Video Article

Healthy Brain-pituitary Slices for Electrophysiological Investigations of Pituitary Cells in Teleost Fish

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Abstract

Electrophysiological investigations of pituitary cells have been conducted in numerous vertebrate species, but very few in teleost fish. Among these, the clear majority have been performed on dissociated primary cells. To improve our understanding of how teleost pituitary cells, behave in a more biologically relevant environment, this protocol shows how to prepare viable brain-pituitary slices using the small freshwater fish medaka (*Oryzias latipes*). Making the brain-pituitary slices, pH and osmolality of all solutions were adjusted to values found in body fluids of freshwater fish living at 25 to 28 °C. Following slice preparation, the protocol demonstrates how to conduct electrophysiological recordings using the perforated whole-cell patch-clamp technique. The patch-clamp technique is a powerful tool with unprecedented temporal resolution and sensitivity, allowing investigation of electrical properties from intact whole cells down to single ion channels. Perforated patch is unique in that it keeps the intracellular environment intact preventing regulatory elements in the cytosol from being diluted by the patch pipette electrode solution. In contrast, when performing traditional whole-cell recordings, it was observed that medaka pituitary cells quickly lose their ability to fire action potentials. Among the various perforation techniques available, this protocol demonstrates how to achieve perforation of the patched membrane using the fungicide Amphotericin B.

Video Link

The video component of this article can be found at https://www.jove.com/video/57790/

Introduction

The pituitary is a key endocrine organ in vertebrates located below the hypothalamus and posterior to the optic chiasm. It produces and secretes six to eight hormones from the specific cell types. Pituitary hormones constitute an intermediate between the brain and peripheral organs and drive a wide range of essential physiological processes including growth, reproduction, and regulation of homeostasis. Similar to neurons, endocrine cells of the pituitary are electrically excitable with the ability to fire action potentials spontaneously [1]. The role of these action potentials is cell dependent. In several cell types of the mammalian pituitary, action potentials can elevate the intracellular Ca^{2+} sufficiently for a sustained release of hormone [2]. In addition, the pituitary receives both stimulatory and inhibitory information from the brain that affects the membrane potential of the cells [3,4,5,6]. Typically, stimulatory input increases the excitability and often involves the release of Ca^{2+} from intracellular stores as well as increased firing frequency [7]. Understanding how the cell utilizes the ion channel composition and adapts to these input signals from the brain is key to understanding hormone synthesis and release.

The patch-clamp technique was developed in the late 1970s by Sakmann and Neher [8,9,10] and further improved by Hamill [11], and allows detailed investigations of electrophysiological properties of cells down to single ion channels. Moreover, the technique can be used for studying both current and voltage. Today, patch-clamping is the gold standard for measuring electrophysiological properties of the cell. Four major configurations of the tight seal patch-clamp technique have been developed [11]; the cell-attached, the inside-out, the outside-out, and the whole-cell patch. The three first configurations are typically used for single ion channel investigations. For the fourth, following the cell-attached configuration, a hole in the cell membrane is made using sub-atmospheric pressure. This configuration also allows investigations of the ion channel composition of the whole cell [12]. However, one limitation of this technique is that cytoplasmic molecules are diluted by the patch pipette solution [13] (**Figure 1A**), thus affecting the electrical and physiological responses of the studied cells. Indeed, some of those molecules may play important roles in the transduction of the signal or in the regulation of different ion channels. To avoid this, Lindau and Fernandez [14] developed a method where a pore-forming compound is added to the patch pipette. Following the cell-attached configuration, the compound will incorporate into the plasma membrane under the patch and slowly perforate the membrane creating electrical contact with the cytosol (**Figure 1B**). Several different antifungals such as nystatin [15] and amphotericin B [16], or surfactants such as the saponin beta-escin [17,18] can be used. These compounds create pores large enough to allow monovalent cation and Cl^- diffusion between the cytosol and the patch pipette while preserving the cytosolic levels of macromolecules and larger ions like Ca^{2+} [15,16].

The challenge of using perforated patch is the potentially high series resistance. Series resistance (R_s) or access resistance is the combined resistance over the patch pipette relative to the ground. During patch-clamp recordings, the R_s will be in parallel with membrane resistance (R_m).

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R_m and R_s in parallel work as a voltage divider. With the high R_s, the voltage will fall over the R_s giving errors in the recordings. The error will become larger with larger currents recorded. In addition, the voltage divider is also frequency dependent creating a low-pass filter, thus affecting the temporal resolution. In effect, the perforated patch may not always allow recordings of large and fast currents like the voltage gated Na^+ currents (for detailed readings see reference [19]). Also, R_s may vary during patch-clamp recordings, again leading to changes in the recorded current. Thus, false positives may occur in situations where R_s changes during drug application.

The electrophysiology on the sliced tissue was first introduced by the Andersen lab to study electrophysiological characteristics of the neurons in the brain [20]. The technique paved the way for detailed investigations of single cells as well as cell-cell communications and cell circuits in a more intact environment. A similar technique for making pituitary slices was introduced in 1998 by Guérineau *et al.* [21]. However, it was not before 2005, that brain-pituitary slice preparation was used successfully for patch-clamp studies in teleost [22]. In this study, the authors also reported the use of perforated patch-clamp recordings. However, by far, most of the electrophysiological investigations of pituitary cells have been conducted in mammals, and only a handful of other vertebrates, including teleost fish [1,2,22,23]. In teleosts, almost all studies were performed on primary dissociated cells [24,25,26,27,28,29,30].

