The zebrafish brain in research and teaching: a simple in vivo and in vitro model for the study of spontaneous neural activity

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CURRENTLY, zebrafish (Danio rerio) have established themselves as a key animal model in neuroscience. Behavioral, genetic, and immunohistochemical techniques have been used to describe the connectivity of diverse neural circuits. However, very few studies have used zebrafish to understand the function of cerebral structures or to study neural circuits. Information about the techniques used to obtain a workable preparation is not readily available. Here, we describe a complete protocol for obtaining in vitro and in vivo zebrafish brain preparations. In addition, we performed extracellular recordings in the whole brain, brain slices, and immobilized nonanesthetized larval zebrafish to evaluate the viability of the tissue. Each type of preparation can be used to detect spontaneous activity, to determine patterns of activity in specific brain areas with unknown functions, or to assess the functional roles of different neuronal groups during brain development in zebrafish. The technique described offers a guide that will provide innovative and broad opportunities to beginner students and researchers who are interested in the functional analysis of neuronal activity, plasticity, and neural development in the zebrafish brain.

extracellular recordings; development; neural circuits

zebrafish brain.

METHODS

Larval and young adult zebrafish (D. rerio, strain AB-3, Zirc; Fig. 1B) were housed in the zebrafish facility at Reykjavik University in a zebrafish aquatic housing system (Aquatic Habitats, Apopka, FL). The housing system is a self-contained bench-top unit with a capacity of 740 zebrafish (1.35 l volume), 3 gallons/min recirculation system, thermal control (28.5°C), ambient light control (fish were maintained on a 14:10-h light-dark cycle), filters, and a ultraviolet sterilizer.

Zebrafish Brain Assay In Vitro

Whole brain preparation. Larval or young adult zebrafish (Fig. 1B) were anesthetized in a solution of distilled water and 0.02% MS-222 (tricaine methanesulfonate, Sigma-Aldrich, St. Louis, MO). Animals were fixed with insect pins in a Sylgard-covered petri dish containing artificial cerebrospinal fluid (aCSF) that consisted of the following (in mM): 131 NaCl, 2 KCl, 1.25 KH2PO4, 2 MgSO4, 10 glucose, 2.5 CaCl2, and 20 NaHCO3. The aCSF was maintained at room temperature and a pH of 7.4. First, the skin and skull bones of the zebrafish head were removed. After the brain was exposed, the cranial nerves permitted the remote manipulation of neurons, neural circuits, and behavior in zebrafish larvae (8). Therefore, zebrafish have become a widely used model, and they have been used in the classroom to teach important biological concepts, particularly in genetics and development (12). The advantages of using zebrafish, which are an excellent model for research and education, include low cost, low maintenance, rapid development, ease of genetic manipulation, genomic similarities with humans, ease of external fertilization, and the possibility to produce eggs and embryos continuously with only a few fish. Other important features include fast development, a clear pattern of development, transparency at early ages, and the relatively large size of the embryos and larvae, which facilitate manipulation. In addition, it is possible to buy wild-type, mutant, and transgenic strains of zebrafish at a low cost (35).

Currently, technical details concerning the techniques used to obtain a workable preparation are not gathered or readily available. In this article, we describe, in detail, in vitro and in vivo protocols for obtaining brain preparations of larval and adult zebrafish to study neural activity during development. These preparations can be used for electrophysiological studies of spontaneous or evoked activity in the zebrafish nervous system, to describe the patterns of activity in specific brain regions, and to track neural circuits during development. The use of our technique, together with other approaches, such as pharmacological tests and genetic manipulations, offers new possibilities for the functional analysis of neuronal communication, plasticity, and neurodevelopment. Consequently, there are an increased number of available resources for researchers interested in zebrafish and the possibility to teach neurophysiology via a simple model.

