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A protocol for heterologous expression and functional assay for mouse pheromone receptors

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Abstract

Innate social behaviors like intermale aggression, fear, and mating rituals are important for survival and propagation of a species. In mice, these behaviors have been implicated to be mediated by peptide pheromones that are sensed by a class of G protein-coupled receptors, vomeronasal receptor type 2 (V2Rs), expressed in the pheromone-detecting vomeronasal organ (VNO) (Chamero et al., *Nature* 450:899–902, 2007; Haga et al., *Nature* 466:118–122, 2010; Kimoto et al., *Curr Biol* 17:1879–1884, 2007; Leinders-Zufall et al., *Nat Neurosci* 12:1551–1558, 2009; Papes et al., *Cell* 141:692–703, 2010). Matching V2Rs with their cognate ligands is required to understand what receptors the biologically relevant pheromones are acting on. However, this goal has been greatly limited by the unavailability of appropriate heterologous tools commonly used to carry out receptor deorphanization, due to the fact that this family of receptors fails to traffic to the surface of heterologous cells. We have demonstrated that calreticulin, a housekeeping chaperone commonly expressed in most eukaryotic cells, is sparsely expressed in the vomeronasal sensory neurons (VSNs). Stable knock down of calreticulin in a HEK293T derived cell line (R24 cells) allows us to functionally express V2Rs on the surface of heterologous cells. In this chapter we describe protocols for maintenance and expansion of the R24 cell line and functional assays for V2Rs using these cells.

Keywords

Heterologous; calcium; V2Rs; R24; cells

1. Introduction

In vertebrates, pheromones are largely detected by the accessory olfactory organ, the vomeronasal organ (VNO). In mice, the VNO has been implicated in detecting a large number of inter and intra specific chemosignals that mediate innate behavior like fear, aggression, mating rituals, pregnancy block, mate recognition, etc (1-5). The VNO contains closely packed sensory neurons that express at least two hundred and fifty putative pheromone receptors spanning three unrelated families of G protein-coupled receptors (GPCRs): vomeronasal receptors classes I and II (V1Rs and V2Rs respectively) and formyl peptide receptors (FPRs)(6-11). Each sensory neuron expresses either one V1R or an FPR or

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a combination of two V2Rs in spatially distinct regions. A key question in studying pheromonal olfaction is what biological ligands activate a receptor or related receptors; however, the complex expression pattern of such a large receptor repertoire makes it challenging to adequately address this question. Heterologous cells provide a convenient platform to carry out large scale functional and deorphanization studies for GPCRs; however, a major road block in studying the pheromone receptors using this tool was the inability to express the receptors functionally on the surface of heterologous cells. We have recently demonstrated that vomeronasal sensory neurons expressing V2Rs show low expression of an otherwise ubiquitous cellular chaperone, calreticulin. Depletion of calreticulin in heterologous cells (HEK293T) enabled export of V2Rs to the surface. Using this knowledge, we established the cell line R24, from HEK293T, with constitutive knock down of calreticulin. We then used this line to carry out V2R ligand binding assays with calcium imaging (12). In this chapter we describe the protocol to maintain and expand the cells and carry out the functional assays.

Heterologous cells have been extensively exploited to study G protein-coupled receptors. The advantages of deorphanizing receptors heterologously in comparison to *in vivo* protocols are many. One can selectively transfect a receptor-expressing plasmid into cells, and examine the response of the transfected cells easily to a ligand or set of ligands by applying a series of ligand pulses. To test the specificity of a particular receptor-cognate ligand pair, one can set up control experiments easily by testing either other ligands with the receptor of choice or other receptors with the ligand of choice. The heterologous system allows for identification of receptors for a set of biologically relevant ligands without performing genetic manipulations and/ or *in vivo* assays. The heterologous cells may be further used to study the signal transduction pathway by addition or subtraction of cofactors in the transfection step and comparing how the activity of the cells is affected. One can also create chimeric receptors, receptors with point mutations, or domain switched receptor constructs, transfect into cells, and test their response to a ligand or set of ligands. It allows us to identify motifs or domains and particular amino acids that are important for either ligand binding or signaling or both. Additionally, the heterologous cells provide a means to study various other cell biological areas, such as mechanisms of endoplasmic reticulum targeting of receptors, surface export and interactions with chaperones (13).