In the present paper, we outline an optimized protocol for preparation of healthy brain-pituitary slices from the model fish medaka. The approach represents several advantages compared to primary dissociated cell cultures. First, the cells are recorded in a relatively preserved environment compared to dissociated cell culture conditions. Second, slice preparations allow us to study indirect pathways mediated by cell-cell communication [22], which is not possible in dissociated cell culture conditions. Furthermore, we demonstrate how to conduct electrophysiological recordings on the obtained tissue slices using the perforated whole-cell patch-clamp technique with amphotericin B as the pore-forming agent.

Medaka is a small freshwater fish native to Asia, primarily found in Japan. The physiology, embryology, and genetics of medaka have been extensively studied for over 100 years [31], and it is a commonly used research model in many laboratories. Of particular importance to this paper is the distinct morphological organization of the hypothalamus-pituitary complex in teleost fish: Whereas in mammals and birds the hypothalamic neurons release their neuro-hormones regulating pituitary endocrine cells into the portal system of the median eminence, there is a direct nervous projection of hypothalamic neurons onto the endocrine cells of the pituitary in teleost fish [32]. Thus, carefully conducted brain-pituitary slicing is of particular importance in fish, allowing us to investigate electrophysiological characteristics of the pituitary cells in a well-preserved brain-pituitary network, and in particular how pituitary cells control their excitability and thereby Ca^{2+} homeostasis.

Protocol

All animal handling was performed according to the recommendations for the care and welfare of research animals at the Norwegian University of Life Sciences, and under the supervision of authorized investigators.

1. Preparation of Instruments and Solutions

NOTE: All solutions should be sterile. Careful attention should be given to the pH and the osmolality (osmol/kg water) of all solutions, which should be carefully adapted to the extracellular environment of the studied species. pH and osmolality should be adjusted with precise electronic equipment such as pH meter and freezing point osmometer respectively.

1. **Make 500 mL of extra-cellular solution without Ca^{2+} (Ca^{2+} free EC): 150 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 4 mM glucose, 10 mM Hepes.**
    1. Dissolve 4.38 g of NaCl, 186.37 mg of KCl, 61.89 mg of $MgCl_2$, 360.32 mg of Glucose and 1.19 g of HEPES in 450 mL of ultrapure (0.055 uS/cm at 25 °C, the resistivity of 18.2 MOhm) H_2O in a glass beaker using a magnetic agitator.
    2. Adjust the pH to 7.75 with NaOH.
    3. Transfer the solution into a 500 mL volumetric flask. Fill the volumetric flask with ultrapure H_2O until 500 mL.
    4. Mix the solution well before measuring the osmolality. Adjust to 290–300 mOsm with mannitol. Filter sterilize the solution using water vacuum system and 0.2 µm filter.
2. **Make 500 mL of extra-cellular solution (EC): 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 4 mM glucose, 10 mM Hepes.**
    1. Dissolve 4.38 g of NaCl, 186.37 mg of KCl, 147.01 mg of $CaCl_2$, 61.89 mg of $MgCl_2$, 360.32 mg of Glucose and 1.19 g of HEPES in 450 mL of ultrapure H_2O in a glass beaker using a magnetic agitator.
    2. Adjust the pH to 7.75 with NaOH.
    3. Transfer the solution into a 500 mL volumetric flask. Fill the volumetric flask with ultrapure H_2O until 500 mL.
    4. Mix the solution well before measuring the osmolality. Adjust to 290–300 mOsm with mannitol. Filter sterilize the solution using water vacuum system and 0.2 µm filter.
3. **Make 500 mL of extra-cellular solution with 0.1% bovine serum albumin (EC with BSA): 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$, 4 mM glucose, 10 mM Hepes.**
    1. Dissolve 4.38 g of NaCl, 186.37 mg of KCl, 147.01 mg of $CaCl_2$, 61.89 mg of $MgCl_2$, 360.32 mg of Glucose and 1.19 g of HEPES in 450 mL of ultrapure H_2O in a glass beaker using a magnetic agitator.
    2. Adjust the pH to 7.75 with NaOH.
    3. Transfer the solution into a 500 mL volumetric flask. Fill the volumetric flask with ultrapure H_2O until 500 mL.
    4. Mix the solution well before measuring the osmolality. Adjust to 290–300 mOsm with mannitol. Filter sterilize the solution using water vacuum system and 0.2 µm filter. Add 50 mg BSA.
4. **Make 250 mL of intracellular solution (IC): 110 mM KOH, 20 mM KCl, 10 mM Hepes, 20 mM sucrose.**
    1. Dissolve 1.54 g KOH, 327.75 mg of KCl, 595.75 mg of HEPES and 1.712 g of sucrose in 450 mL of ultrapure H_2O in a glass beaker using a magnetic agitator.
    2. Adjust the pH to 7.2 with (N-morpholino) ethane sulfonic (MES) acid.

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    3. Transfer the solution into a 250 mL volumetric flask. Fill the volumetric flask with ultrapure H_2O until 250 mL.
    4. Mix the solution well before measuring the osmolality. Adjust to 280–290 mOsm (10 mOsm lower than EC) with sucrose. Filter sterilize the solution using water vacuum system and 0.2 µm filter.

