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INSTRUMENTS AND TECHNIQUES

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"In vivo" monitoring of neuronal network activity in zebrafish by two-photon Ca^{2+} imaging

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Abstract The zebrafish larva is a powerful model for the analysis of behaviour and the underlying neuronal network activity during early stages of development. Here we employ a new approach of "in vivo" Ca^{2+} imaging in this preparation. We demonstrate that bolus injection of membrane-permeable Ca^{2+} indicator dyes into the spinal cord of zebrafish larvae results in rapid staining of essentially the entire spinal cord. Using two-photon imaging, we could monitor Ca^{2+} signals simultaneously from a large population of spinal neurons with single-cell resolution. To test the method, Ca^{2+} transients were produced by iontophoretic application of glutamate and, as observed for the first time in a living preparation, of GABA or glycine. Glycine-evoked Ca^{2+} transients were blocked by the application of strychnine. Sensory stimuli that trigger escape reflexes in mobile zebrafish evoked Ca^{2+} transients in distinct neurons of the spinal network. Moreover, long-term recordings revealed spontaneous Ca^{2+} transients in individual spinal neurons. Frequently, this activity occurred synchronously among many neurons in the network. In conclusion, the new approach permits a reliable analysis with single-cell resolution of the functional organisation of developing neuronal networks.

Keywords Ca^{2+} imaging · In vivo · Neuronal network activity · Two-photon excitation · Zebrafish

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Introduction

The development of neuronal networks is regulated by spontaneous and experience-dependent activity [36]. Better understanding of the assembly and functional organization of neuronal networks during development requires the ability to follow simultaneously the spatio-temporal distribution of activity in a large population of neurons "in vivo". However, it is difficult to gain access to tissue deep in intact preparations such as the mammalian brain [20]. Recently, the mechanisms underlying the activity patterns during embryonic stages of development have been examined in chick, *Xenopus* and zebrafish [12, 18, 21], vertebrates with easily accessible embryos [27]. In these models of embryonic development neuronal activity has been investigated mostly with Ca^{2+} imaging, as action potentials trigger Ca^{2+} transients due to the activation of voltage gated Ca^{2+} channels [29].

A frequently used approach for the labelling of neurons in these preparations is the injection of dextran-conjugated Ca^{2+} indicator dyes. These dyes label identified but restricted neuronal populations that have processes at the site of dye ejection. The method was introduced by O'Donovan and colleagues in the isolated spinal cord of chick embryos and showed rhythmic motor activity in motoneurons and interneurons in vitro [21, 22]. This labelling technique was first employed in vivo in the zebrafish [12]. The zebrafish is transparent during early stages of development and therefore ideally suited for imaging techniques. After the labelling of selected groups of spinal cord and hindbrain neurons by the injection of dextran-conjugated Ca^{2+} indicators into the muscle or the spinal cord, the dynamics of intracellular Ca^{2+} can be imaged [12]. This has facilitated the examination of the neuronal basis of motor behaviour in zebrafish larvae [12, 13, 23, 25, 32] and provided interesting information, especially during escape responses, by demonstrating strong population discharges. Another useful approach enabling the labelling of a larger population of cells is the injection of dextran-conjugated Ca^{2+} indicator dyes into individual blastomeres of zebrafish embryos [8]. This

technique has been used to detect spontaneously occurring neuronal Ca^{2+} transients in embryos [2,37] or during escape behaviour in larval spinal neurons [8].These approaches are limited, however, by the number of arbitrarily stained neurons and the length of the incubation period, lasting 12–24 h. This prolonged incubation period is of particular concern when studying developmental issues, especially because chronic exposure to Ca^{2+} indicators can interfere with normal developmental processes [3, 9].

As an alternative to these approaches, the membrane-permeable acetoxymethyl (AM) ester derivatives of Ca^{2+} indicator dyes have also been used for labelling neurons in living animals. For example, the spinal cord of semi-intact *Xenopus* embryos has been loaded by bath incubation with AM Ca^{2+} indicators after the removal of the skin and muscle bulk overlaying the somites. Using this procedure, it could be shown that spontaneously occurring Ca^{2+} transients in growth cones regulate the rate of axonal growth [18]. Recently, Stosiek et al. have succeeded in using these AM-dyes for labelling neurons in anaesthetized mice [31]. By injecting a bolus of AM Ca^{2+} indicators into the cortex they were able to stain a large population of neurons. This so called "multi-cell bolus loading" enabled simultaneous two-photon recordings of spontaneous and sensory-evoked activity in hundreds of cortical neurons with single-cell resolution [31].

