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Recording of chromaffin cell electrical activity *in situ* in acute adrenal slices

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Abstract

Because catecholamines secretion mainly relies on the excitable nature of adrenal chromaffin cells, monitoring their electrical activity is an essential step in assessing the adrenal medullary tissue function. The difficult access to the gland *in vivo* allows only population activity to be recorded in this condition. *In vitro* preparations allow recordings of spontaneous or evoked activity from single or multiple cells, depending on the biological samples used (dissociated chromaffin cells *versus* adrenal tissue preparations). In this chapter, I provide a detailed description of the techniques used for electrophysiological recordings in rodent chromaffin cells in acute adrenal slices, using the patch-clamp technique. This methodology allows preservation of the tissue integrity and detection of action potentials, synaptic activity and secretory events, it is thus suitable for the study of adrenomedullary activity-secretion coupling.

Key words: Chromaffin cell, Acute adrenal slice, Excitability, Action potential, Synaptic activity, Stimulus-secretion coupling, Rat, Mouse

1 Introduction

Adrenal chromaffin cells are the main drivers of catecholamine release and as such they critically contribute to the adaptive response to stress. To efficiently cope with stressful life events, the organism needs to adapt the amounts of catecholamines (epinephrine and norepinephrine) released in the blood circulation. Catecholamine secretion is a finely regulated process, through the continuous remodeling of the adrenomedullary stimulus-secretion coupling [1-3]. Among the 'intra-adrenal' parameters undergoing dynamic changes during stress, the electrical activity of chromaffin cells is undoubtedly one of the most critical [4], as it chiefly patterns the release of catecholamines [5,6]. To study the physiological/pathological functions that occur *in vivo*, it is necessary to work on biological preparations that maintain, as much as possible, the structural integrity of the adrenal gland (*i.e.* including cortical and medullary tissues). Such a level of tissular organization is attainable in *ex vivo* preparations, and especially in acute slices, in which the cellular composition and tissular architecture are maintained and the exchange of biological signals between cells is preserved [7]. While recordings of the electrical activity from single isolated chromaffin cell have been described in the literature for many decades, the use of acute adrenal slices is comparatively more recent.

The procedures reported here describe both the preparation of rodent acute adrenal gland slices and the electrophysiological recordings from chromaffin cells in these slices. Additional information on other experiments that can be carried out with this methodology is also provided.

2 Materials

2.1 Dissection of the adrenal glands

1. Fine scissor to remove the adrenal glands from the animal
2. Two fine forceps to unsheathe each gland from the surrounding fat tissue
3. A carbogen gas cylinder (95% O₂/5% CO₂)

4. Ringer's saline: 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 12 mM glucose. The saline is continuously bubbled with carbogen (95% O₂/5% CO₂) and buffered to pH 7.4 (pH adjusted with NaOH). The osmolarity is adjusted to 310-320 mOsm. Milli-Q water is used for preparing solutions. The extracellular solution is used on the day of the experiment. However, a stock solution (ex: Ringer 10X) can be prepared in advance and stored at 4°C for 4-5 days. CaCl₂, MgCl₂ and glucose are added on the day of the experiment and only when the pH is adjusted (to prevent divalent ions from precipitation). Fill a glass beaker with Ringer's saline (100 mL). To reach the Ringer's saline optimal temperature (0-3°C) required for adrenal gland survival during slicing, the beaker is placed in a mixture of crushed ice and salt (NaCl) in a polystyrene box.

5. Filter paper Grade 1 (circles, 90 mm diameter)

2.2 Adrenal slice preparation

1. Extracellular medium for slicing: cooled Ringer's saline bubbled with carbogen.

2. A vibratome (many models are commercially available). To maintain the optimal temperature of the Ringer's saline during slicing (0-3°C), the vibratome chamber must be cooled, either by an automatic cooling unit included in the vibratome, or by enclosing the chamber in a mixture of ice and salt.

3. A razor blade (no need for a special blade, use a simple blade commonly used for shaving, Fig. 1c). Before use, clean the razor blade with alcohol (70%) to remove traces of grease.

4. A tube of rapid glue. The use of surgical glue is not mandatory. Prefer using gel glue which is easier to manipulate rather than liquid glue.

5. An agarose block. Prepare 100 ml of an agarose solution (6 % in Milli-Q water) and pour the whole solution into a petri dish (100 mm diameter) to obtain a final thickness of about

1 cm. After cooling, cut a 1 cm cube and hemisection it. The half-cube shape is optimal for stabilizing the adrenal gland during slicing without affecting free floating of slices in the chamber (Fig. 1d).

6. A plastic Pasteur pipette used to manipulate the slices. Cut the pipette tip with a scissor and fire-polish it to a diameter slightly larger than the slice size.

2.3 Slice storage after cutting

1. A storage chamber maintained at 35-37°C (Fig. 1e), filled with Ringer's saline and continuously bubbled with carbogen to maintain a constant pH of 7.4.