5. Make 100 mL of 2% low melting agarose solution in Ca^{2+} and BSA free EC (2 g of low melting agarose in 100 mL of Ca^{2+} free EC). Dissolve using a microwave and place the melted agarose in a water bath at 40 °C. Let it cool down until it reaches 40 °C before use on the tissues.
NOTE: EC, Ca^{2+} free EC may be stored at 4 °C for several weeks if sterile and IC solutions at -20 °C, for several months. Agarose can be stored for a week at 4 °C and can be reused 3–5 times by briefly microwaving. Excessive reuse will lead to evaporation and alteration of agarose and salt concentration and quality.
6. **Make agar bridge:**
    1. Heat 7.5 mm long borosilicate glass capillaries with outside diameter (O.D.) 2 mm and inside diameter (I.D.) 1.16 mm using a Bunsen burner at a focal point about 1/3 from one end until the glass starts to bend. Remove the glass from the flame and allow the glass to bend with an angle of about 120 degrees (**Figure 2A**).
    2. Melt 2% agar in normal EC without glucose using a microwave, and while the agarose is liquid fill the pre-made glass capillary using capillarity forces by putting one of the ends of the glass into the liquid and waiting for few minutes. Store bridges until use at 4 °C in EC without glucose.

7. Prepare borosilicate glass with filament patch pipettes using a suitable pipette puller. Refer to the pipette puller manual to obtain the desired electrode resistance.
NOTE: The taper of the pipette should be as short as possible. Short pipette taper is achieved by using 4–6 pulling steps. The electrode resistance should be between 2 and 6 MΩ depending on cell size.
8. **To avoid movement of tissue during the patch-clamp experiments coat the surface of the recording chamber with 0.1% of polyethylenimine (PEI) in borate buffer.**
    1. Prepare 500 mL of borate buffer (25 mM):
        1. Dissolve 4.768 g of $Na_2B_4O_7/10H_2O$ in 450 mL of ultrapure H_2O in a glass beaker using a magnetic agitator.
        2. Adjust pH to 8.4 with HCl.
        3. Transfer the solution into a 500 mL volumetric flask. Fill the volumetric flask with ultrapure H_2O until 500 mL and 0.2 µm filter sterilize the solution using water vacuum system.

    2. Prepare a 1% stock solution of PEI (1 mL 50% PEI to 49 mL 25 mM borate buffer) and a 0.1% PEI dilution by diluting 10 µL of 1% stock solution in 100 µL of borate buffer for final use.
    3. Add 2 mL of 0.1% PEI on the glass of the slice holder and let the coating incubate for 1 minute. Then briefly wash the glass twice with 5 mL of ultrapure H_2O and let air dry until use.

9. **Prepare amphotericin B solutions.**
    1. Make 60 mg/mL stock solution by dissolving 3 mg of amphotericin B powder in 50 µL dimethyl sulfoxide (DMSO), in a 1 mL tube protected from light. Vortex at the maximum speed for 30 s and sonicate for 15 min to homogenize. Store aliquots into 3–5 tubes at -20 °C and use one aliquot per day.
    NOTE: If the amphotericin B stock solution is not clear and yellow color but milky (still yellow) after 15–20 min sonication make a new stock solution.
    2. Make IC solution with amphotericin B by pipetting 2 mL of the IC into a clean glass beaker with more than 10 mL capacity. Add 8 µL of amphotericin B stock solution into the pipette solution and mix by pipetting. Wrap the beaker with aluminum foil and place on ice until use and renew it every 3 h.

2. Dissection and Slicing with Vibratome

1. Prepare the dissection tools before dissecting, including one sharp and one strong forceps with scissors. Clean the tools (tissue holder, brush, forceps) with ethanol and make sure that they are used only for dissection purposes and never in contact with fixed tissues. Keep them in a separate box to avoid contamination.
2. Euthanize the fish in cold ice water for 1 min.
3. **Follow the subsequent steps to quickly dissect the medaka brain and pituitary with sharp forceps (use no more than 3 min).**
    1. While holding the fish with the strong forceps severe the two optic nerves behind the eyes with scissors or sharp forceps.
    2. Open the dorsal part of the skull from the posterior to the anterior side by breaking the skull bones step by step with strong forceps.
    3. Peel off the skull on one side with strong forceps.
    4. Severe the spinal cord with scissors or sharp forceps.
    5. Gently, grab the spinal cord with the forceps, flip the brain from posterior to anterior and place it into a dish with the ice-cold Ca^{2+} free EC.

4. Fill a 1.5–2 cm^3 metal mold with liquid agarose and place it on the ice.
5. Just before the agarose solidifies (at around 25–30 °C), gently grab the brain by the spinal cord with the forceps, dry the forceps with a piece of fine paper and put the tissue into the agarose. Quickly mix the agarose to dilute the traces of EC left around the tissue and orientate the tissue and let the mold on ice for a few seconds until the agarose hardens.
NOTE: It is essential for this step to be fast. The agarose solidifies quickly as the metal mold is cooled down by the ice.
6. Trim a square block of agarose containing the tissue with a scalpel blade and glue it on the vibratome specimen holder using surgery (non-toxic) glue.
7. Fill the cuvette of the vibratome receiving the specimen (brain-pituitary) holder with ice-cold Ca^{2+} free EC.
8. Place the specimen holder into the vibratome and cut the agarose block to make parasagittal sections of 150 µm, using high frequency and the low speed for the sectioning. Collect the selected sections and place them into the patch-clamp recording chamber with 3 mL of ice-cold Ca^{2+} free EC.