Vargas R, Jóhannesdóttir I, Sigurgeirsson B, porsteinsson H, Karlsson KÆ. The zebrafish brain in research and teaching: a simple in vivo and in vitro model for the study of spontaneous neural activity. Adv Physiol Educ 35: 188–196, 2011; doi:10.1152/advan.00099.2010.—Recently, the zebrafish (Danio rerio) has been established as a key animal model in neuroscience. Behavioral, genetic, and immunohistochemical techniques have been used to describe the connectivity of diverse neural circuits. However, very few studies have used zebrafish to understand the function of cerebral structures or to study neural circuits. Information about the techniques used to obtain a workable preparation is not readily available. Here, we describe a complete protocol for obtaining in vitro and in vivo zebrafish brain preparations. In addition, we performed extracellular recordings in the whole brain, brain slices, and immobilized nonanesthetized larval zebrafish to evaluate the viability of the tissue. Each type of preparation can be used to detect spontaneous activity, to determine patterns of activity in specific brain areas with unknown functions, or to assess the functional roles of different neuronal groups during brain development in zebrafish. The technique described offers a guide that will provide innovative and broad opportunities to beginner students and researchers who are interested in the functional analysis of neuronal activity, plasticity, and neural development in the zebrafish brain.

extracellular recordings; development; neural circuits

zebrafish brain.
were cut, and the brain was extracted using microdissecting tweezers and the tips of insect pins (Fig. 1, C and D). Particular care was taken not to damage the olfactory bulbs and telencephalon because they can detach easily from the remainder of the brain. To avoid this, we began the dissection by cutting at the level of the junction between the spinal cord and brain stem. The brain stem was gently lifted with an insect pin, and the ventral roots of the cranial nerves were cut. The optic nerve was cut with small scissors before the whole brain was lifted for the dissection of the olfactory lobe (see APPENDIX A). A large amount of practice was necessary to gain expertise and to reduce tissue damage. For the electrophysiological recordings, the whole brain was fixed to the bottom of the recording chamber with Vetbond tissue adhesive (3M, St. Paul, MN). The preparation was maintained in aCSF that was bubbled with 95% O2 and 5% CO2 at room temperature for 1 h before their transfer to the recording chamber (see APPENDIX A).

**Brain slice preparation.** Young adult zebrafish were anesthetized in a solution of cold distilled water and 0.02% MS-222 (Sigma-Aldrich) and killed by decapitation. The skull was immersed and washed in 4–8°C aCSF for 2–3 min. To generate slices, the brain was suspended in a block of 2% agarose gel (50% distilled water and 50% aCSF, agarose type VII-A, Sigma-Aldrich) and fixed to the holder with Vetbond tissue adhesive (3M). This method was used because of the small size of the adult brain (3 mm long, 2 mm thick, and 2.5 mm wide; Fig. 1D). It was not possible to fix the brain with glue, which is common in large brain preparations. A vibratome (HM650 V, Thermo Fisher Scientific, Waltham, MA) that was programmed to a high vibration frequency and a slow blade advance speed produced the best slices with long-term durability. Recordings were obtained from the brain slices for 6 h. The slice orientation was mostly transverse, and three slices were obtained from each brain. Insome thickness ranged from 300 to 400 μm, the slice orientation was mostly

**Staining.**

Adult zebrafish were first anesthetized and the brains were then removed according to the procedure described above. Brains were immersion fixed in 2% paraformaldehyde in 0.1 M PBS at 4°C for 24 h. After fixation, brains were washed and stored in 0.1 M PBS (pH 7.4). Coronal (Fig. 2, A and C), transversal, and parasagittal brain slices (50–100 μm) were generated using a vibratome (Vibratome Microm HM650 V, Thermo Fisher Scientific). Subsequently, slices were fixed to microscope slides and immersed in 1% methylene blue in 0.1 M PBS (pH 7.4) for 3 min (see APPENDIX C). Brain sections were washed three times for 1 min each and coverslipped. Slices were visualized using an upright microscope (FN1 eclipse, Nikon Instruments), and micrographs were obtained with a DS-L2 digital camera (Nikon Instruments; Fig. 2C).

