. Because the recording area covered a small portion of the tectum only (figure 3b), the tectum data are not representative and are excluded from further discussion. Hence, we focus on the habenula and pallium OSR data. As illustrated in figure 3d, more pallium OSR neurons were observed in the red block after the stimulus duration was increased from 20 to 30 s, which was confirmed by a significant interaction between colour and block ( $p = 0.013$ ) and post-hoc comparisons ( $p < 0.01$  by Fisher LSD). Thus, the effects shown in figure 3d were largely driven by the increase of red OSR neurons. As illustrated in figure 3e, a significant increase of the number of OSR neurons was observed as a function of colour ( $p < 0.05$ ) and block



**Figure 3.** (a) Schematic diagram and time line of the experiment design. The stimulus is turned on four times for 20 s in Blocks 2 and 3 and 30 s in Blocks 4 and 5, all with a fixed ISI at 20 s. Block 1 is baseline recording, during which no stimuli were presented. The missing fifth stimulus (the empty dashed rectangular box) is the time window in which we identify the OSR neurons throughout the brain. In Block 1, the first empty dashed rectangular box (labelled AA) was used as the time window to obtain the baseline number of neurons meeting the OSR criteria for comparison with Blocks 2 and 3. The second empty dashed rectangular box in Block 1 (labelled BB) was used as the baseline window to obtain the OSR for comparison with Blocks 4 and 5. (b) Schematic diagram of cell segmentation of the whole brain using IMAGEJ automated algorithm (see the electronic supplementary materials for details). The brain is dissected into five different regions—blue for the habenua, red for the pallium, yellow for the anterior-dorsolateral pallium, light blue for the olfactory bulb and green for the tectum. The entire brain was scanned at five different z-planes with a distance of 12  $\mu\text{m}$  between planes. With a range of 48  $\mu\text{m}$  from top to ventral, the imaging covers the entire habenua and most of the pallium (1 pixel = 0.215  $\mu\text{m}$ ). (c) Mean ( $\pm$  s.e.m.) number of segmented neurons as a function of five recording blocks in each of the five subregions. There was no significant difference ( $p = 0.91$ ) in the factor block and no significant interaction ( $p = 0.31$ ) between block and brain regions across the five blocks indicating that the number of identified neurons remain at the same level across blocks. (d) Mean ( $\pm$  s.e.m.) pallium OSR neurons for colour and block conditions. The symbol  $\dagger\dagger$  indicates a significant difference for the factor of colour ( $p < 0.01$ ) while the symbol  $**$  indicates a significant difference for the factor of block ( $p < 0.01$ ). The symbol  $\ddagger$  indicates a significant interaction between colour and block ( $p < 0.05$ ). (e) Mean ( $\pm$  s.e.m.) habenua OSR neurons for colour and block conditions. The symbol  $\dagger$  indicates a significant difference for the factor of colour ( $p < 0.05$ ), while the symbol  $**$  indicates a significant difference for the factor of block ( $p < 0.01$ ). (f) Ratio heatmap ( $F/F_0$ ) of habenua OSR neurons from all five fish in the 30-s UV, red and baseline conditions. The green rectangle bar in the beginning of the block indicates the time window (6–40 s) where the mean baseline fluorescence level ( $F_0$ ) was taken. The list of neurons from top to bottom is sorted by ascending standard deviation (s.d. from min to max) during the stimulus sequence, thus neurons with lesser response during the stimulus sequence appear at the top of each colour condition. There were 81 habenua OSR neurons (Rows 1–81) in the UV condition and 83 habenua OSR neurons (Rows 101–183) in the red condition. In the baseline, only 27 habenua neurons (Rows 201–227) met the OSR criteria owing to spontaneous activity in the habenua.

( $p < 0.01$ ), but no significant interaction was found. This suggests that the habenua showed an equivalent increase in OSR neurons in both the red and UV blocks ( $p = 0.92$  by Fisher LSD) when the stimulus duration was increased to 30 s.