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9. Place the grid harp (**Figure 2B**) on the tissue section.
   NOTE: The harp will together with the coating stabilize the tissue and prevent it from moving during the patch-clamp recordings.
10. After the harp is in place, change the Ca^{2+} free EC medium to the normal EC with Ca^{2+} and BSA. Let the tissue rest for 10 min.

3. Perforated Patch-clamp and Electrophysiological Recordings

1. Before starting the experiment, chloride the silver wire electrodes by the following steps: First, use a fine sandpaper (p180) to clean the silver wire electrodes. Second, rinse with 2 mL, 70% ethanol. Third, dip the wires in 2 mL, 2–5% chlorine for 10 min. Finally, rinse with 2 mL, ultrapure H_2O.
2. Place the recording chamber with the brain-pituitary slice in the microscope stage and locate the target area (pituitary) using 10X objective and inspect the cells using 40X objective.
   NOTE: If the preparation is successful, very few round cells should be visible. These round cells are usually damaged cells that are detaching from the tissue.
3. Place the grounding electrode through the 2% agar bridge into the EC bath.
4. Locate a healthy cell using the 40X objective.
   NOTE: A healthy cell should be firmly attached to the tissue slice and not rounded up and detached from the slice.
5. Set the amplifier in voltage-clamp (VC; **Figure 4A** point 1) mode. Open the seal test window and press **the bath** configuration (**Figure 4B** point 1 and 2. Use a 5-mV pulse to monitor pipette resistance (**Figure 4B** point 3).
6. Backfill the tip of the patch pipette with the antifungal free IC solution before adding the IC solution with amphotericin B using a micro-filler syringe (see **Figure 3**).
7. Add a slightly positive air pressure into the patch pipette using 1 mL syringe.
   NOTE: This makes a small liquid flow from the patch pipette that avoid contamination of the tip.
8. Once in the bath, assess the patch pipette resistance and make sure no particles are attached to the tip of the pipette (**Figure 4C**).
   NOTE: A small piece of tape is attached onto the monitor displaying the video recording form the field of view. This makes the positioning of the patch pipette relative to cell easier when the objective is not focusing on the cell.
9. Guide the patch pipette down to the cell using micromanipulators.
   NOTE: Make sure that the pipette is clean by looking for small particles that may have attached to the tip of the pipette. Sudden changes in resistance may also be a result of particles stuck in the pipette tip.
10. Readjust the pipette when a few microns above the cell so that the tip of the pipette will touch the cell 1/3 from the middle of the cell, as shown in **Figure 5**. When touching the cell, release the pressure and apply the gentle suction to make a seal.
    NOTE: The time from the patch pipette enters the bath to a successful seal should be kept as short as possible. Not more than 1–1.5 min in order to avoid amphotericin B reaching the tip of the pipette and leak into the bath.
11. Immediately switch to the **patch** window in the patch-clamp software (**Figure 4D** point 1) and have a holding potential between -50 and -60 mV (**Figure 4D** point 2). Zero out the fast capacitance made up by the glass pipette (**Figure 4D** point 3).
12. Switch to the **cell** window in patch-clamp software (**Figure 4E** point 1 and 2) and start monitoring access resistance, **Ra**, displayed on the window. Zero out the membrane capacitance in the amplifier software (**Figure 4E** point 3) after sufficient access.
    NOTE: Sufficient access might take 10 to 30 min (**Figure 4E** point 2). A good access for current clamp recordings should be below 20 M .
13. Switch to current clamp, **IC**, in amplifier software window (**Figure 4F** point 1). Adjust the fast-capacitive currents in the **I-clamp** window of the amplifier software (**Figure 4F** point 2 and 3) to the same value as in 3.12. Check membrane potential (**Figure 4F** point 1).
    NOTE: Importantly, if the membrane potential is shallow (typical above -35 mV) the cell might be damaged. Cancel the recording and find a new cell.
14. After finding a healthy cell (firmly attached to the tissue with membrane potential below -40 mV), start the experimental recordings as detailed in the manufacturer's protocol [33,34].

Representative Results

This protocol demonstrates a step by step protocol of how to achieve reliable electrophysiological recordings from pituitary (gonadotrope) cells, using a medaka transgenic line [Tg(*lhb*-hrGfpII)] where the target cells (Lh-producing gonadotropes) are labeled with green fluorescent protein (GFP).

Initially, the electrophysiological investigations were conducted using whole-cell configuration. However, spontaneous action potentials were not observed in any of the studied cells. In a subset of cells, action potentials could be triggered using small current injections (5-9 pA) from a resting potential between -50 and -60 mV (**Figure 6**). These action potentials had a fast rundown, and after about 4–6 min triggered action potentials were no longer possible. The particularly fast rundown observed may be explained by the small cell size. In general, pituitary cells are smaller than neurons [30]. For instance, average membrane capacitance of gonadotrope cells ranges between 4 and 10 pF [3,30,35]. Similar to these findings medaka gonadotrope cells had an average membrane capacitance of (mean ± S.D) 3.4 ± 0.9 pF (n = 67).

Switching to the perforated patch configuration using amphotericin B, spontaneous action potentials were observed in about 50% of the recorded cells (**Figure 7A**, n = 63 cells from 21 animals). Moreover, action potentials could be triggered in 95% of these cells with no observable rundown even after prolonged recordings (up to 1 h). Importantly, in order to achieve reliable and high-quality recordings, it is necessary to first fill the tip with antifungal-free IC solution. If small amounts of antifungals escape the pipette tip before making the gigaseal, it can damage the target cell as well as surrounding cells.