DiI, a soluble fluorescent dye that marks cell membranes retrogradely and anterogradely, was used to evaluate the possibility of tracing neuronal circuits. DiI crystals were placed over specific zones of fixed whole brains, and brains were maintained for 7–10 days at 4°C in 0.1 M PBS. Sections were obtained according to the procedure described above. The localization and distribution of DiI were examined using an epifluorescence microscope (FN1 eclipse, Nikon Instruments) with a specific filter set (G-2B, excitation filter: 510/560 nm, barrier filter: 610 nm, Nikon Instruments; Fig. 2D).

**Recording Technique.**

Recording electrodes were generated from borosilicate glass (WPI, New Haven, CT) using a vertical puller (PC-10, Narishige Group, Tokyo, Japan). A single-stage pull was used to make pipettes with a long tapered end and with tip resistances of 1–2 MΩ (5-to 10-μm-wide tips). An upright microscope (Nikon Eclipse FN1, Nikon, Tokyo, Japan) and micromanipulator assembly (MIH-3, Narishige Group) were used to obtain the recordings. Microelectrodes filled with aCSF were lowered into the tissue while a positive pressure was applied through the pipette tip using a 10-ml syringe. The microelectrode was carefully inserted into the tissue under visual guidance (Figs. 1C, 3C, 4A, and 5B). A seal test (brief voltage steps of 1 mV, 10 ms, 100 Hz) permitted observation of the electrode during its advancement. Upon noting a decrease in current (reflecting an increase in resistance as a cell was approached), the pressure at the pipette tip was reversed via the application of mild suction through the syringe. Seal resistances in
the range of 10–20 MΩ were obtained, and recordings were collected for up to several hours. The recordings were amplified and filtered (3 Hz–10 kHz) through a Multiclamp 700B amplifier (MDS Analytical Technologies, Sunnyvale, CA) and digitized with a data-acquisition interface Power 1401 analog-to-digital converter (CED, Cambridge, UK). Extracellular recordings were obtained from the following different areas of the whole brain: tectum opticum, telencephalon, and brain stem. We emphasized the telencephalon because we were particularly interested in this structure (Figs. 1C and 3C). The acquisition and analysis of signals were performed using Signal 4 software (CED). Additionally, we tested the effect of a chemical stimulus on telencephalic electrical activity, i.e., caffeine (1 mM), which is a general-purpose excitatory agent (10).

Zebrafish Brain Assay In Vivo

Zebrafish larvae were anesthetized in a solution of distilled water and 0.02% MS-222 (Sigma-Aldrich). Larvae were suspended in 2% agarose gel (type VII-A, Sigma-Aldrich), and each embedded larva was fixed to the bottom of a petri dish with Vetbond tissue adhesive (3M) in circulating aCSF at room temperature. Next, the soft tissue was removed to expose the following brain structures: the telencephalon, tectum opticum, and cerebellum (Figs. 5, A and B). Using this method, larvae survived for > 1 h (see APPENDIX B).

Statistical Analysis

Spontaneous electrical activity was analyzed in recordings obtained from the telencephalon of adult whole zebrafish brains under basal
conditions and after exposure to 1 mM caffeine (Fig. 6, A–D). Averages of rate, amplitude, and duration of spike-like events were evaluated before and after caffeine administration. The effect of caffeine was analyzed using Student’s paired t-test.

RESULTS

Zebrafish Brain Assay In Vitro

Whole brain preparation. The in vitro preparation was performed for both larvae and adults (Figs. 1, C and D, and 3, A–C). Dorsal and ventral areas were explored on one side of the isolated whole brain in each experiment because the brain was fixed with adhesive tissue to the chamber. Therefore, we explored dorsal areas of the telencephalon and tectum opticum and ventral areas of the telencephalon and brain stem (36) (Fig. 3C). The brain dissection was not complicated, and the viability of the preparation was high (>90%). In adult brains, the size and tissue opacity made it difficult to explore deeper zones such as the thalamus and brain stem nuclei; therefore, a slice preparation was required (Figs. 3, A–C and 4A). Spontaneous basal activity was found in all areas of the brain as rhythmic oscillatory waves or as discrete spike-like events. This activity was absent in damaged brains.