In the present study we ejected a bolus of membrane-permeable Ca^{2+} indicator dyes into the spinal cord of living zebrafish ranging from 4-day-old larvae to 2-month-old fry. At earlier stages this resulted in robust staining of the entire spinal cord. Using two-photon microscopy we could make simultaneous, long-lasting recordings of Ca^{2+} transients from spinal neurons with single-cell resolution. These Ca^{2+} transients were evoked either by pharmacological and sensory stimulation or occurred spontaneously. Advantages of this technique are the rapid and robust staining combined with high-resolution Ca^{2+} imaging from a large population of neurons during early stages of development.

Materials and methods

Animal preparation and dye loading

Experiments were performed on zebrafish (*Danio rerio*) larvae raised at 28.5 °C and obtained from a zebrafish colony maintained according to established procedures [33]. All experiments were carried out in compliance with institutional guidelines. Intact zebrafish larvae (4–60 days old) were anaesthetized or paralysed in Evans solution (in mM): 134 NaCl, 2.9 KCl, 2.1 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.8, 290 mOsm, containing 0.2% tricaine (MS-222, Sigma, Deisenhofen, Germany). For experiments on spontaneous and sensory-evoked activity, tricaine was replaced by 0.01–0.02% mivacurium chloride (Mivacron, Glaxo Smith Kline, Germany). The effect of both reagents was reversible as the larvae rapidly recovered a well-coordinated swimming activity when transferred back to a drug-free solution. The immobilized larvae were then embedded in 2% low-melting-point agarose (Gibco BRL, Burlington, Canada) and placed on their sides in the recording chamber.

All membrane-permeable Ca^{2+} indicator dyes used (Fura PE3 AM, TefLabs, Austin, Tex., USA; Calcium Green-1 AM, Indo-1 AM or Magnesium Green AM, Molecular Probes, Eugene, Ore., USA) were, for each experiment, freshly dissolved in DMSO with 20% pluronic (Molecular Probes) to yield a 10 mM stock solution and further diluted in Evans solution [10] to a final concentration of 1 mM, as reported previously [31]. A fine borosilicate glass pipette (Hilgenberg, Malsfeld, Germany; 6–8 MΩ resistance when filled with the Evans solution) was used to eject the dyes directly into the spinal cord. Under visual control (60× water-immersion objective of an upright microscope), the pipette was slowly advanced into the spinal cord through the overlaying skin, muscle and dura at the level of the anus (Fig. 1B) using a custom-made, standard, patch micromanipulator. Once the pipette reached its destination in the centre of the spinal cord, the Ca^{2+} indicator solution was ejected repetitively (10–20 times) by brief (50 ms), low pressure (7 psi) pulses using a Picospritzer II (General Valve Fairfield, N.J., USA). Ca^{2+} recordings started 30–60 min after the dye ejection.

Ca2+ imaging

Fluorometric Ca^{2+} measurements were performed at room temperature using a custom-built two-photon laser-scanning microscope based on a mode-locked laser system operating at 720–870 nm wavelength, 80 MHz pulse repeat, <100 fs pulse width (Tsunami and Millenia, Spectra Physics, Mountain View, Calif., USA) and a laser-scanning system (MRC 1024, Bio-Rad, UK) coupled to an upright microscope (BX50WI, Olympus, Tokyo, Japan) and equipped with a 60×1.0 NA water immersion objective (Fluor 60×, Nikon, Tokyo, Japan) [15]. Excitation wavelengths ranged from 750 nm for Indo-1, 790 nm for Fura PE3 and Calcium Green-1 to 800 nm for Magnesium Green. For imaging spontaneous Ca^{2+} transients, a population of spinal neurons, usually within an entire somite (>100 neurons at a time), was monitored simultaneously for up to four periods lasting 10–40 min, respectively. The image in Fig. 1A from a zebrafish in which the spinal cord was stained with Calcium Green-1 AM is the superimposition of a transmitted light and a fluorescence image each taken with a digital camera (Coolpix 950, Nikon Corporation, Tokyo, Japan). For the acquisition of the fluorescence image the zebrafish larva was excited at 488 nm with an argon laser using a confocal scanner unit (CSU-10, Yokogawa, Tokyo, Japan).