In order to test if cells in the perforated patch configuration are able to respond to their main releasing hormone, we applied 1 µM Gnrh1 puff ejected on the target cells. The experiments were performed in the current clamp to allow us to monitor changes in voltage. These experiments revealed a biphasic response (**Figure 7B**). The first phase is a hyperpolarization where the release of Ca^{2+} from internal stores activate Ca^{2+} activated K^+ channels causing the hyperpolarization. The first phase is followed by a depolarization and increased action potential frequency from 1–2 Hz to around 3 Hz. In some of the recordings, the second phase had pseudo plateau potentials where 2 or 3 small spikes were observed before repolarization.

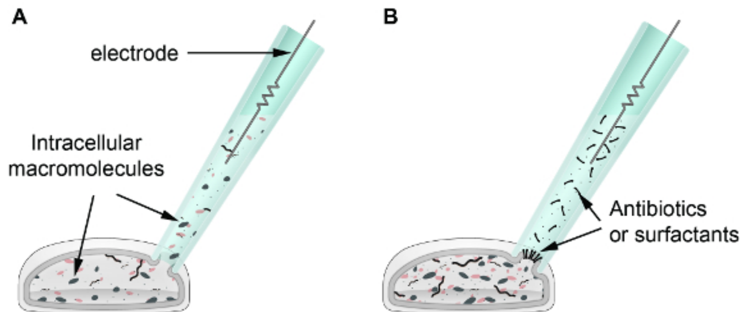


Figure 1: Difference between the whole-cell (**A**) and the perforated patch (**B**) configurations of the patch-clamp technique. In the whole-cell configuration (**A**), after gigaseal is in place, a hole is made in the membrane by applying a small negative pressure into the patch pipette. In this configuration, important intracellular molecules such as signaling molecules can be diluted into the patch pipette solution thus affecting the electrical and physiological responses of the cell. This phenomenon is even more important in small cells, such as pituitary cells. In contrast, in perforated-patch configuration (**B**), following gigaseal, electrical contact between the cytosol and the patch pipette is made up of antifungals or surfactants. The antifungal or surfactant is loaded into the patch pipette before patching and will be incorporated into the plasma membrane under the patch, thereby slowly perforating the membrane, allowing only small molecules like monovalent ions to pass. Please click here to view a larger version of this figure.

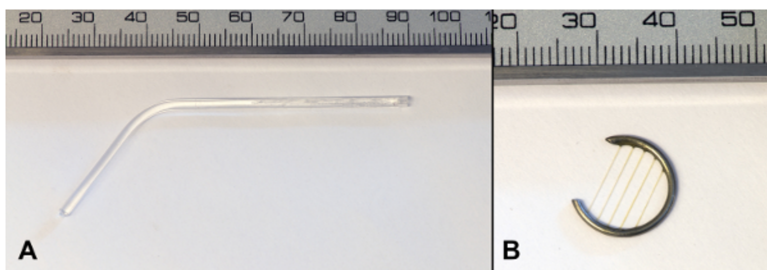


Figure 2: Pictures of the Agar bridge (**A**) and the Harp (**B**) used in the protocol. Scale in mm. Please click here to view a larger version of this figure.

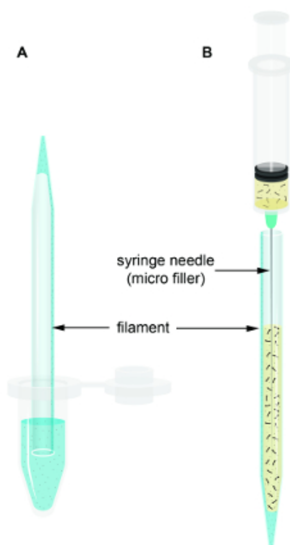
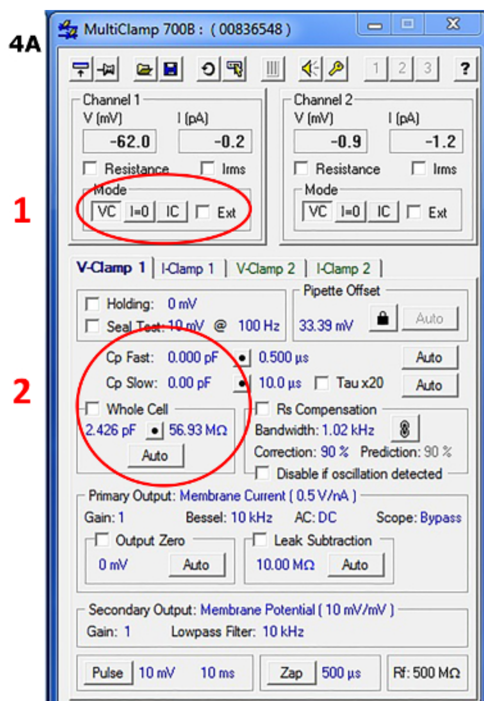


Figure 3: Filling of the patch-pipette. (**A**) Antifungal free IC solution (blue liquid) is backfilled by capillary forces due to the filament inside the pipette. (**B**) After filling the pipette tip with antifungal free IC, the posterior part of the pipette is filled with IC solution containing amphotericin B (yellow liquid) using a syringe needle (micro filler). Please click here to view a larger version of this figure.