2. A nylon sieve on which the adrenal slices are deposited. For an optimal preservation of the slices, control carefully that they lie flat on the sieve and do not stick to it or to each other. Under these conditions, adrenal slices can be used up to 6-8 hours after slicing.

2.4 Electrophysiological recordings (patch-clamp)

1. Extracellular solution: 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 12 mM glucose. The saline is continuously bubbled with carbogen (95% O₂/5% CO₂) and buffered to pH 7.4 (pH adjusted with NaOH). The osmolarity is adjusted to 310-320 mOsm with sucrose. Milli-Q water is used for preparing solution. Prepare a 1 L solution. The composition indicated here corresponds to a standard solution, which can be modified with pharmacological drugs to assess the contribution of specific components to the electrical activity recorded (Na⁺ currents, Ca²⁺ currents, K⁺ currents, excitatory/inhibitory synaptic currents, gap junction currents...).

2. Borosilicate glass for the patch pipettes (thin wall with filament, 1.5 mm outer diameter, 1.17 mm inner diameter).

3. A glass puller (many models are commercially available). For recordings in the whole-cell configuration, patch pipettes are pulled to have a resistance of 5-7 M Ω when filled with the intracellular solution of K-gluconate (see below). For perforated patch-clamp recordings, the resistance of the pipettes is 2-5 M Ω . For loose patch-clamp recordings, increase the pipette resistance to 5-10 M Ω when filled with extracellular medium. Except for recordings in perforated patch [8-10], there is no specific need to polish the pipette tips after pulling. Also, since the signals recorded are not very small, there is no need for further electrical isolation.

4. An upright microscope equipped with differential interference contrast optics, and with low magnification x5 (or x4) and high magnification (x60, water immersion) objective lenses. Notice that the magnification value can slightly differ between microscope's manufacturers.

5. An electrophysiology set-up (patch-clamp amplifier + one or two 3-axis micromanipulators + an anti-vibration air table and a Faraday cage).

6. A recording chamber equipped with perfusion and temperature control systems. A peristaltic pump can be used for perfusing the recording chamber (perfusion rate of about 2 ml/min).

7. Intra-pipette solutions. All solutions are prepared in Milli-Q water. The composition of solutions differs between the recording configurations (conventional whole-cell recording *versus* perforated whole-cell recording, patch-clamp *versus* loose patch), the recording modes (current-clamp *versus* voltage-clamp) and the ion current recorded (Na⁺, Ca²⁺, K⁺... currents). Three examples of intra-pipette solutions are listed below:

a. Intra-pipette milieu for whole-cell conventional patch-clamp recordings: 140 mM K-gluconate, 2 mM MgCl₂, 1.1 mM EGTA, 5 mM HEPES, 3 mM Mg-ATP, 0.5 mM Na-GTP. The solution is titrated to pH 7.2 with KOH. Osmolarity is adjusted at 285-295 mOsm with K-Gluconate [11].

b. Intra-pipette milieu for perforated patch-clamp recordings: Differing from the standard internal solution used for conventional patch-clamp recording is the presence of a pore-forming antibiotic (typically amphotericin B, nystatin or gramicidin). The most commonly used agent in rat/mouse adrenal slices is amphotericin B (0.1 to 1 mg/ml prepared in dimethylsulphoxide) [7,8,12]. See [13] for a detailed description of the methodology using amphotericin B. When deciding which permeant agent to use, it is important to take into consideration the ion selectivity of the pores formed. Indeed, most antibiotics create an anion-selective channel when incorporating the cell membrane and chloride ions will equilibrate between intra and extracellular compartments.

c. Intra-pipette milieu for loose patch recordings: 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose and buffered to pH 7.4 with NaOH. Osmolarity is adjusted at 285-295 mOsm with NaCl [11].

All internal solutions are maintained at 4°C before use. Solution containing the permeabilizing agent is prepared on the day of the experiment and stored at 4°C. Patch-pipettes are filled using a fine catheter from a 1 ml- plastic syringe (or a yellow pipette tip), for which the tip has been pulled with a flame. For perforated patch-clamp, pipettes are tip-filled with standard internal solution and then back-filled with the solution containing the pore-forming agent.

3 Methods

3.1 Acute slice preparation

1. After removal, keep the adrenal glands in ice-cold saline for 2 min. Before slicing, the glands have to be unsheathed from the surrounding adipose tissue (Fig. 1a). Transfer one gland on filter paper and remove the fat engulfing the adrenal with two fine forceps. A well-dissected gland will facilitate the slicing procedure, as the slices will themselves release from

the gland. During the dissection process, be careful to maintain the integrity of the capsule. This then helps to generate healthy slices, with cortex and medulla.