Pharmacology

Glutamate (100 mM, Na-glutamate, Sigma), glycine (1 M, Roth, Karlsruhe, Germany) and GABA (0.5 M, Sigma) were applied iontophoretically (MVCS-02, npi, Tamm, Germany) from a fine glass pipette (20–30 MΩ) for 50–100 ms with retaining and ejection currents of 2–5 nA and 40–70 nA, respectively. In the experiments illustrated in Fig. 4, a second glass pipette was used to pressure eject strychnine (1 μM in Evans solution, Sigma).

Data analysis

Background-corrected images were analysed off-line with a LabView-based software package (Fast Analysis 1.0, National Instruments, Austin, Tex., USA) and Igor software (WaveMetrics, Lake Oswego, Ore., USA). Changes in Ca^{2+} levels were calculated as $\Delta F/F$ (Calcium Green-1 AM and Magnesium Green AM) or $-\Delta F/F$ (Fura PE3 AM and Indo-1 AM), which is the ratio between the fluorescence change (ΔF) and the baseline fluorescence before stimulation (F).

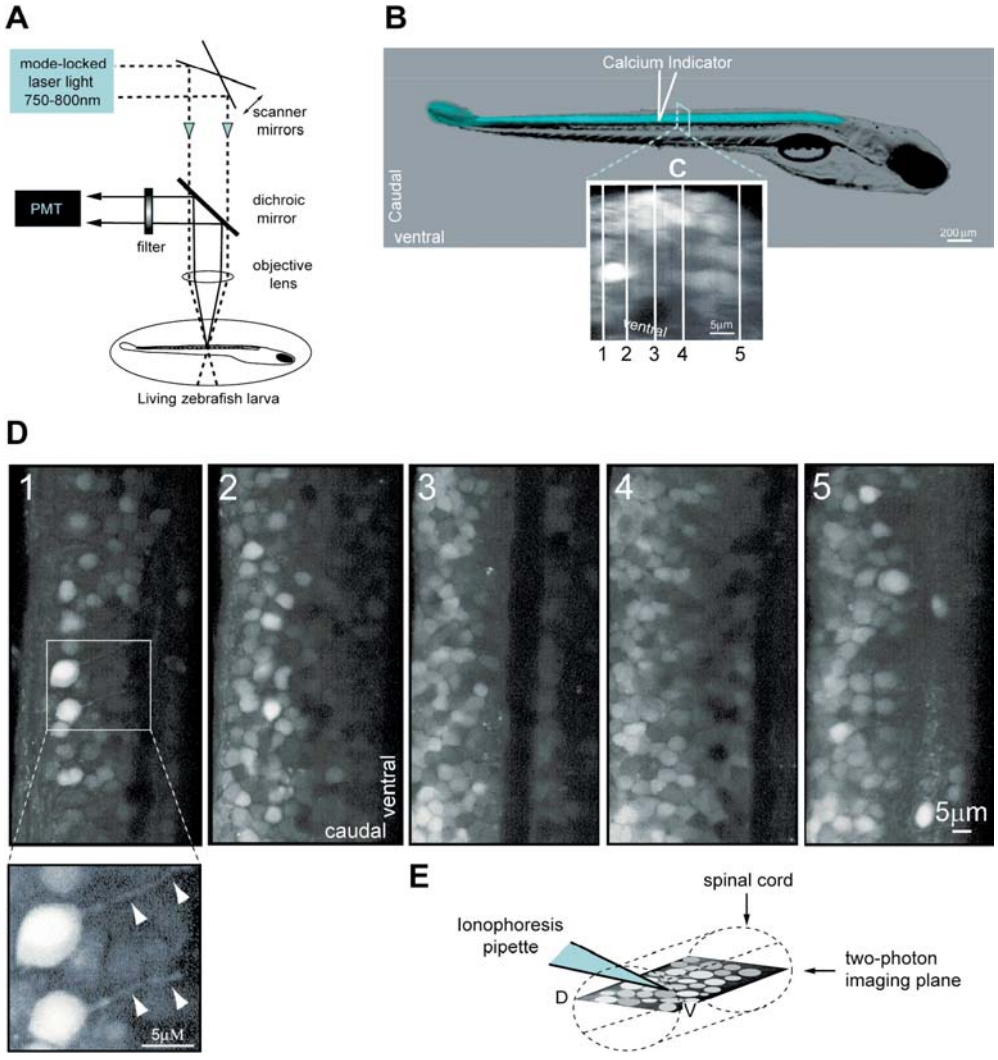

Fig. 1A–E The experimental approach. **A** Illustration of the experimental arrangement for two-photon imaging of living intact zebrafish larva. The different Ca^{2+} indicators are excited by the appropriate wavelength of pulsed laser light generated by a tunable Ti:Sapphire laser system. The emitted light is collected by a photomultiplier tube (*PMT*). **B** Superimposition of a transmitted light and a confocal fluorescence image of an intact living zebrafish larva (7 days old). Injection of Calcium Green-1 AM through a pipette results in bright staining of the whole spinal cord (*green*). **C** Two-photon vertical section through a Fura PE3 AM loaded spinal cord from a 11-day-old larva. The location of the section within the larva is illustrated in **B**. *White, numbered lines* indicate the position of the sagittal images illustrated in **D**. **D** Two-photon sagittal images demonstrating the loading of virtually all neurons of an entire somite. *Inset*: single neurons and even fine processes (*arrowheads*) are clearly visible. **E** Schematic illustration of the experimental arrangement for the iontophoresis of glutamate, glycine or GABA from a fine pipette inserted directly into the spinal cord of the living intact zebrafish. Ca^{2+} signalling was imaged in neurons in the vicinity of the pipette tip (*D* dorsal, *V* ventral)

Results

For investigating neuronal network activity during development in vivo we established a procedure for Ca^{2+} imaging in zebrafish larvae based on a loading technique previously described for brain slices [24, 35] and, more recently, in anaesthetised mice [31]. Intact living zebrafish larvae (4–60 days old) were immobilised and embedded on their sides in a recording chamber containing agarose (Fig. 1A, see Methods). Membrane-permeable Ca^{2+} indicators were then ejected as a bolus through a fine glass pipette by repetitive short pressure pulses (see Methods) directly into the spinal cord at somite No. 14, at the level of the anus (Fig. 1B, 7-day-old larva). At early developmental stages this procedure stained the entire spinal cord in 30–60 min. This is shown in the composite photomicrograph in Fig. 1B in which the spinal cord of a 7-day-old zebrafish larva is visible as a green band after staining with Calcium Green-1 AM. Imaging the spinal cord at high resolution using two-photon microscopy (experimental set-up illustrated in Fig. 1A) showed that a large proportion of spinal neurons was brightly stained (Fig. 1D). These selected sagittal images (localised along the vertical two-photon image in Fig. 1C) show the

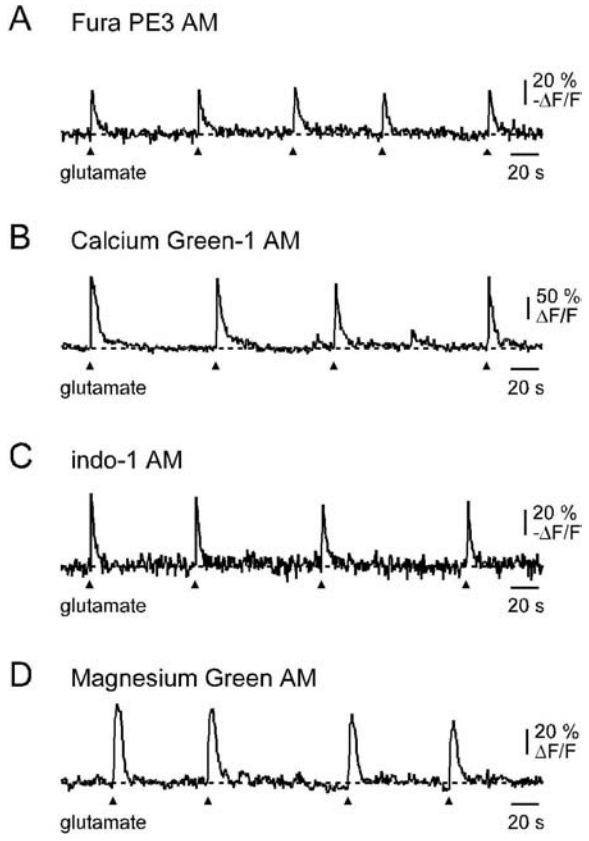

Fig. 2A–D Glutamate-evoked Ca^{2+} transients. Repetitive iontophoretic application of glutamate (*arrowheads*) evoked robust Ca^{2+} transients in spinal neurons loaded with Fura PE3 AM in **A**, Calcium Green-1 AM in **B**, Indo-1 AM in **C** and Magnesium Green AM in **D**