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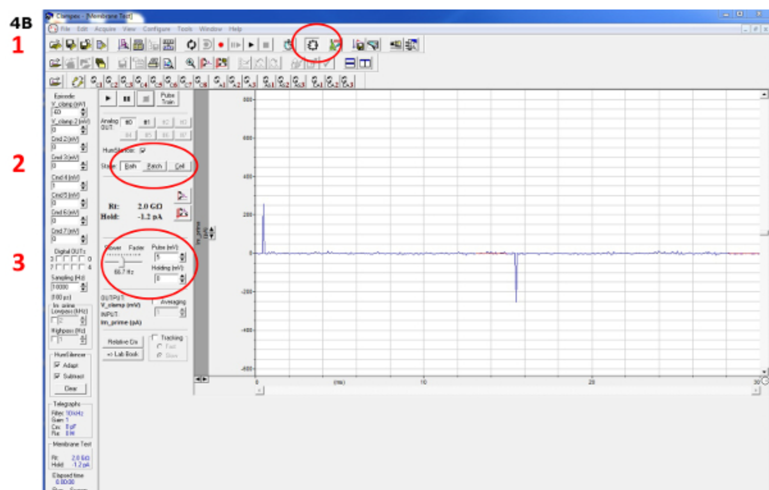

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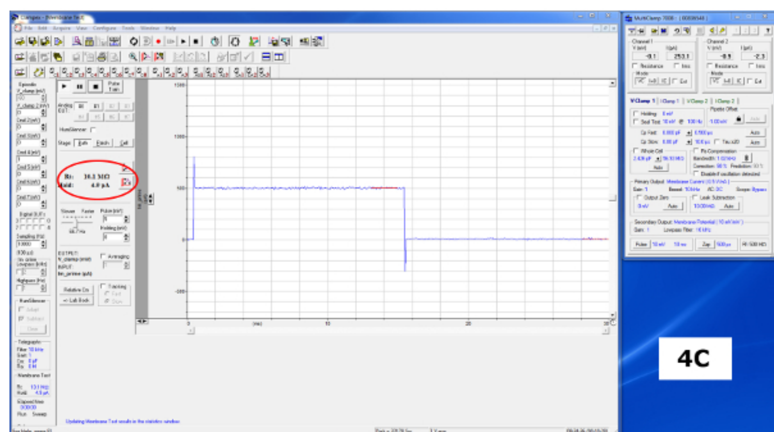

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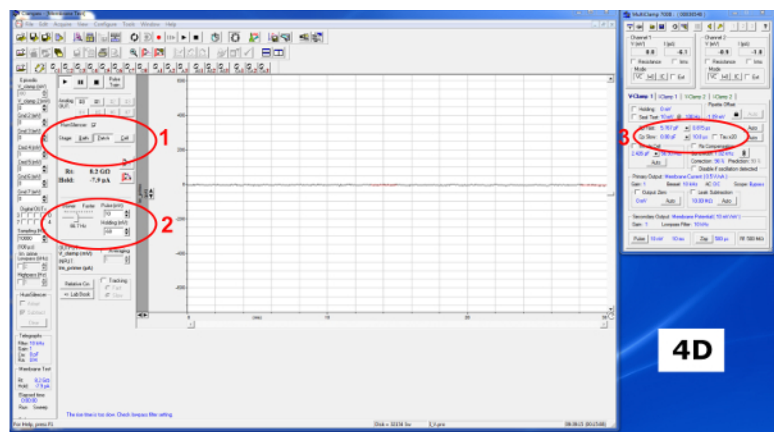

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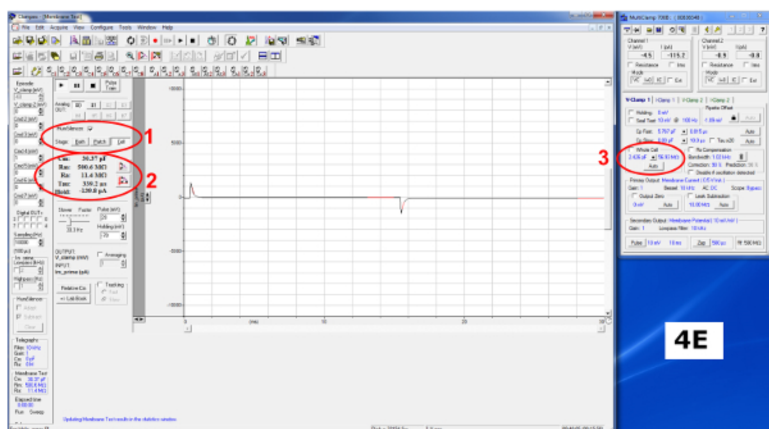

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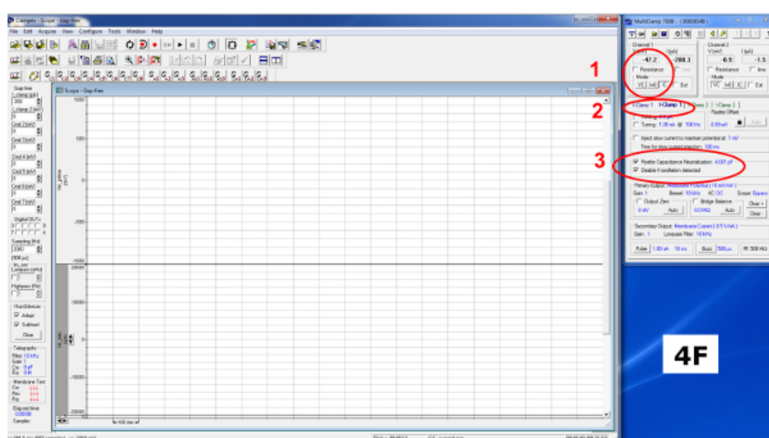