To assay for ligand binding, a V2R receptor of interest is co-transfected with H2M-10.4 and B2m as well as G α 15, a Gq class of G protein widely used in functional assays of GPCRs. Activation of G α 15, which couples to many but not all of the GPCRs, leads to a transient intracellular calcium release (14, 15). Changes in intracellular calcium can be easily detected by chemical dyes with high affinity for calcium which change their spectral properties upon binding calcium ions. Using this principle, we perform calcium dye based imaging because this provides an extremely sensitive and efficient method. We use a combination of two calcium dye indicators, Fluo-4 and Fura Red, which are both visible light excitable (488nm). The green-fluorescent emission (~525 nm) of Fluo-4 increases on binding calcium while the red fluorescence (~660nm) of the Fura Red indicator decreases once the indicator binds Ca²⁺. Using two dyes in combination enables one to carry out ratiometric quantification for changes in cytosolic calcium concentration.

R24 cells, like most other heterologous cell lines pose their own set of disadvantages, which should be closely considered in parallel with the advantages of deorphanizing V2Rs. In an attempt to mimic the vomeronasal sensory neurons, this heterologous cell line was established by knocking down calreticulin, a very essential endoplasmic reticulum chaperone that plays multiple roles in sustenance of cell health and calcium homeostasis. As a result, the cells are slower growing than the parent cell line (HEK293T), an important parameter to bear in mind while planning experiments. The cell must be handled with extreme caution -- over trypsinization should be avoided, cells should be triturated slowly, and all media changes must be done gently and in a timely way. Each batch of cells may be used for a limited number of passages.

Keeping these limitations in mind, one can design experiments using the R24 heterologous cells and calcium imaging to study the ligand specificities and functions of mouse V2Rs.

2. Materials

Use cell culture grade commercially available reagents. Prepare all solutions in a sterile environment, preferably in sterile laminar flow chambers. Diligently follow all waste disposal rules when disposing waste materials.

2.1 R24 cell culture and maintenance

1. Minimum essential medium (MEM) containing Earle's salts and L-glutamine.
2. Fetal bovine serum (FBS): heat inactivated.
3. 10% supplemented medium (M10): Mix 45ml MEM, with 5ml FBS to obtain 10% supplemented medium (M10). Store M10 in 4°C.
4. Penicillin-Streptomycin-Amphotericin (PSF) culturing medium: Prepare M10 as above and add Penicillin-Streptomycin and Amphotericin to final concentrations 100µg/ml and 1.25µg/ml respectively.
5. Puromycin containing PSF, R24 maintenance medium: Prepare PSF culturing medium as described. Prepare two R24 maintenance media by adding puromycin to final concentrations 5µg/ml and 20µg/ml respectively.
6. 100mm coated sterile cell culture dishes
7. Centrifuge tubes

2.2 Cell transfer and plating for calcium imaging

1. 1× Phosphate Buffer Saline, PBS
2. Trypsin (0.05%)
3. 35 mm sterile cell culture dishes with 10mm optical glass well.

2.3 Transfection

1. Transfection reagent

2. Plasmid DNA: V2R pheromone receptor, major histocompatibility factor 10.4, β 2 microglobulin and mouse G α 15 complete open reading frames cloned in mammalian expression vector pCI.