Brain slice preparation. To generate the slices, the brain was suspended in a block of agarose type VII-A and fixed to the holder with cyanoacrylate glue. This method was used because of the small size of the adult brain (3 mm long, 2 mm thick, and 2.5 mm wide; Figs. 1D and 3, A–C). It was not possible to fix the brain with glue, which is a commonly used procedure in large brain preparations. Agarose was prepared using 50% distilled water and 50% aCSF to avoid hyperosmolarity, which
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Fig. 6. Effect of caffeine on spontaneous electrical activity in the telencephalon. Electrical activity in the anterior dorsal lobe of the telencephalon in adult brains was recorded before (A) and after (B) caffeine application (1 mM) in the artificial cerebrospinal fluid (aCSF) bath solution. A: regular spontaneous activity, which was detected as spikes, was observed at rest. B: caffeine (1 mM) increased the frequency, amplitude, and duration of the recorded spikes. A–D: the horizontal bars in the top traces (A and B) indicate the segments that were expanded in the middle traces (C and D). *Segment that were expanded in the bottom traces (E and F).

causes the tissue to become nonviable. A high vibration frequency and a slow blade advance speed produced viable slices with thicknesses that ranged from 300 to 400 μm. Brain slices were conserved for 6 h. Three slices were obtained in the transverse orientation (n = 3; Fig. 4), and parasagittal (n = 2) and coronal (n = 2) slices were also obtained. The slice orientation was selected according to the area of interest.

Staining. To identify the structural organization of the zebrafish brain, we used a simple histological protocol with 1% methylene blue, which is a dye that marks cellular bodies of neurons in fixed brain slices (Fig. 2C). In some cases, larvae were fixed and stained according to the procedure used for adult brains (Fig. 2A). To evaluate the possibility of tracing neuronal circuits and neuronal projections, we used DiI, a soluble fluorescent dye that marks cell membranes retrogradely and anterogradely (Fig. 2D). The application of this marker was simple, and the circuits were visible after 1 wk of brain incubation with the dye; no additional treatment was necessary to obtain slices from brains marked with DiI.

Zebrafish Brain Assay In Vivo

The in vivo brain preparation was performed only in larvae. Larvae were submerged in agarose gel, in which they survived for >1 h. The recorded brain regions included the dorsal telencephalon, tectum opticum, and cerebellum (Fig. 5, A and B). These areas were easily exposed by removal of the surrounding soft tissue with little damage. To accomplish this dissection, the tip of a micropipette or an insect pin was used as a scalpel with excellent results (see APPENDIX B).

Recording Technique

In general, spontaneous electrical activity was detected in all brain regions explored: the telencephalon (n = 5, activity in 80% of brains explored), tectum opticum (n = 5, activity in 40% of brains explored), and brain stem (n = 5, activity in 80% of brains explored). The olfactory lobe demonstrated spontaneous activity that had a rhythmic, oscillatory pattern or displayed spikes. In some cases, the oscillatory activity showed an amplitude-modulated pattern. This type of activity has been previously reported in a study (7) of the olfactory neuroepithelium. Spontaneous electrical activity was more frequent and easier to detect in larval brains than in young adult brains. Spontaneous spikes were observed in the telencephalon, tectum opticum, and brain stem, and rhythmic activity was observed in the olfactory lobe, tectum opticum, and cerebellum.

Spontaneous activity was not detected in brains with mild damage.

In the adult slice preparation, spontaneous electrical activity, which again appeared as a rhythmic, oscillatory pattern or as spikes (Fig. 4), was not consistently present.