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Figure 4: Software screenshots. (**A**) showing amplifier software window. 1. highlights (red circle) the mode area that allows switching between voltage clamp (VC), current clamp without any current injection (I=0) and current clamp with possibility for current injections (IC). 2 highlights (red circle) the area for adjusting fast capacitive current in voltage clamp as well as whole-cell capacitive current adjustments. (**B**) shows the patch-clamp software with the membrane test window open. 1. highlights (red circle) the membrane test button. 2. highlights (red circle) the different pulse configurations, Bath, Patch, and Cell. 3. highlights (red circle) the pulse configuration amplitude and frequency. (**C-F**) shows a combined widow of the patch-clamp software (left) and amplifier (right) software. (**C**) highlights (red circle) the resistance when the pipette is in the bath and proper grounding using agar bridge (in Bath mode). (**D**) 1. highlights (red circle) the switching to Patch mode following gigaseal. 2. highlights (red circle) the pulse configuration (10 mV pulse) and holding potential adjusted to -60 mV. 3. highlights (red circle) the window for correcting or zeroing the fast-capacitive currents following a giga seal. (**E**) 1. highlights (red circle) the Cell mode used for monitoring access resistance. 2 highlights (red circle) the cell parameters, membrane capacitance (Cm), seal quality (Rm), access resistance (Ra), the time constant (Tau) and holding current (Hold). 3. highlights (red circle) the button for correcting the membrane capacitive currents. (**F**) 1. Highlights (red circle) the IC mode for monitoring membrane potential. 2. Highlights (red circle) the button to switch to current clamp adjustments (I-clamp). 3. Highlights (red circle) the window for correcting the fast-capacitive currents.

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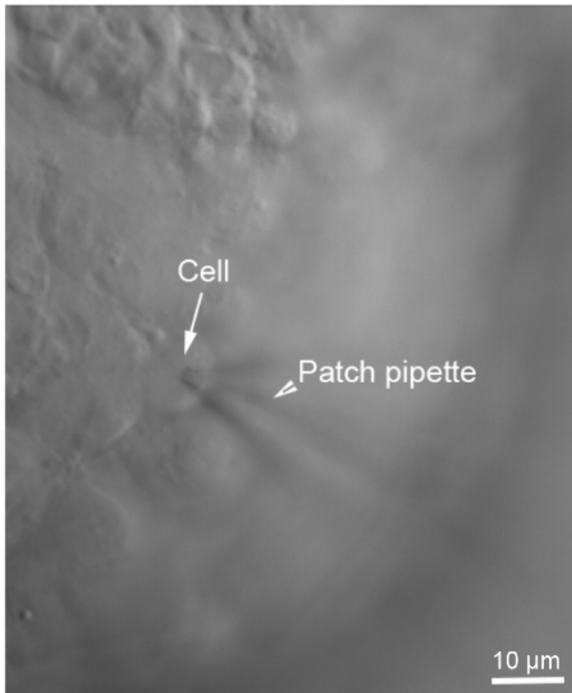

Figure 5: Micrograph from a patch-clamp recording on a pituitary cell using the perforated patch configuration. Please click here to view a larger version of this figure.

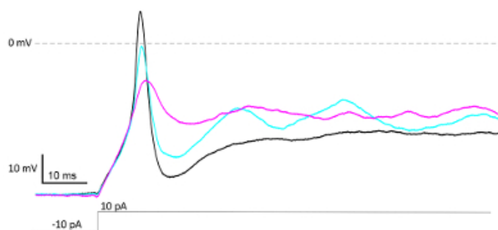

Figure 6: Current clamp recordings from a GFP-labeled Lh-producing cell following current injections using normal whole-cell configuration. The cells were kept between -50 and -60 mV. Typical action potentials could be triggered in a subset of cells using 5–10 pA current injections immediately after achieving access to the cell (black trace). After 1–3 min the action potential amplitude started to decrease, a typical sign of rundown (cyan trace). After 4–6 min the action potential amplitude almost completely disappeared (magenta trace). Please click here to view a larger version of this figure.

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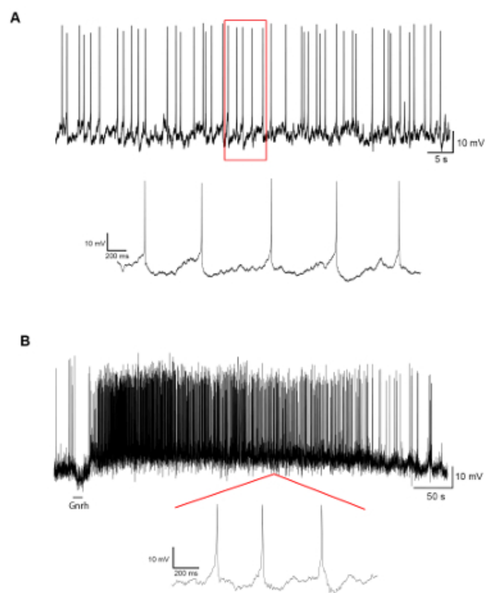

Figure 7: Current clamp recording from a GFP-labeled Lh-producing cell using the perforated patch configuration, following stimulation with the Gonadotropin-releasing hormone Gnrh1. (**A**) Current clamp recording demonstrating spontaneous action potentials from a Lh producing gonadotrope cell. (**B**) A biphasic response where Gnrh1 stimulation for 10 s first induced a hyperpolarization of the cell membrane followed by a depolarization and increased firing frequency (from 1–2 Hz to 3 Hz) of action potentials in a cell which previously fired spontaneous action potentials. Please click here to view a larger version of this figure.