2. Transfer the dissected gland to the stage of the vibratome (Fig. 1b). Apply a small amount of glue on the stage and glue the gland first and then the agarose block. Position the agarose cube just behind the gland to stabilize it during slicing (Figs. 1c and 1d). As they are generated, handle the slices with a plastic Pasteur pipette and gently release them on the nylon sieve in the storage chamber (maintained at 35-37°C and bubbled with carbogen). The acute adrenal slices obtained using the above protocol are suitable for electrophysiological recording immediately after cutting and for 6-8 hours after cutting (*see Note 1*). Fig. 1f illustrates an example of six serial slices prepared from the same adrenal gland (adult male rat). As expected from the tissular organization of the adrenal gland, the first slices are exclusively composed of the cortex and the medullary tissue gradually appears with the next slices. With a little knowledge of the adrenal gland, it is easy to recognize the two tissues by eye. The labeling of living slices with a dye can help a novice person to be trained (*see Note 2*).

Note that acute adrenal slices can be used to investigate the amount of catecholamines released from the entire medullary tissue, as reported in rat [14].

3.2 Electrophysiological recordings (patch-clamp)

The reader can find a detailed description of the patch-clamp technique in the pioneer article authored by Hamill and colleagues [15]. Regarding chromaffin cells, electrophysiological recordings in acute slices have been successfully performed on both rat and mouse, male and female, adult and newborn animals [16-19,11,20].

1. Collect a slice with a plastic Pasteur pipette from the storage chamber and carefully transfer it to the recording chamber attached to the stage of the upright microscope. Ensure that

the perfusion is switched off during the transfer procedure to avoid aspiration of the slice in the vacuum.

2. Once the slice is positioned into the chamber, place a nylon mesh on the edge of the slice (see **Note 3**) to keep it flat in the bottom of the chamber. Restart the perfusion system and check that the temperature of the extracellular saline is maintained at 30-32°C. Importantly, make sure that the slice is well positioned on the bottom of the chamber and does not drift during perfusion. Electrophysiological recordings will be not stable with submerged slices not secured to the bottom of the chamber.

3. Locate the adrenal medullary tissue as a whole using the low magnification objective lens. To observe chromaffin cells, switch to the water immersion objective lens. Choose a cell for recording and move the stage of the microscope to position it at the center of the optic field (see **Note 4 and Note 5**).

4. Switch again to the low magnification objective and mount a patch pipette (filled with the internal solution) in the holder of a patch-clamp amplifier. Apply a small amount of positive pressure to the patch electrode as approaching the target cell (see **Note 6**). This allows i) keeping the tip of the pipette clean and ii) facilitating high resistance seal.

5. Use the micromanipulator ('macro-travel' mode) to move the pipette down to reach the surface of the adrenal medulla (without touching the tissue).

6. Switch to the high magnification objective. Locate the patch pipette above the slice surface and use the 'micro-travel' mode of the micromanipulator to carefully position the patch electrode above the chosen cell. Move slowly the pipette down to delicately touch the cell. Note that a light halo can sometimes be viewed on the cell surface. Stop to move down. From this position, several recording configurations are possible (Fig. 2):

a. Conventional patch-clamp recording: As soon as the patch electrode touches the cell, apply a continuous but slight negative pressure (suction) until the formation of a high-resistance (>

G Ω) electrical seal, known as a gigaseal, between the pipette and the cell (Fig. 2a). From this configuration (cell-attached configuration) and to switch to the whole-cell configuration, apply an additional suction pressure, shorter but stronger (*see Note 6 and Note 7*, Fig. 2d). Breaking of the cell membrane fragment under the pipette leads to a gradual 'washout' of the cytoplasm by the intra-pipette solution. Depending on the experiment conducted, this can be seen as an advantage (possibility to load the recorded cell with an extrinsic agent (pharmacological drug, calcium/voltage/...-sensitive dyes, fluorescent tracer... (*see Fig. 4d*)) or a disadvantage when considering the dialysis-induced loss of internal compounds which are crucial for cell function. Signals are sampled at 10 kHz and filtered at 3 kHz. In the current-clamp mode of the whole-cell configuration, changes in membrane potential are monitored and spontaneous discharges of action potentials can be recorded (Figs. 3a and 3b). In the voltage-clamp mode, the membrane potential is maintained at a holding value (-50 mV for example, corresponding to chromaffin cell resting membrane potential) and changes in membrane currents (Na⁺, Ca²⁺, K⁺ currents...) can be monitored (with appropriate composition of both extracellular and internal recording solutions).

b. Perforated patch-clamp recording (Fig. 2c): The first steps until obtaining the gigaseal are identical to those described above for conventional patch-clamp recording. From the cell-attached configuration, the access resistance gradually decreases upon the action of the permeabilizing agent to reach a stable value within a couple of minutes to about 20 minutes [7]. A low access resistance (<20 M Ω) is required before starting recording. The time to reach this resistance value depends on many parameters, such as the extent to which the tip of the pipette is filled with amphotericin-free solution, the quality of the gigaseal (resistance), or the accessibility of the cell surface. While the use of a cleaning pipette to remove loose material from the cell surface is not necessary required for conventional patch-clamp, this can improve the quality of recording in the perforated conditions [8]. Typical chart recordings of membrane

potential/current changes can be found in [7,8,12,21,10,9,22]. Electrophysiological recordings in this configuration have been successfully performed on both adult rat and mouse, male and female.

