Video Article

HSV-Mediated Transgene Expression of Chimeric Constructs to Study Behavioral Function of GPCR Heteromers in Mice

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Abstract

The heteromeric receptor complex between 5-HT_{2A} and mGlu2 has been implicated in some of the behavioral phenotypes in mouse models of psychosis[1,2]. Consequently, investigation of structural details of the interaction between 5-HT_{2A} and mGlu2 affecting schizophrenia-related behaviors represents a powerful translational tool. As previously shown, the head-twitch response (HTR) in mice is elicited by hallucinogenic drugs and this behavioral response is absent in 5-HT_{2A} knockout (KO) mice[3,4]. Additionally, by conditionally expressing the 5-HT_{2A} receptor only in cortex, it was demonstrated that 5-HT_{2A} receptor-dependent signaling pathways on cortical pyramidal neurons are sufficient to elicit head-twitch behavior in response to hallucinogenic drugs[3]. Finally, it has been shown that the head-twitch behavioral response induced by the hallucinogens DOI and lysergic acid diethylamide (LSD) is significantly decreased in mGlu2-KO mice[5]. These findings suggest that mGlu2 is at least in part necessary for the 5-HT_{2A} receptor-dependent psychosis-like behavioral effects induced by LSD-like drugs. However, this does not provide evidence as to whether the 5-HT_{2A}-mGlu2 receptor complex is necessary for this behavioral phenotype. To address this question, herpes simplex virus (HSV) constructs to express either mGlu2 or mGlu2ΔTM4N (mGlu2/mGlu3 chimeric construct that does not form the 5-HT_{2A}-mGlu2 receptor complex) in the frontal cortex of mGlu2-KO mice were used to examine whether this GPCR heteromeric complex is needed for the behavioral effects induced by LSD-like drugs[6].

Video Link

The video component of this article can be found at https://www.jove.com/video/53717/

Introduction

Hallucinogens, such as LSD, psilocybin and mescaline cause significant changes in human consciousness, cognition and emotion[7-9]. Inactivation of serotonin 5-HT_{2A} receptor signaling by either genetic or pharmacological approaches causes markedly attenuated behavioral responses to hallucinogens in both rodent models[3,10] and humans[11]. Although hallucinogens bind other receptor subtypes[8], the 5-HT_{2A} receptor is considered as necessary for the unique behavioral activity of these chemicals.

Group II metabotropic glutamate receptors (*i.e.*, mGlu2 and mGlu3) have been the target of considerable attention regarding the molecular mechanism of hallucinogens and their integral role underlying psychosis[12]. Previously, it has been demonstrated that mice with no expression of mGlu2 protein (mGlu2-KO mice) are insensitive to the cellular and behavioral effects of hallucinogens[5]. It has also been suggested that the 5-HT_{2A} and the mGlu2 receptors form a specific heteromeric complex through which serotonin and glutamate ligands modulate the pattern of G protein coupling in living cells [1,2].

Structurally, transmembrane (TM) domains 4 and 5 of mGlu2 play a fundamental role in heteromeric formation with the 5-HT_{2A} receptor[5]. Additionally, further investigation demonstrated that three residues located at the intracellular end of TM4 of mGlu2 are necessary to form the 5-HT_{2A}-mGlu2 receptor heterocomplex in living cells[6].

Based on these findings observed in heterologous expression systems, here we describe the use of HSV-mediated expression of wild-type mGlu2 and mGlu2/mGlu3 chimeric constructs in the frontal cortex of mGlu2-KO mice to test whether heteromeric formation between 5-HT_{2A} and mGlu2 is necessary for the head-twitch behavior induced by hallucinogenic 5-HT_{2A} receptor agonists.

Protocol

NOTE: All procedures for animal breeding and cares were conducted according to the Institutional Animal Care and Use Committee (IACUC) regulation of Icahn School of Medicine at Mount Sinai. Be sure to use sterile gloves throughout the procedure.