2.4 Calcium imaging assay

1. Fura Red solution: Dissolve 50 μ g Fura Red in 12.5 μ l DMSO to obtain 4 μ g/ μ l Fura Red.
2. Fluo-4: Dissolve 50 μ g Fluo-4 in 12.5 μ l DMSO to obtain 4 μ g/ μ l Fluo-4
3. Pluronic acid: Weigh out 20mg pluronic acid (amorphous solid) in an eppendorf tube, add 100 μ l cell culture grade DMSO and incubate at 37°C for 10 – 15 minutes, intermittently tapping the mixture to dissolve the pluronic acid (see Note 1).
4. Bovine serum albumin (BSA), 7.5%
5. Glucose, 45%
6. Hanks Balanced Salt Solution (HBSS) 1 $\times$, with glucose: anhydrous calcium chloride 140 mg/ L, magnesium chloride -6H₂O 100mg/L, magnesium sulfate-7H₂O 100mg/ L, potassium chloride 400mg/ L, potassium phosphate monobasic 60mg/ L, sodium chloride 8000mg/L, sodium phosphate dibasic -7H₂O 48mg/L, Dextrose 1000mg/L; pH = 7.4
7. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1M.
8. Imaging buffer: HBSS, 10mM HEPES, 0.45% glucose. Mix 5ml 1M HEPES and 5ml 45% glucose in 500ml HBSS to obtain imaging buffer.
9. Loading buffer: Add 13.3 μ l, 7.5% BSA to 1ml imaging buffer to obtain 0.1% BSA in imaging buffer.
10. Dye mixture: Mix well 0.5 μ l 20% pluronic acid, 0.5 μ l Fluo-4 and 1 μ l Fura Red with vigorous pipeting, and add 500 μ l loading buffer to obtain dye mixture (see Notes 2, 3).

2.5 Equipments

1. 37°C cell culture incubator with 5% carbon dioxide.
2. Certified class II biological safety cabinet with laminar flow.
3. Centrifuge with swinging bucket rotor for 15 ml conical tubes.
4. Phase contrast microscope with 10 $\times$, 20 $\times$ objectives.
5. Fluorescence microscope with Fluo 4 and Fura Red filters (excitation, 488 nm; emission, 500–560 nm for Fluo-4 and 605–700 nm for Fura red), 40 $\times$ objective.
6. Peristaltic pump.

3. Methods

All cell culture procedures must be carried out in a sterile way, in class II biological safety cabinet with laminar flow, at room temperature unless otherwise mentioned.

3.1 R24 cell culture and maintenance

1. Quickly thaw a frozen vial of R24 cells in a 37°C water bath.
2. Immediately after thawing, transfer cells from vial to sterile 15 ml tube containing 10 mL M10.
3. Centrifuge at 1000 rpm for 5 minutes.
4. Aspirate supernatant and resuspend cells in 8mL M10 with gentle trituration.
5. Plate cells in a 100mm sterile cell culture dish.
6. Observe the cells closely in a phase contrast microscope, they should appear like round particles in suspension in the media.
7. Incubate overnight in a cell culture incubator at 37°C, 5% CO₂.
8. Observe the cells again after overnight incubation, they should have settled to the bottom of the cell culture dish and attached to it.
9. If the cells appear to be firmly attached, aspirate M10 plating medium and replace with 8ml PSF culture medium (see Note 4).
10. 24 hours later, change medium was to PSF containing 5µg/mL puromycin (see Note 5).
11. 24 hours after addition of 5µg/mL puromycin, change the medium to 20µg/mL puromycin containing PSF (see Note 6).
12. Thereafter maintain the cells in 20 µg/ml puromycin and PSF containing medium for experiments.

3.2 Transfer R24 cells for transfection

1. Allow cells to grow till confluent or up to desired confluency, checking cell density periodically (see Note 7).
2. Prior to cell transfer, aspirate media and gently add 8mL sterile PBS for washing out media (see Note 8).
3. Incubate cells in 3mL trypsin (0.05%) at room temperature; when all cells are loosened from the sterile cell culture dish, gently triturate them a few times to detach them.
4. Stop trypsinization by adding 5 mL M10; gently triturate the mixture several times to dissociate the cells from each other and make a homogeneous cell suspension (see Note 9).