c. Dual patch-clamp recording: The maintenance of the tissular architecture in adrenal slices allows recording of electrical events occurring in chromaffin cell pairs, such as junctional currents flowing through gap junction channels [23]. The procedure is roughly similar to that described for 'single' patch-clamp recordings. The following sequence is recommended: gigaseal in cell #1, gigaseal in cell #2, whole-cell in cell #1 and whole-cell in cell #2. Like for 'single' patch-clamp, both membrane potential and membrane current changes can be monitored. The principle of dual patch-clamp to record junctional currents is to clamp the two cells engaged in the pair at the same potential and then change the potential (depolarization/hyperpolarization) in only one cell. If the two cells are electrically coupled, the current in cell #1 results from the addition of the membrane current evoked in response to depolarizing/hyperpolarizing voltage steps and the junctional current. The current in cell #2 corresponds to the junctional current (same amplitude as recorded in cell #1 but opposite direction). Representative chart recordings of junctional currents in chromaffin cell pairs in rodent adrenal slices can be found in [17-19,24].

The whole-cell configuration of the patch-clamp technique offers the possibility to record various classes of electrical signals (Fig. 4 and *see Note 8*). The most common ones are membrane potential changes driven by action potential firing and membrane current changes in response to activation/inhibition of ion channels (Fig. 4a). In chromaffin cells, the synaptic activity induced by innervating axons (excitatory/inhibitory postsynaptic potentials/currents) can be easily recorded (Fig. 4b). The electrical coupling in a cell pair can also be investigated through patch-clamp recording (Fig. 4c). One can assess the intercellular communication through passive diffusion of fluorescent tracers (Fig. 4d).

d. Loose cell-attached patch recording (Fig. 2b): This method allows investigating of membrane excitability under physiological conditions since there is no rupture of the cell membrane. In the absence of intracellular milieu dialysis by the intra-pipette medium, long-lasting recordings (>10 minutes) are possible. Position the tip of a patch electrode at the surface of a chromaffin cell and apply a minimal suction pressure to get a 'weak' seal resistance (<500 M Ω .) This configuration using a loose seal is well-appropriate for recording cell spiking activity. Under the voltage-clamp mode, the electrical activity is viewed as action potential currents. To record the electrical firing occurring at the resting membrane potential (a condition for which no current is injected by the patch amplifier), adjust the holding potential of the recording pipette to get a holding current equal to zero. Note that a giga-seal seal (resistance >G Ω as describe above) is required for triggering action potentials in the patched cell and for recording resting and synaptic potentials. The theory and the experimental applications of this technique are detailed in [25]. A representative example of action potential currents recorded in chromaffin cells in acute adrenal slices is illustrated in Fig. 3c.

4 Notes

1. The adjustment of the cutting parameters depends on the equipment used. The following values are given as a guide: slicing speed approximately 0.05-0.1 mm/s, vibration amplitude 1.2 mm and vibration frequency 30-100 Hz. Slices can be generated at various thickness (from 100 to 250 μ m), depending, at least in part, on the species and the age of animals. The small size of adrenal glands in neonates is a limiting factor for the number of slices containing medulla. In our hand, slices of 100 μ m thickness appear the best compromise in rat neonates, taking into account the two parameters (adrenal size and number of slices) [17,18].

2. To distinguish between the cortical and the medullary tissues in a living slice, a labeling with hematoxylin (1g/l for 2 minutes) can be helpful.

3. Meshes of nylon threads used to maintain the slices in the recording chamber are commercially available as grids. Choose an appropriate size and format to allow the recording pipettes to fully access the recording area. It is possible to use homemade grids with a nylon sock. The main advantage, if we except the cost, is the possibility to stretch the sock to adjust the spacing between threads to the size of the slice.

4. The use of a microscope eyepiece equipped with an ocular micrometer can be useful to precisely locate the cell of interest.

5. For some reasons (tissue structure, cell composition...), the access *via* the patch pipette to chromaffin cells located deeply in the tissue is tricky or even impossible. As such, most of the electrical signals recorded in chromaffin cells come from cells one or two layers deep into the slice.

6. All pressures (positive when approaching the cell of interest, or negative to induce the cell-attached and whole-cell configurations) can be generated either from the mouth of the experimenter or using plastic syringes (1-10 ml).

7. Coating and fire polishing of the patch electrode tips are generally not required for recording in the conventional patch-clamp condition.