In our laboratory, we are interested in exploring the spontaneous electrical activity produced by the telencephalon. Therefore, in the present study, we emphasized the recording of spontaneous electrical activity in the telencephalon of young adult zebrafish brains. Spontaneous electrical activity became visible as spikes (Fig. 3D). To evaluate tissue excitability in the telencephalon, we tested the effect of a chemical stimulus on telencephalic activity using caffeine (1 mM), which is a general-purpose excitatory drug. Caffeine increased the frequency and amplitude of spikes (Fig. 6). After the application of caffeine, the spike frequency increased from 0.029 ± 0.01 to 0.154 ± 0.05 Hz [(n = 7, time (t) = −2.6, P = 0.041)], the amplitude increased from 0.036 ± 0.01 to 0.107 ± 0.031 nA (n = 7, t = −3.1, P = 0.021), and the duration increased from 88.7 ± 23.3 to 165.4 ± 19.4 ms (n = 7, t = −4.4, P = 0.005).

DISCUSSION

In the present article, we describe the preparation of zebrafish larvae in vivo and of the brain of zebrafish of different ages in vitro (from the larval stage to the young adult). These preparations are viable for many hours (a minimum of 1 h in the present study). The zebrafish brain is transparent during early development but not in adults (Figs. 1D and 3, A–C). Therefore, it is practical to use a slice preparation of the adult brain. We demonstrate that it is possible to obtain slices from the adult zebrafish brain that are viable for hours (Fig. 4, A–D).

After standardizing the protocols for the in vitro (APPENDIX A–C) and in vivo (APPENDIX B) models, we explored the electrical activity that is produced by the zebrafish brain. We observed spontaneous electrical activity in the youngest larvae at 5 days postfertilization (Fig. 5, A–E). Spontaneous basal activity was found in all areas of the brain as rhythmic oscillatory waves or as discrete spike-like events; this latter activity could represent excitatory or inhibitory postsynaptic currents. Typically, more prominent spontaneous activity is observed in younger brains compared with adult brains. The differences in neural activity between young and adult brains may be explained by differences in maturation; for example, the high proportion of glial and endothelial cells in adult brains results in difficulties of obtaining clean recordings due to a decrease in the signal-to-noise ratio. Furthermore, the maturation of dif-
different neurotransmitter systems could give rise to differences in the activation and deactivation of specific neural circuits. These phenomena should be studied in detail using a variety of techniques. Brains that exhibited severe damage or were obtained from dead animals did not demonstrate electrical activity. A great amount of practice was necessary to gain expertise in the tissue dissection and to reduce tissue damage.

Traditionally, the classical models used to teach electrophysiology use frogs, chickens, and rats. The most common demonstrative experiments that are performed in colleges and schools use the sciatic nerve and gastrocnemius muscle to explain the biophysical properties of muscles and neurons and synaptic transmission at the neuromuscular plate. These models are used to teach concepts such as membrane potential, resting potential, action potential, latency, threshold potential, conduction velocity, and synaptic transmission at the neuromuscular plate and its associated properties. In only a few cases has the brain been used to teach central nervous system phenomena. In general, brain activity has been explored by behavioral experiments (20). Electrophysiology instruction using the brain has been difficult because rats, mice, and chickens are expensive, difficult to manipulate, and, in many cases, require specific temperature conditions. The large size of their brains also necessitates the use of brain slice preparations for the experiments.

Using a pilot training program, we have trained graduate and postgraduate students attending a biomedical engineering program in the different steps needed to acquire expertise in a complete method for extracting zebrafish brain (details in APPENDIX A–C) and performing basic electrophysiological recordings (see APPENDIX D). After practical training, students without a previous biological background in neuroscience gained the skills necessary to perform a complete experiment. The procedures outlined in this article can be replicated at very low cost, with custom-built equipment. Researchers, universities, and research institutions around the world, especially in developing countries, can establish these protocols quickly and easily to the benefit of present and future generations of students and researchers. Although our goal with the use of the brain extraction method has been to obtain electrophysiological recordings, we have used the brain extraction protocol in histological, genetic, and neuroplasticity studies. Clearly, the zebrafish is an ideal model for teaching and research, because it permits students to develop skills in areas such as small-animal surgery, neuroanatomy, and neurophysiology. Additionally, in research, the brain extraction technique is fundamental for neurophysiology, immunohistochemistry, neurogenesis, neurogenetics, and neurochemistry studies.