In summary, our data confirm the possibility of using whole-brain calcium imaging at single-cell resolution in temporal processing experiments. Specifically, the number of habenua OSR neurons in the red and UV blocks was

significantly increased in later recording blocks, either because of more training experience with the stimulus sequence or because the stimulus duration was increased from 20 to 30 s. By looking at the ratio heatmap in figure 3f, the habenula OSR appears to result from timing of the ISI as some of the OSR neurons began showing excitation at the offset of each stimulus presentation, and reached the highest response level during the omitted stimulus time period. The OSR response observed here is conceptually similar to the anticipatory response shown in the lateral habenula in previous primate studies [35], but differs in that there was no reward or punishment in our paradigm. Therefore, stimulus valence through conditioning is probably not required to evoke a habenula anticipatory response, but a sequence of visual stimuli with a fixed ISI is sufficient. On a related note, the detection and prediction of periodic visual stimulus patterns has also been shown in the retina ganglion cells [72] and the tectum [70], but our experiment differs in the length of the stimulus duration, ISI duration (in the millisecond range in [72]), and the number of stimulus repetitions (a minimum of 10 repetitions in [70]) that are required to evoke an OSR. Here, three exposures of the fixed ISI were enough to evoke an OSR in the habenula after the fish had some experience with the same stimulus sequence. Our data also indicate that a 6–8-dpf larval zebrafish can time up to 20 s within 1 h of training.

## 6. Future interval-timing studies using zebrafish

Zebrafish offer several advantages for the study of the neural mechanisms underlying interval timing in vertebrates. For example, genetically encoded calcium indicators that report calcium-dependent neural activity can be easily developed and maintained in zebrafish to establish reliable transgenic lines, as demonstrated by our data. By using the Gal4/UAS system [73], specific neural populations can be labelled with Gal4 driver lines based on these cells' genetic properties [7]. On the behavioural side, there are two ways to conduct timing studies—motor timing or perceptual timing. The motor timing approach, such as swimming in a certain location of a tank at a certain time window, may not be plausible in young larval zebrafish because of two concerns. First, it may require a sustained amount of training for a larval zebrafish to learn a visuospatial task, as demonstrated by Valente *et al.* [66], such that by the time the fish has acquired the task, the brain is no longer accessible for non-invasive imaging techniques, unless the skull is removed, and even then only the brain surface is accessible for imaging [9]. Second, a behaving and moving zebrafish head is certainly not ideal for imaging. However, there are exceptions to the second concern. A recent study successfully applied calcium imaging of the tectum while a 5-dpf larval fish was capturing a moving paramecium (a protozoan that larval fish hunt for food) [74]. This study was possible because larval fish often swim in

intermittent bouts, thereby allowing the calcium signals in a non-moving tectum to be recorded in between the swimming bouts while the prey-capturing behavioural sequence was still ongoing. This new experimental approach may provide some hints for developing future behavioural and cognitive tasks, including timing tasks.

The use of perceptual timing tasks that allow investigation of how the fish perceives the duration of a stimulus or a sequence of stimuli is more plausible given the current technical limitations. A perceptual timing task can also be combined with restricted tail movement as some previous studies have shown promising results (CS–US conditioning in [69] and CS-entrained rhythms in [70]) in young larval fish, with minimum training required. It is also possible to train a larval fish in a different environment before imaging and then record the brain calcium signals to the same stimulus under a microscope, but this depends on how well a fish can generalize what it learned across different contexts.

State-of-the-art research on interval timing suggests that it is a cognitive function that relies on multiple brain regions, with cortico-striatal circuits currently under the research spotlight [75,76]. However, the details of how neurons in cortico-striatal circuits generate the sense of time remain to be elucidated. Using traditional electrophysiological recording techniques to record only a subset of neurons in the circuits is not sufficient to reveal the entire timing network. Imaging studies of the entire zebrafish forebrain at single-cell resolution (e.g. figure 3b) may provide this knowledge. For example, by combining whole-brain calcium imaging in conjunction with exposing fish to psychoactive drugs that alter the neurotransmission of dopamine or ACh, we can examine how clock-speed and temporal memory consolidation/retrieval are modulated at cell and circuit levels.

In addition, a developing brain following intense behavioural training may also be dynamically changing by itself owing to, for example, neurogenesis, synaptogenesis and synaptic pruning. As traditional *in vivo* extracellular electrophysiological recording techniques cannot distinguish between new and old cells over the course of several days, learning-dependent cellular and synaptic modification cannot be represented by frequency information of action potentials recorded near the electrodes. In contrast, whole-brain calcium imaging in zebrafish will be able to preserve these dynamic changes as there are calcium indicators for imaging cell bodies [77] and synaptic terminals [78] and structural changes owing to development or behavioural training [79]. As interval timing is a complex cognitive function that usually requires several days of training to adjust behaviour properly, its underlying cellular and synaptic change over the course of training will become visible under a microscope.

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