Discussion

Electrophysiological recordings using the patch-clamp technique on brain-pituitary slices require careful optimization. Well-optimized protocols for conducting live-cell investigations specifically in teleosts are limited, with the majority of publications using protocols based on mammalian systems. In this regard, it is important to be aware of the fact that several physiological parameters like pH and osmolality are not only species dependent, but also very much dependent on whether the organism in question lives on land or in water. For fish, it is also necessary, to take into account, if they live in a marine or freshwater environment. For example, the CO_2 partial pressure is much lower in fish compared to mammals with levels ranging from 1.7–3.4 mmHg pCO_2 in fish and 40–46 mmHg pCO_2 in mammals [36]. Based on this, we adjust the pH to 7.75 [37,38,39,40] in all solutions used in the current protocol. We also use HEPES buffer as it has been shown to possess excellent buffering capacities in the pH region of 7.4–7.8 [41]. For the osmolality, we use 290–300 mOsm which is what has been measured in the extracellular environment of medaka [42]. The temperature used during the electrophysiological recordings has been selected according to the environment of the fish. Indeed, medaka is living in waters with a wide temperature range (from 4 to 40 °C), while in our laboratory they are raised in a controlled environment at 26–28 °C. Based on this, we performed our recordings at room temperature (around 25 °C), different from what is used for mammalian tissues (37 °C) or for cold water fish species such as Atlantic cod (12 °C) [18].

To be able to patch pituitary cells, it is critical to generate healthy tissue slices. It is imperative to use clean tools (tissue holder, brush, forceps, etc) that are only in contact with live tissue (free of fixatives). A quick dissection of the brain and pituitary and keeping the tissue at low temperature (4 °C) while dissecting and slicing are other key factors to provide viable sections. Specific attention should also be given to the temperature of the agarose upon tissue embedding. Too warm agarose could damage the tissue while too cold agarose will not leave you time to orientate the tissue for proper sectioning. In addition, slicing should be done with EC solution without Ca^{2+} to avoid the damaged cells following sectioning to enter into apoptosis. Bubbling is not necessary, but the tissue slices should be collected immediately after sectioning. Leave the slice about 15 min following sectioning to let the cells rest a bit before patching.

Teleost fish are excellent models to investigate electrophysiological properties of pituitary cells. Indeed, contrary to mammals and birds, fish do not possess a median eminence, meaning that the hypothalamic neurons controlling the pituitary directly project their axons onto their target cells [32]. Thus, in a brain-pituitary slice, the pituitary cells are maintained in a more intact environment compared to primary pituitary cell cultures where the cells are dissociated using chemical and mechanical treatments. Interestingly, using brain-pituitary slices of medaka, we could observe that the action potential firing frequency in Lh cells, upon Gnrh1 activation, increased (**Figure 5**). These observations are in agreement with what has been reported in pituitary cell culture studies from our own lab [29] indicating that using primary pituitary cell cultures are still relevant to characterize the membrane properties of pituitary cells. However, brain-pituitary slices allow us to study also indirect effects of different compounds as well as interactions between cells, as it maintains structural connections that are lost in primary pituitary cell cultures. In addition, slice preparation is faster to prepare compared to a dissociated primary cell culture, and electrophysiological recordings can be conducted in the following 30 min after dissection. This means that, contrary to a primary cell culture, circadian rhythms can be addressed in a meaningful way using freshly prepared slices.

Because of the small size of the pituitary cells in fish (membrane capacitance around 3 - 10 pF), and our own observations showing that gonadotrope cells lose their ability to fire action potentials (**Figure 3**) and thus lose their ability to respond to releasing hormones when recording in whole-cell configuration, we decided to use and present the perforated patch technique herein. Indeed, it has been shown that whole-cell

configuration could lead to the diffusion of important cytoplasmic molecules thus changing the electrophysiological cell properties [13]. Using instead the perforated patch configurations with amphotericin B to perforate the cell membrane into the patch pipette, making only small holes allowing only small ions to pass through [15,16,43,44], we could avoid this dilution and record the electrical activity of the cell for a prolonged time.

One important point in the perforated patch technique is to first back-fill the pipette with IC solution without antifungals in order to avoid releasing antifungals into the medium and damage all cells of the section while approaching with the patch pipette. Indeed, because of the positive pressure applied to the patch pipette while approaching the tissue, some liquid is leaking through the patch pipette until the patch is made. This flow helps to keep the tip clean until you reach the targeted cell but if antifungal is released in the medium before the patch is made, it can perforate all membranes of all cells, changing dramatically the permeable characteristics of the cell membranes and thus their electrical properties.

The presented procedure has been optimized and successfully used to study the electrical activity of medaka gonadotrope cells. In addition, the protocol may be used to study all (endocrine) cell types found in the pituitary of medaka and other teleost fish species. Keep in mind only that the osmolality and pH of all solutions must be adjusted to that of the body fluids of the species in question.

Disclosures

The authors have nothing to disclose

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