Therefore, we propose that the zebrafish brain be used as an inexpensive and accessible model to study the electrophysiological phenomena that occur in the central nervous system and the activity of neural circuits.

APPENDIX A: ADULT ZEBRAFISH WHOLE BRAIN IN VITRO

Materials

The following materials are needed:
- Adult zebrafish
- Beakers (100 and 300 ml)
- Insulin syringes
- Needles (21 gauge, 1 in.)

Microtweezers
- Microscissors (3½ in.)
- Glass microcapillaries
- Pasteur pipettes (1–2 ml)
- Plastic petri dishes
- Plastic box and sponge
- Spoon
- Dissecting stereomicroscope

aCSF

aCSF is composed of the following:
- Distilled water (100 ml)
- NaCl (7.656 g)
- KCl (0.0149 g)
- KH2PO4 (0.017 g)
- MgSO4·7 H2O (0.0493 g)
- Glucose (0.1802 g)
- CaCl2 (0.0.277 g)
- NaHCO3 (0.1608 g)

aCSF is bubbled with a gas mixture of 95% O2-5% CO2 (pH 7.4).

Anesthetic

The anesthetic is MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate salt). The stock solution is 2%. It is composed of the following:
- Distilled water (100 ml)
- MS222 powder (2 g)

Protocol

The protocol for adult zebrafish brain extraction is shown in Fig. 7.

APPENDIX B: LARVAL ZEBRAFISH BRAIN IN VITRO AND IN VIVO

Materials

The following materials are needed:
- Larval zebrafish
- Beakers [20 ml (agarose), 100 ml (anesthetic), and 300 ml (aCSF)]
- Insulin syringes
- Insect pins
- Glass microcapillaries
- Pasteur pipettes (1–2 ml)
- Plastic petri dishes
- Cyanoacrylate glue (Vetbond)
- Dissecting stereomicroscope

Solutions

The following solutions are used:
- aCSF (100 ml)
- MS-222 (0.02%, 100 ml)

Agarose

Agarose is composed of the following:
- Low-melting agarose type VII-A powder (200 mg)
- aCSF (5 ml)
- Distilled water (5 ml)

Protocol

The protocol for larval zebrafish brain extraction is shown in Fig. 8.
APPENDIX C: BRAIN SLICE PREPARATION

Materials

The following materials are needed:

- Adult zebrafish
- Beakers [20 ml (agarose), 100 ml (anesthetic), and 300 ml (aCSF)]
- Insulin syringes
- Needles (21 gauge, 1 in.)
- Microtweezers
- Microscissors (3¼ in.)
- Glass microcapillaries
- Pasteur pipettes (1–2 ml)
- Plastic petri dishes
- Plastic box and sponge
- Spoon
- Cyanoacrilate glue (Vetbond)
- Dissecting stereomicroscope
- Vibratome
- Petri dish with nylon net
- Gas mixture of 95% O₂ and 5% CO₂ at room temperature