8. Secretory events such as hormone release (= catecholamine release in the case of chromaffin cells) can be monitored by an electrochemical approach based on amperometry. The technique uses a carbon fiber electrode to detect ion currents generated by the oxidizing reaction of catecholamines (dopamine, norepinephrine, epinephrine). The instrumentation usually found on an electrophysiological setup (microscope, electrode holder, micromanipulators, patch-clamp amplifier...) is quite suitable for the amperometric detection of secretory events. The carbon fiber used for amperometry is placed in close vicinity of the cell (use a micromanipulator to precisely position the electrode). The exocytotic currents generated by oxidation of catecholamines at the surface of the carbon fiber are measured at a constant potential of +800 mV and recorded by a patch-clamp amplifier (voltage-clamp mode). Note that the detection of catecholamine exocytosis by amperometry does not discriminate between dopamine, norepinephrine and/or epinephrine secretion. Examples of exocytotic events recorded in chromaffin cells in rat adrenal slices are illustrated elsewhere [7,16]. A variant of this technique, called 'patch amperometry', combines, as its name suggests, the two techniques of patch-clamp and amperometry. The insertion of a carbon fiber electrode into a patch pipette (Fig. 2e) allows monitoring of exocytotic events of individual chromaffin granules by using capacitance measurements (a cell surface-related electrophysiological parameter) combined with electrochemical detection of catecholamines. This technique has been first described in isolated bovine chromaffin cells using the cell-attached configuration of the patch-clamp technique [26] and then extended to cell-free membrane patches in the inside-out configuration [27]. To learn more, step-by-step instructions for the application of this method are described in [28]. To my knowledge, the use of patch amperometry in adrenal slices has not yet been reported.

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Figure legends

Fig. 1: Protocol for acute adrenal slice preparation. (a) Dissection of an adrenal gland and fat removal. (b) Illustration of a vibratome chamber. (c) Positioning of an adrenal gland in the chamber with the agarose block and the razor blade. Inset: a slice in progress. (d) Schematic representation of the agarose block (note its half-cube shape) placed behind the adrenal gland. (e) Top view of the storage chamber. Note the nylon sieve on which slices (see arrow heads) are stored before use. (f) Photographs of six serial slices from a rat adrenal gland. Each slice was labeled with hematoxylin (1 g/l, 2 min), as such the cortical and medullary tissues can be

distinguished by differential staining intensity (the dashed line delimits the surface of the medullary tissue). The inset illustrates a schematic representation of the two (neuro)endocrine tissues (cortex and medulla) of an adrenal gland.

Fig. 2: Schematic representations of some patch-clamp configurations. (a) Cell-attached configuration with the gigaseal. (b) Loose cell-attached configuration. Note the formation of a loose seal rather than the tight gigaseal. Blue arrows indicate the weak contact between the electrode tip and the cell membrane. (c) Whole-cell configuration in perforated patch-clamp. The presence of a pore-forming antibiotic in the intra-pipette solution (orange circles) allows to permeabilize the cell membrane, allowing electrical access between the cell and the patch-pipette. (d) Whole-cell configuration in conventional patch-clamp. Under this experimental condition, the membrane fragment under the patch pipette is broken, which leads to a gradual dialysis of the cytosol by the intra-pipette solution (blue arrows). (e) Cell attached Configuration for patch amperometry. This technique combines amperometric detection of secretory events and capacitance measurement. Note the carbon-fiber electrode inserted into the patch pipette.

Fig. 3: Representative examples of spontaneous electrical activity in chromaffin cells recorded in mouse adrenal slices. Regular discharge (a) and discharge in bursts (b) of action potentials recorded in conventional whole-cell patch-clamp condition. (c) Discharge of action potential currents recorded in loose patch-clamp configuration (holding potential = 0 mV). The detailed methodology is available in [\[11\]](#).

Fig. 4: Whole-cell configuration of the patch-clamp: a versatile technique for recording many parameters. The different possibilities are illustrated on a schematic cluster of three

chromaffin cells releasing catecholamines. **(a)** Recording of action potential firing discharges in a mouse adrenal slice (current-clamp mode, conventional patch clamp). To assess the electrical activity at resting membrane potential, the injected current by the amplifier is held at 0 pA. **(b)** Recording of synaptic activity. Example of excitatory postsynaptic cholinergic currents recorded in a rat adrenal slice (voltage-clamp mode, holding potential = -60mV, conventional patch-clamp). **(c)** Recording of junctional coupling in a pair of electrically coupled chromaffin cells in a rat adrenal slice. Cell #1* is the stimulated cell. The depolarizing injected current triggers action potentials in cell#1* (black traces). In the unstepped cell (#2), a small depolarization resulting from gap junctional communication between cell #1 and cell#2 is elicited (red traces). **(d)** Monitoring of the dye diffusion between gap junction-coupled chromaffin cells. Example of Lucifer yellow (LY) diffusion between a chromaffin cell triplet within a lobule. The patch pipette is filled with Lucifer yellow and the passage in the whole-cell configuration (*i.e.* disruption of the membrane fragment under the patch pipette) allows the passive diffusion of the dye into the patched cell and neighboring gap junction-coupled cells.