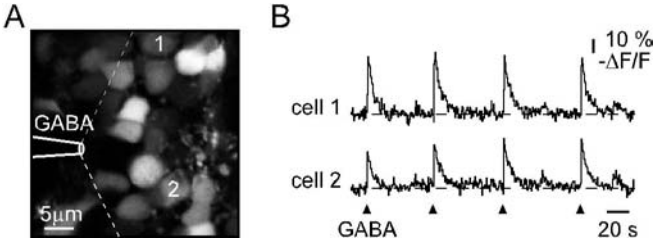

Fig. 3A, B GABA-mediated Ca^{2+} transients. **A** Two-photon image from a sagittal section of the spinal cord stained with Fura PE3 AM (10-day-old zebrafish larva). GABA was applied iontophoretically from a pipette (location illustrated schematically). **B** Repetitive iontophoretic applications of GABA (*arrowheads*) triggered robust Ca^{2+} transients in the spinal neurons marked with *1* and *2* in **A**

neuronal population of an entire somite (No. 12) in an 11-day-old zebrafish larva stained with Fura PE3 AM. Some of these spinal neurons extended fine processes (Fig. 1D and inset below). As reported previously, zebrafish exhibit intrinsic fluorescence arising from pigmentation [32]. In the trunk of zebrafish larvae the pigments are however distributed only on the skin. Fluorescence recordings from the spinal cord thus suffer no interference from intrinsic fluorescence signals.

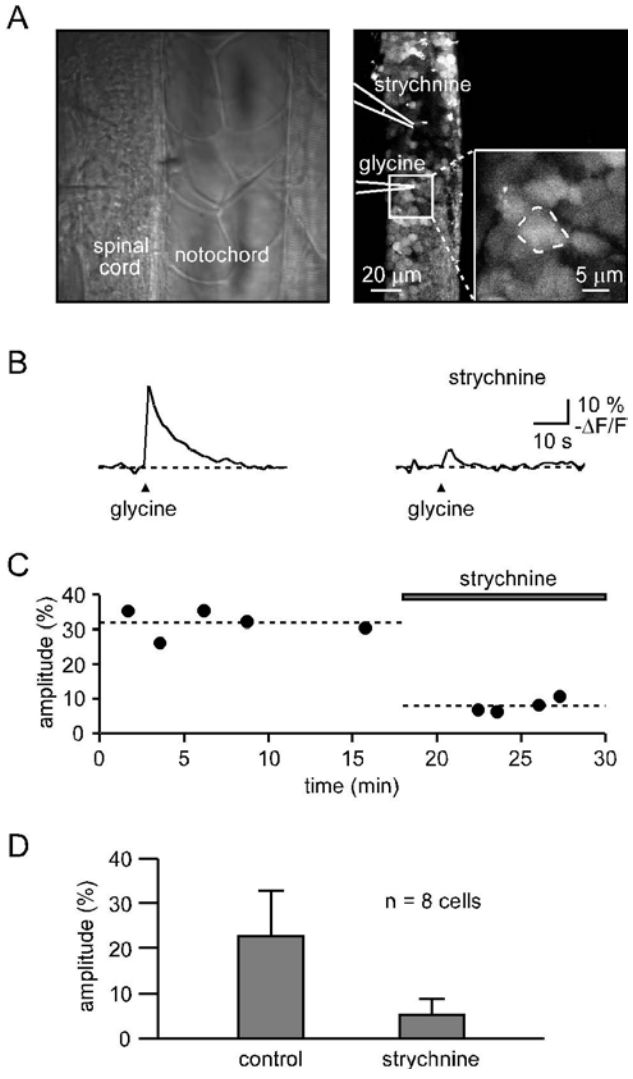

Fig. 4A–D Glycine-mediated Ca^{2+} transients. **A** Transmitted light (*left*) and two-photon fluorescence image (*right*) of the spinal cord in an intact living zebrafish larva (7 days old) loaded with Fura PE3 AM. The position of the glass pipettes containing glycine (*bottom*) and strychnine (*top*) is visible on the *left* and drawn in the *right* image. The *inset* in the *right bottom* of the two-photon image (*right*) shows a high-power view of the spinal neurons around the pipette containing glycine. **B** Iontophoretic application of glycine evoked Ca^{2+} transients in the neuron illustrated in **A** (*right*, *inset*). These Ca^{2+} transients were blocked by pressure ejection of the glycine receptor antagonist strychnine (1 µM). Signals are averages of four individual signals. **C** Plot of peak amplitudes of Ca^{2+} transients during repetitive glycine applications (same experiment as in **B**). The transients were blocked by the injection of strychnine. *Dashed lines* represent the mean amplitudes before and after the ejection of strychnine. **D** Summary of the effect of strychnine on glycine-mediated Ca^{2+} transients (n=8 cells taken from n=4 larva, t-test P<0.001)