5. At this point, the cells need to be plated for assay and maintenance. Depending on anticipated frequency of future uses, transfer a portion of the trypsinized cells into a sterile conical tube; for the immediate assay, depending on the number of dishes to be set up, transfer another portion into a second sterile conical tube.
6. Centrifuge both aliquots at 1000rpm, for 5 minutes at room temperature.
7. Resuspend the aliquot for maintenance in 8ml 20 μ g/ ml puromycin containing PSF media by gentle trituration and transfer to a sterile 100mm cell culture dish. Maintain the dish in the cell culture incubator at 37°C, 5% CO₂ till further use.
8. Resuspend the aliquot of cells for imaging in M10 (antibiotic free), 1ml per 10mm dish.
9. Transfer 1ml of media containing resuspended cells into each glass bottomed dish.
10. Incubate dishes overnight in 37°C, 5% CO₂.

3.3 Transfection of cells for imaging

1. Observe the cells closely using a phase contrast microscope 24 hours after plating. If cells appear to have settled on the glass bottomed dish, they are ready for transfection. R24 cells should be ~40% confluent at the time of transfection in the 35mm cell culture dishes with 10mm optical glass wells.
2. Mix plasmid DNA in the following amounts (per glass bottomed dish): V2R pheromone receptor 1000ng, H2-M10.4 1200 ng, β 2- microglobulin 200 ng and G α 15 400 ng (see Note 10).
3. For transfection, follow instructions from the transfection reagents manufacturers' manual.
4. Incubate the cells for ~36 hours in 37°C, 5% CO₂.

3.4 Calcium imaging

1. Approximately 36 hours after transfection, slowly aspirate the medium out of the glass bottomed culture dish.
2. Gently add 1ml imaging buffer to wash remaining media, taking care to avoid pipetting the buffer in the glass bottom.
3. Slowly aspirate the buffer from the culture dish.
4. Pipette 50 μ l dye mixture to the glass bottom.
5. Incubate the cells in the dye mixture for 45 minutes in dark (see Note 11).
6. Dilute stimulant to desired strength in imaging buffer.
7. Wash the stimulant/ buffer delivery tubing thoroughly with distilled water.
8. For imaging, expose the cells to a constant flow of imaging buffer (see Note 12).
9. Collect the data at wavelengths appropriate for Fluo-4 and Fura Red (excitation 488nm, emission 500-560nm for Fluo-4, 605-700nm for Fura-red) at regular

intervals. Apply stimulants for desired length of time punctuating with buffer flow to allow the cells to recover in case they respond to a particular pulse of test ligand (see Note 13, 14).

10. At the end of the ligand pulses, apply a pulse of 10nM isoproterenol to use for positive control (see Note 15).

3.5 Preparation of R24 cell frozen stocks

1. Pre cool an isopropyl alcohol bath to 4°C.
2. To prepare frozen stocks of R24 cells, start culture and grow them in PSF containing medium as discussed in Section 3.1.
3. When confluent, split them as discussed in Section 3.2. After aspirating out the trypsin medium, add 2ml freezing medium and gently triturate.
4. Transfer 1 ml of resuspended cells to one freezing vial (two vials of frozen stocks from each confluent 100mm plate of R24 cells).
5. Freeze cell vials in -80°C.
6. Store frozen vials of R24 cells for long term at -80°C.
7. Cells split as discussed and resuspended after spinning in freezing mixture (each 100mL confluent plate of cells resuspended in 1mL freezing mixture, frozen in a single vial for usage next time) and frozen in -80°C in isopropyl alcohol bath.

3.6 Data analysis

Base fluorescence intensities, changes in the Fluo-2 and Fura Red intensities, and the ratio of the changes can be plotted with graphing softwares like Microsoft Excel or ImageJ. Use the ratio to analyze the data for responses (Figure 1).

Determine cells that have responded to a positive control stimulant applied at the end of the experimental run, for example 10nM Isoproterenol (see Note 16).

Response to ligand pulse should appear within 90 seconds of application of the stimulant (see Note 17).

Responses that appear before the stimulant front reaches the cells should be counted as non specific.

A cell that responds non-specifically outside the range of time interest should be discounted from analysis.

A cell must show an increase of Fluo-4 intensity and a concomitant decrease in Fura Red signal intensity; ratio of Fluo-4 to Fura Red signals must exceed 50% of the base line (see Note 18).