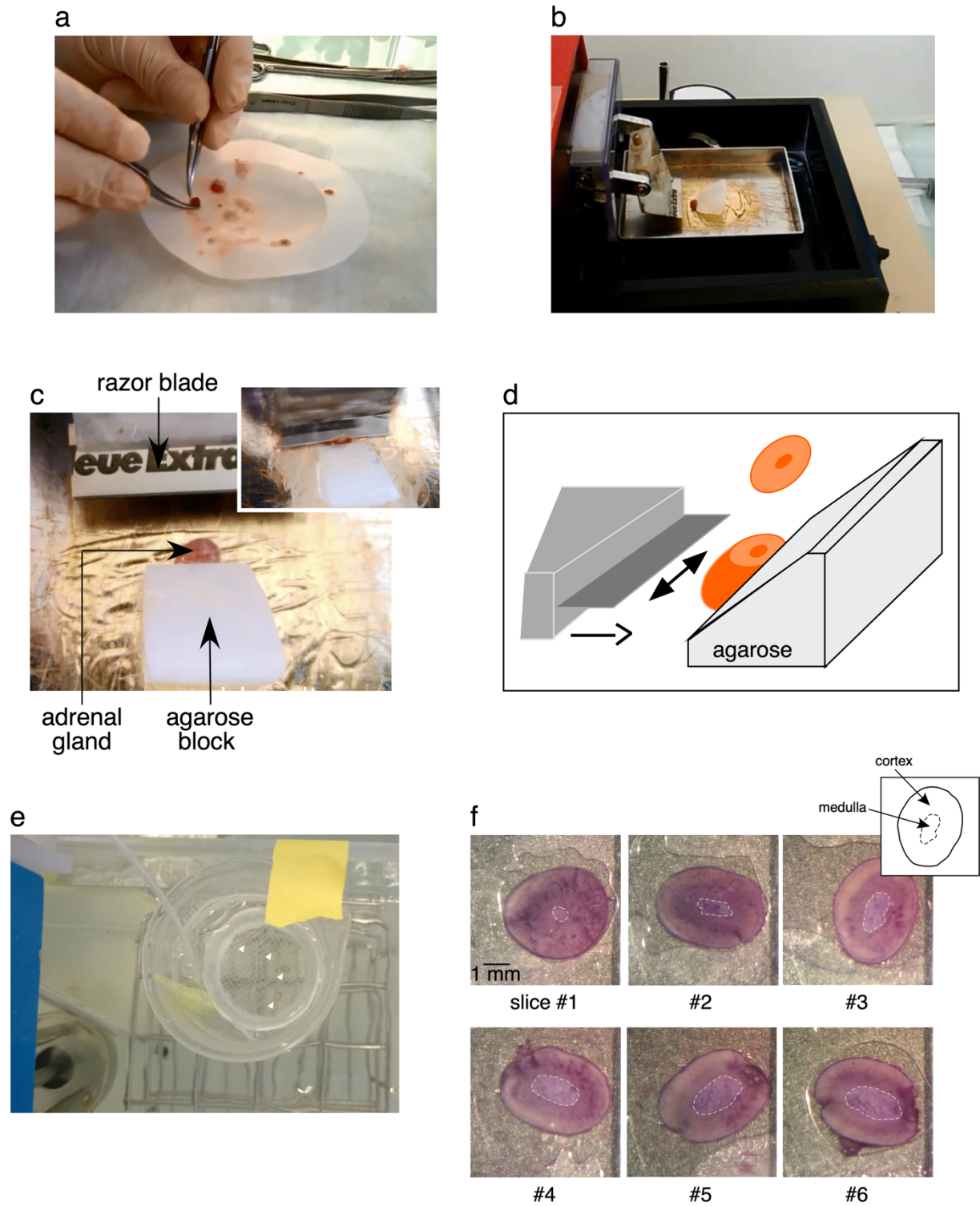


FIGURE 1

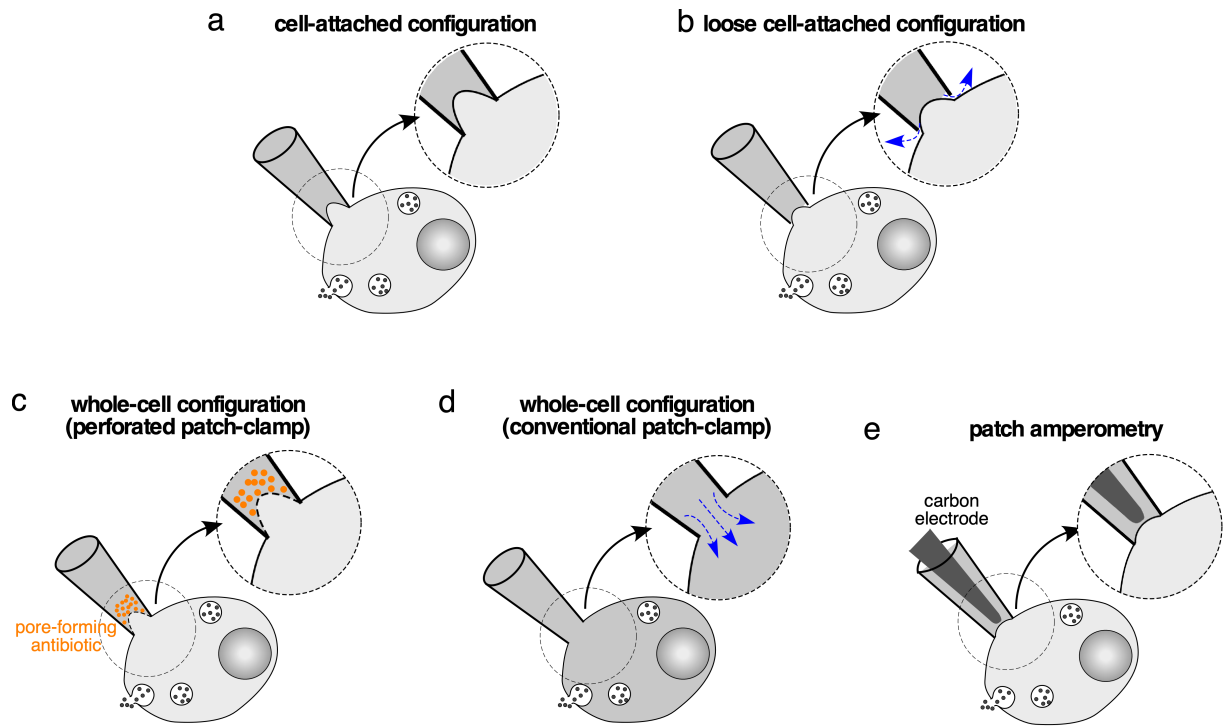


FIGURE 2

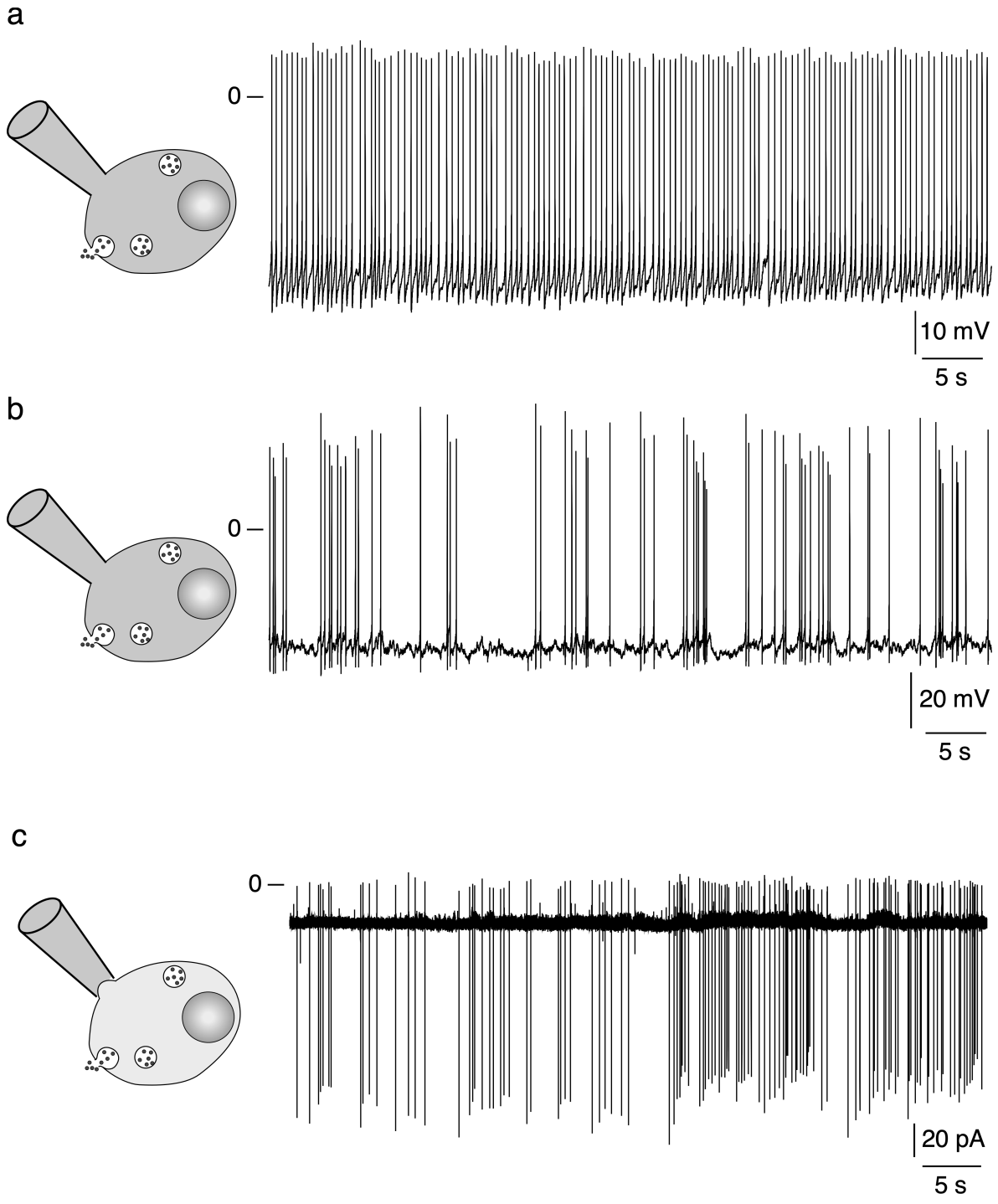