Besides Fura PE3 AM and Calcium Green-1 AM, the labelling of spinal neurons was also feasible using Indo-1 AM and Magnesium Green AM (see Fig. 2, described below). From all indicators tested, Fura PE3 AM (excitation at 790 nm) and Indo-1 AM (750 nm) were brightest at resting conditions. In contrast, neurons stained

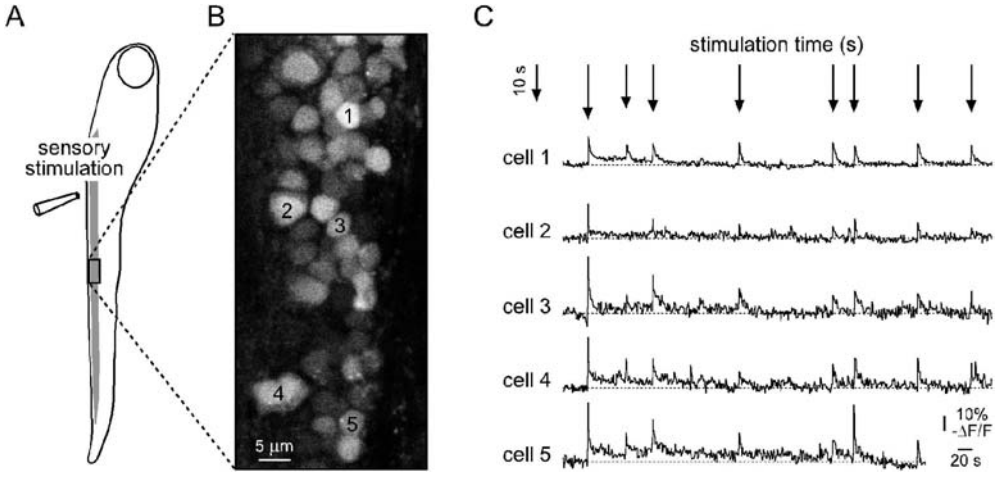

Fig. 5A–C Sensory-evoked Ca^{2+} transients. **A** Schematic illustration of the experimental arrangement. The zebrafish larva was stimulated repetitively with an air puff from a glass pipette placed four somites away from the imaging site. **B** Two-photon image demonstrating spinal cord neurons (loaded with Fura PE3 AM) in which the Ca^{2+} transients were measured in response to the sensory stimulation. **C** Traces of the fluorescent changes evoked in the five neurons indicated in **B**, due to sensory stimuli applied repetitively as indicated by the *upper arrows*