Efficiency of transfection may be calculated by expressing the number of isoproterenol responsive cells as a percentage of the total number of cells in the field of view (see Note

19). Number of cells responding to a particular ligand should be normalized by expressing as a percentage of the total number of isoproterenol responsive cells. Normalization enables us to compare responses between different dishes, conditions, and days of experiments.

Notes

1. For best results, make fresh solutions of 20% pluronic acid every week.
2. After mixing pluronic acid, Fluo-4, and Fura Red thoroughly, consider incubating in a 37°C water bath or heating block for 5 minutes. This increases the total uptake of the dyes by each cell.
3. Once loading buffer is prepared, keep wrapped in aluminum foil in the dark till the end of the experiment, since the fluorescent dyes are light sensitive.
4. Observe the cells closely before replacing the medium. If cells look unsettled, wait until the cells are attached to the dish before replacing the M10 medium.
5. Some cells usually die after addition of lower concentration of puromycin.
6. A larger number of cells usually die after adding media containing higher concentration of puromycin.
7. R24 cells grow and divide approximately every 36 hours.
8. It is important to wash out supplemented medium before adding trypsin since any trace of medium will inhibit trypsin activity.
9. R24 cells attach less robustly to cell culture dishes than the parent cell line HEK293T; therefore while carrying out cell transfer, maintain caution to not over trypsinize. Stop the enzymatic reaction as soon as the cells detach from the culture dish.
10. These amounts of plasmid DNA have been optimized for pCI (mammalian vector) based constructs and transfections carried out with Lipofectamine 2000 (Invitrogen) mediated transfections. For other expression and transfection systems, the relative amounts of the different constructs in the transfection mixture may have to be optimized.
11. For best results, soak transfected cells in dye solution, one dish at a time so that cells can be imaged promptly after loading with dye. Letting the loaded cells wait longer than the required incubation time results in formation of intracellular dye aggregates that appear as tiny fluorescent specks while carrying out the assay and may interfere with the readout.
12. R24 cells attach lightly to the glass bottomed dish; delivery must be minutely regulated such that buffer or stimulants are delivered at a steady rate and at the same time does not dislodge the cells from the dish. We optimized our assays at delivery rate of ~50µl/sec, regulated by a peristaltic pump (Rainin).
13. We used Leica confocal microscope and the live imaging mode of Leica confocal software for data acquisition. Data was collected at 3s interval.

14. We pulsed up to 100nM of ESP family of peptides to stimulate cells transfected with test receptors; ligand pulses were applied for 10 seconds and buffer bath was applied for 2 minutes following ligand pulse.
15. Isoproterenol is a cognate ligand for the beta adrenergic receptor, endogenously expressed in the HEK293T cells or cells derived from it. On binding isoproterenol, the receptor couples with transfected G α 15, resulting in an increase in intracellular calcium.
16. Applying the pulse of isoproterenol at the end of the assay enables the experimenter to ensure that cells are healthy and responsive throughout the experiment. Cells not responding to isoproterenol in the end should not be counted.
17. We empirically determined responses of R24 cells transfected with V2Rp receptors to applied concentrations of ESP ligands appeared within 90 seconds time frame. This may have to be optimized for different ligand-receptor pairs and instrument set up.
18. A concomitant increase in fluorescence intensity of one fluorophore and decrease in the other ensures real signals are selected for analysis over noise. We empirically determined an increase in ratio of Fluo-4 to Fura Red by 50% from the base line enables us to segregate noise from real signals. This may have to be optimized for different ligand-receptor pairs, calcium dyes and instrument set up.
19. R24 cells are transfection efficient; typically 80 – 100% of cells respond to 10nM isoproterenol within seconds of application. An assay that shows less efficient calcium response to the positive control should not be considered for statistical purposes.

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Figure 1.

Response of single R24 cell transfected with V2Rp1 to serial application of stimulants.

Upper panel: change of Fluo-4 and Fura Red fluorescence intensities (B, before application of stimulant indicated in X axis; A, after application of stimulant indicated in X axis). Lower panel: Calcium concentration trace of the cell on application of recombinant peptide stimulants (ESP15, 36, 3, 5 and 6 respectively), 100nM each and positive control (isoproterenol, 1nM).