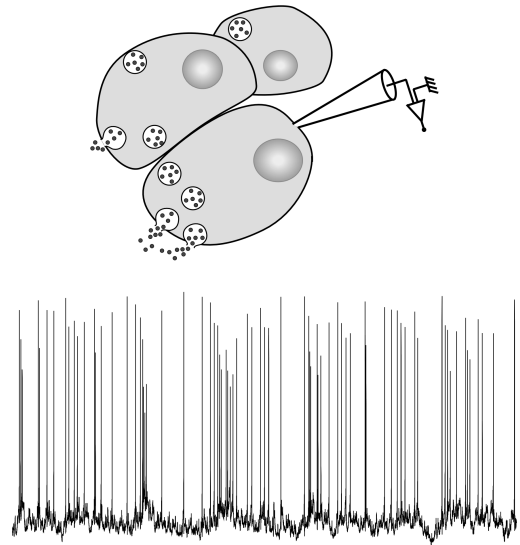
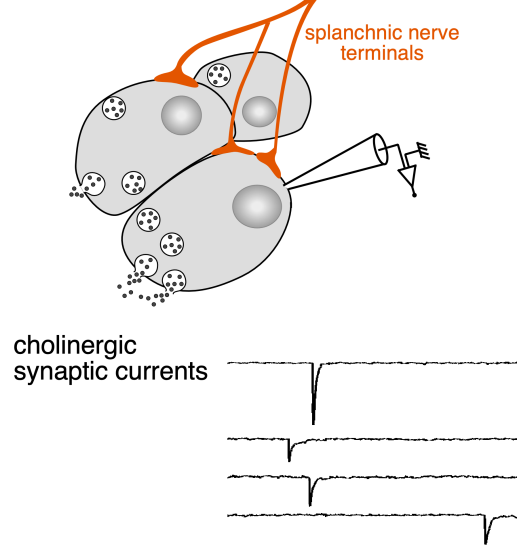


FIGURE 3

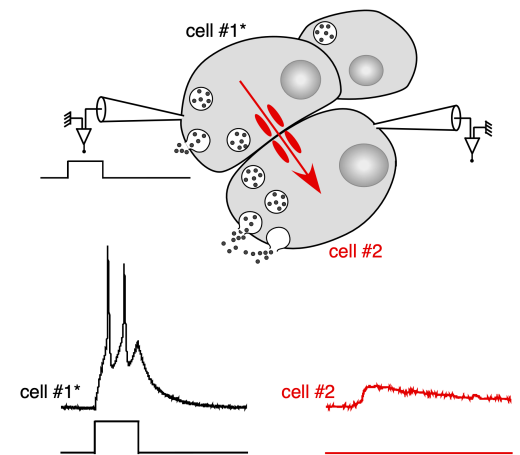
a Action potential discharges



b Spontaneous excitatory synaptic events



c Electrical coupling in a cell pair



d Dye diffusion between gap junction-coupled cells

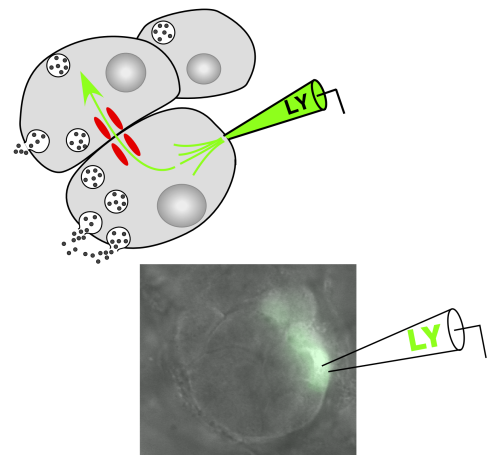


FIGURE 4