Fig. 7. Protocol for adult zebrafish brain extraction. A: the instruments used for the dissection are inexpensive and include syringe needles, glass microcapillaries, insect pins, petri dishes, pipettes, scalpels, tweezers, and microscissors. B: initially, adult zebrafish are anesthetized in a solution of distilled water and 0.02% MS-222. A Sylgard petri dish or box with sponge are used to hold the fish during the brain extraction. A sponge is commonly used to fix the fish during intraperitoneal drug microinjections. C: spoons are used to manipulate the fish and avoid injury. D: the fish is fixed with insect pins or syringe needles in a Sylgard-covered petri dish containing aCSF. E: the skin and skull bones of the zebrafish head are removed from one side to avoid telencephalon damage. F: the other side of the skull is removed to expose the complete brain; the telencephalon, tectum opticum, and cerebellum are now clearly visible. G: the spinal cord is cut with a syringe needle at the junction between the spinal cord and brain stem. The brain is then lifted with a capillary glass, and the cranial nerves are exposed and cut. H: the optic nerve (*) is cut with small scissors before the whole brain is lifted for dissection of the olfactory lobe. Particular care is taken not to damage the olfactory bulbs and telencephalon because they can detach easily from the remainder of the brain. I: finally, the brain is extracted using microdissecting tweezers or the tips of insect pins. The brain is moved from one place to another using a Pasteur pipette. For electrophysiological recordings, the whole brain is fixed to the bottom of the recording chamber with Vetbond tissue adhesive. The preparation is maintained in aCSF that is bubbled with 95% O₂ and 5% CO₂ at room temperature.

Fig. 8. Protocol for larval zebrafish brain extraction. A: the instruments are inexpensive and include the following: needles, glass microcapillaries, insect pins, and petri dishes. We used MS-222 as the anesthetic. B: initially, zebrafish larvae are anesthetized in a solution of distilled water and 0.02% MS-222. C: the larva is suspended in a 2% agarose gel, and each agarose-embedded larva is fixed to the bottom of a petri dish with Vetbond tissue adhesive in circulating aCSF at room temperature. D: agarose is removed to expose the head of the larva. E: glass capillaries (*) or insect pins are used as scalpels. The soft tissue is removed. F and G: the following brain structures are exposed: the telencephalon, tectum opticum, cerebellum, and brain stem. When we work with in vivo preparations we stop the dissection at this point; for brain extraction, we continue with the next step. H: we cut the spinal cord with a capillary tip at the junction between the spinal cord and brain stem. The brain stem is gently lifted, and the roots of the cranial nerves are cut with glass capillaries. I: finally, after the optic nerve and olfactory nerve are cut, we extract the complete brain.
Solutions

The following solutions are used:
- aCSF (100 ml)
- MS-222 (0.02%, 100 ml)

Agarose

Agarose is composed of the following:
- Low-melting agarose type VII-A (200 mg)
- aCSF (10 ml)

Protocol

The protocol for adult zebrafish brain slices is shown in Fig. 9. The process for adult brain extraction is the same as described in APPENDIX A; here, the difference is that we used cold solutions during the process. Adult zebrafish are anesthetized in a solution of cold (4–8°C) distilled water and 0.02% MS-222 (Sigma-Aldrich) and killed by decapitation. The head is immersed in 4–8°C aCSF, which is bubbled with 95% O2-5% CO2 at room temperature for 1 h before the slices must be transferred to a holding reservoir filled with aCSF. The brain is carefully removed according to the procedure described above. The brain, the cranial nerves are cut, and the brain is suspended in a block of 2% agarose gel (0.1 M PBS, pH 7.4). The brain is suspended in a block of 2% agarose gel (0.1 M PBS, agarose type VII-A, Sigma-Aldrich). Slices are obtained using the protocol described above.

APPENDIX D: BASIC GUIDELINES FOR EFFECTIVE PRACTICE FOR THE INSTRUCTOR

The goal of this outline is to facilitate the implementation of a workshop for undergraduate and graduate students and young scientists who are interested in zebrafish brain research. The course is organized in five modules that can be scheduled individually or consecutively. In addition, the course can be carried out as an intensive short-term or long-term course and for training that includes a major emphasis on experimental strategies and the manipulation of instruments and equipment.