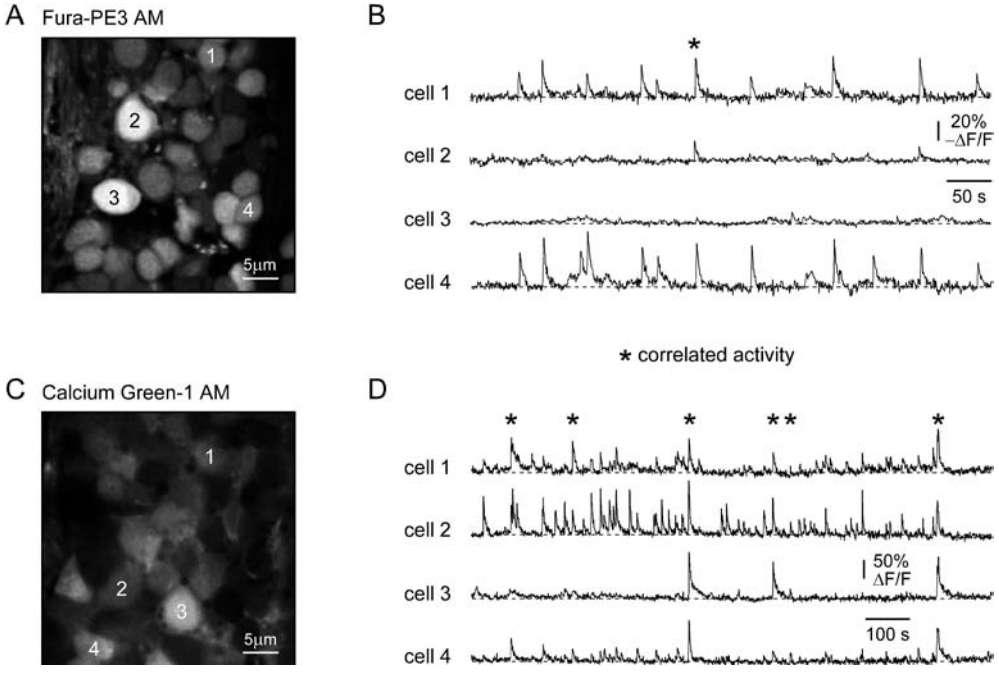

Fig. 6A–D Spontaneous Ca^{2+} transients. **A** Two-photon image of spinal neurons in an intact living zebrafish larva loaded with Fura PE3 AM. **B** Spontaneous Ca^{2+} transients observed in the neurons indicated in **A**. *Correlated activity in all examined cells. **C** Two-photon image of spinal neurons in an intact living zebrafish larva loaded with Calcium Green-1 AM. **D** Spontaneous Ca^{2+} transients observed in the neurons indicated in **C**. *Correlated activity in all examined cells

with Calcium Green 1 AM (790 nm) and the low-affinity Ca^{2+} indicator Magnesium Green AM (800 nm) were dimmer at basal Ca^{2+} levels and required more than 4 times higher excitation levels for equal fluorescence signals than Fura PE3 AM and Indo-1 AM.

The vitality of the neurons stained with this loading method and the dynamic properties of the dyes were examined by evoking Ca^{2+} transients in spinal neurons upon iontophoretic application of glutamate (illustrated in Fig. 1E). Fig. 2 shows that brief applications of glutamate (50–100 ms) evoked robust Ca^{2+} transients in spinal neurons stained with Fura PE3 AM (Fig. 2A), Calcium Green-1 AM (Fig. 2B), Indo-1 AM (Fig. 2C) or Magnesium Green AM (Fig. 2D). The dynamic behaviour of these dyes depended on their affinity for Ca^{2+}. Ca^{2+} transients recorded with the high affinity Ca^{2+} indicator dyes Fura PE3, Indo-1 and Calcium Green-1 had similar decay time constants (decay time constants of 3.7±0.4, 3.1±0.5 and 3.1±0.3 s, respectively; n=5). In contrast Ca^{2+} transients recorded with Magnesium Green had smaller decay time constants (1.7±0.2 s, mean±SEM, n=5) as expected from the lower affinity for Ca^{2+}.

In addition to glutamate, which contributes to the underlying rhythmic synaptic drive during swimming [6],

iontophoretic applications of GABA (Fig. 3B) evoked robust Ca^{2+} transients reliably in spinal neurons of a 10-day-old zebrafish larva. Similarly glycine, which also contributes to the synaptic drive during swimming [6], evoked Ca^{2+} transients in a 7-day-old zebrafish larva (Fig. 4A and B) and these signals were blocked by strychnine (Fig. 4B and C).

In zebrafish larvae, touch responses trigger a well-known escape reflex [12]. We visualized the associated Ca^{2+} transients in the spinal network of paralysed zebrafish (see Methods) in response to single sensory stimuli delivered to the skin (Fig. 5A). Figure 5 shows Ca^{2+} transients evoked in distinct neurons of the spinal network. These responses were produced by sensory stimuli given four somites rostral to the site of imaging using brief pressure puffs from a pipette (experimental arrangement shown in Fig. 5A). The Ca^{2+} transients in these spinal neurons (Fig. 5B) occurred repetitively in response to applied sensory stimuli (upper arrows, Fig. 5C) and represent the largest Ca^{2+} signals recorded under these conditions.

Interestingly, in addition to sensory-evoked Ca^{2+} transients in spinal neurons, we also observed spontaneously occurring Ca^{2+} transients. For the analysis of such network activities during early developmental stages we made long-term recordings (30–40 min) in paralysed zebrafish larvae stained with Fura PE3 AM (Fig. 6A and B), Calcium Green-1 AM (Fig. 6C and D) or Indo-1 AM (not shown). As shown in Fig. 6B and D, individual spinal neurons showed asynchronously occurring Ca^{2+} transients which were interrupted by recurrently occurring synchronous Ca^{2+} transients that involved the entire network (indicated by asterisks, Fig. 6B, D). Similar spontaneous activity patterns were observed in 7- to 20-day-old zebrafish larvae (n=6 animals).

Discussion

In this study we introduce a simple and reliable approach for staining large numbers of neurons, if not the entire spinal population, in the intact, living zebrafish at key developmental stages. We demonstrate that the combination of this approach with two-photon imaging permits an in vivo functional analysis at the level of individual neurons.

In vivo labelling of neuronal networks in developing zebrafish

Compared with the currently used labelling methods with dextran-conjugated dyes in developing zebrafish, the bolus loading technique for the staining of neuronal networks gave much faster labelling. Neuronal staining was achieved quite rapidly (within 30 min) compared with at least 12–24 h in the case of dextran-conjugated Ca^{2+} indicators [8, 9, 12]. Moreover it has been shown that the prolonged presence of Ca^{2+} indicators during embryonic development can disrupt motoneuron development by buffering intracellular Ca^{2+} [3, 9]. The rapidity of the bolus staining procedure may avoid such interference with Ca^{2+}-dependent processes of neuronal development. Another advantage of the bolus injection approach is the loading of most, if not all, spinal neurons, thus revealing the Ca^{2+} dynamics of essentially the entire spinal network. This is in contrast to injection of dextran-conjugated Ca^{2+} indicators in the blastomere stage, which generates chimeric staining patterns in a fraction of the neural population, and to retrograde staining of neurons which selectively labels neurons that extend axons to the site of injection where they may be damaged. The bolus labelling approach allows the resolution of neuronal somata and is thus well suited for population imaging, but only could occasionally fine cellular processes be observed. The dextran-conjugated dyes may thus be preferable for subcellular imaging, e.g. of fine processes [17]

An additional, new aspect of our approach with zebrafish is the use of two-photon microscopy. With this imaging technique we could follow, for the first time, neuronal network activity in zebrafish larvae continuously during prolonged recording times (30–40 min), because of the limited phototoxicity and photobleaching. This duration is far greater than the acquisition intervals of previous studies using confocal microscopy [25].

Ca^{2+} imaging of neuronal network activity

In this study we demonstrate for the first time GABA- and glycine-mediated Ca^{2+} transients in vivo. Glycine- or GABA-mediated Ca^{2+} transients arise presumably because the reversal potential of the glycine- and GABA-mediated Cl^- conductance is above the resting potential. The phenomenon of depolarizing Cl^- has been reported previously in a wide variety of developing networks (for review see [4]) and was also suggested for spinal neurons of the zebrafish embryo recorded during non-invasive cell-attached recordings [26] and in preliminary perforated-patch experiments in the zebrafish larva [7]. Another possibility is that in our imaging experiments, during which we cannot use TTX to block synaptic activation, the observed Ca^{2+} transients were due to disinhibition and thus activation of other neurons that do not respond directly to glycine. This possibility is, however, unlikely for several reasons. The first is the limited spread of glycine or GABA, due to the very short iontophoretic pulse (~50 ms). Second, there was an immediate and tight temporal correlation between the occurrence of the Ca^{2+} transient in response to glycine or GABA iontophoresis (see Figs. 3 and 4). Third, depolarizing glycinergic responses (blocked by strychnine) as large as 10 mV are observed in the presence of TTX and of glutamatergic and cholinergic antagonists in preliminary perforated-patch recordings from spinal neurons in situ (E. Brustein, P. Drapeau, unpublished observations). We conclude therefore that our non-invasive imaging approach provides

direct evidence for the depolarizing effect of glycine and GABA in the developing locomotor network of the zebrafish in vivo and is a novel finding in a living, intact animal. We found spontaneous, asynchronously occurring rises in intracellular Ca^{2+} in individual spinal cells that were interrupted by synchronous recurrent Ca^{2+} transients involving the entire spinal network. The presence of Ca^{2+} transients has been shown in many developing neuronal circuits in vitro [11, 14, 15, 34]. In addition, recent studies have detected spontaneous glycinergic and GABAergic synaptic activity in the zebrafish [1, 19], such as during fictive locomotion [6]. Together, these results support the hypothesis that GABA and glycine could synaptically trigger Ca^{2+} transients in spinal neurons of zebrafish larvae.

These spontaneous activity patterns are thought to contribute to the refinement of neuronal networks [36] by the regulation of neuronal gene expression [16], differentiation [30] and synaptic maturation [28]. In fact, buffering intracellular Ca^{2+} in embryonic zebrafish disrupts the axonal outgrowth in spinal motoneurons [3]. The spontaneously occurring Ca^{2+} transients may also reflect activation of spinal neurons that may be part of the neural network producing behaviourally relevant output such as locomotion [5, 6, 10].

In conclusion, the combination of the bolus loading technique and two-photon microscopy in paralysed zebrafish larvae is a simple and powerful approach for recording neuronal network activity during embryonic stages of development. In combination with genetic manipulations that are well established in the zebrafish, this imaging approach will facilitate the study of developmental processes related to neuronal network assembly and its functional organization.

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