The training starts with a practice class explaining the basic concepts of the laboratory instruments and their manipulation, the preparation of solutions, optic microscopy, and the manipulation of stereomicroscopy and upright microscopy. This instruction is followed by the preparation of anesthetics and surgery for brain extraction. The next step comprises the preparation of microelectrodes for recording, practice with the micromanipulators, and familiarization with the amplifier, signal conditioner, and software used for the acquisition and analysis of signals. Finally, a complete electrophysiological recording is performed. The practice is complemented by instructions concerning the slice preparation.

Modules

Module 1: laboratory instrument manipulation and preparation of solutions. In this part of the course, the following instruments are used: micropipettes, scale, stirrer, vortex, glass, and salts. Instructions regarding the use of these instruments are followed by the manipulation and preparation of the aCSF. Students will also learn to make drug stock solutions and dilutions.

Module 2: training in the use of the stereomicroscope and upright microscope and brain extraction. The aim of this part of the course is to acquire skill with respect to the use of microscopes. To achieve this aim, students will practice using the stereomicroscope and upright microscope. This practice is followed by the preparation of the anesthetic and extraction of a zebrafish brain from a young adult fish using the following surgical instruments: scalpels, tweezers, needles, insect pins, and petri dishes.

Module 3: microelectrode preparation and set up of the in vitro brain preparation. This part of the course is dedicated to the preparation of glass microelectrodes for recordings using the puller and polisher. In addition, the students will learn to set up the in vitro brain preparation using the bath chamber and holder, to fill the microelectrodes with aCSF for extracellular recordings, and to fix the brains to the preamplifier head stage.

Module 4: electrophysiological recordings in the brain. This part of the course is dedicated to performing the extracellular recordings. The students will learn to manipulate the micromanipulators, upright

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Fig. 9. Protocol for adult zebrafish brain slices. A: to prepare slices, we use a vibratome with a stereomicroscope attached to improve visualization of the tissue. B: to generate slices, the brain is suspended in a block of 2% agarose gel. C: the agarose block with the brain is fixed to the holder (H) with Vetbond tissue adhesive. This method is used because of the small size of the adult brain (3 mm long, 2 mm thick, and 2.5 mm wide). It is not possible to fix the brain with glue, which is a common procedure used in large brain preparations. D: the holder is then attached to the vibratome tray, which contains aCSF at 4°C. E and F: the vibratome is programmed to a high vibration frequency and a slow blade (B) advance speed to produce the best slices with long-term durability. G: the slice thickness ranges from 300 to 400 µm. H: the slice orientation is according to experimental requirements. Here, we show a transverse slice. In this case, 3 slices (300–400 µm) were obtained from each brain. I: for recovery, the slices must be incubated in aCSF aerated with 95% O2 and 5% CO2 at room temperature for 1 h before their transfer to the recording chamber. To avoid tissue damage by bubble movements, slices are placed in a petri dish with a nylont net.
microscope, amplifier and signal conditioner, software for acquisition, and signal analyzer.

Module 5: slices and staining. The goal of this part of the course is to acquire skills with the vibratome and to prepare brain slices for recordings and staining. For the electrophysiological recordings, the students will extract the brain and generate slices while the brain is supported in agarose blocks. For the staining procedure, the brains are extracted and fixed in 2% paraformaldehyde in 0.1 M PBS. Slices can be used immediately or saved for the fluorescent dye experiments. In DiI experiments, the brain is maintained in 2% paraformaldehyde in 0.1 M PBS solution for a minimum of 7 days, and brain slices are generated at later time points. The epifluorescence microscope is used with specific filters for the DiI marker to observe the traced circuits.

Advanced students and researchers can find more detailed information about sophisticated electrophysiological techniques in the references, which include intracellular recordings, Ca²⁺ imaging, channelrodopsin, and photoactivation (4, 6, 8, 11, 32, 34). Also, additional information can be found on the use of slices for immunohistochemistry, tracing neural circuits with fluorescent dyes and green fluorescent protein expression (17, 21, 24, 28), and genetic studies (17, 31, 32).

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