1. Drug and Virus Preparation

1. **Drug Preparation**
    1. Prepare 15.0 ml ketamine/xylazine anesthetic by dissolving 1.35 ml of 100 mg/ml Ketamine and 0.75 ml of 20 mg/ml xylazine in 12.9 ml of 0.9% Saline solution. Thoroughly mix solution.

2. **Virus Preparation**
    1. Clone the mGlu2 and mGlu2ΔTM4N constructs into a bicistronic herpes simplex virus (HSV) vector following standard protocols previously described[6]. Package the viral particles as previously described[6,13,14]. Substitution of residues Ala-677$^{4.40}$, Ala-681$^{4.44}$ and Ala685$^{4.48}$ in mGlu2 for Ser686$^{4.40}$, Phe690$^{4.44}$ and Gly-694$^{4.48}$ in mGlu3 (HA-mGlu2ΔTM4N) have been described previously[6].
    NOTE: It was previously demonstrated that the chimeric construct HA-mGlu2ΔTM4N is expressed at the plasma membrane with intact G protein-dependent signaling[6].
    2. Store viral vectors in -80 °C when not in use. Thaw viral vector on ice, and then aliquot into 10 µl aliquots. For the surgical procedure, keep on ice.

2. Surgery

1. **Surgery Preparation**
    1. Weigh mouse and inject mouse with appropriate dose of ketamine/xylazine cocktail (for details, see 1.1.1).
    2. Check mouse to see if properly anesthetized, squeeze foot and tail for pain response, if unable to elicit a response, the mouse is properly anesthetized.
    3. Shave mouse head from the base of the skull to the tip of the nose using clippers. Apply ophthalmic gel to the mouse afterward to prevent blindness of the mouse.
    4. Load each syringe onto the stereotaxic frame. Then tilt perpendicular portion of each arm of the stereotaxic frame so that they are 10 degrees away from the normal. Ensure that the arms are tilted, such that the needles are facing each other.
    5. Clean each syringe by filling the needle with 70% ethanol. Fill the needle at least three times to ensure that the syringe is clean.
    6. Once the needle has been cleaned, flush the needle by filling the needle with double distilled H_2O. Once flushed, fill each needle with 1.3 µl of double distilled H_2O. Twist the plunger of the syringe to release 0.3 µl of double distilled H_2O. If water beads at the tip of the needle, carefully wipe away water. If nothing comes out of the syringe, push the plunger completely down and then repeat cleaning of syringe.
    7. After filling with water, then pull up the syringe filling the syringe with 0.5 µl of air.
    8. Once the air and water are in the syringe, carefully fill the syringe with 1.3 µl of virus solution. At this point ensure that the total volume in the syringe is 2.8 µl. Again twist the tip of the syringe to release 0.3 µl of virus. If liquid beads at the tip of the needle, carefully wipe away liquid. If nothing comes out of the syringe, push the plunger completely down and then repeat cleaning of syringe.

2. **Surgery**
    1. Attach the mouse to the stereotaxic frame, making sure to adjust the stereotaxic frame so that the skull is level and flat. Apply povodine-iodine to the exposed scalp. Using a scalpel, make a sagittal incision along the midline of the skull within the exposed shaved area. Then attach the buret clamps to the skin at the incision site to make sure that the skull remains exposed.
    2. Use H_2O_2 to dissolve away the periosteum to expose the sutures of the skull. Now that the bregma and sutures are visible, be sure to adjust the stereotaxic frame to make sure that the skull is level.
    3. Align the needle tips of the syringes with the bregma and record the coordinates of the bregma. Calculate the coordinates of the where the needles are going to be inserted.
        1. For the Rostral-Cauldal (R-C) plane, add 1.6 mm to the recorded R-C bregma coordinates (+1.6 from Bregma). For the Dorsal-Ventral (D-V) plane, subtract 2.4 mm from the recorded D-V bregma coordinates (-2.4 from Bregma).
        2. Finally, for the Medial Lateral (M-L) plane, add 2.6 mm to the recorded M-L bregma coordinates (+2.6 from Bregma). For all coordinates be sure to record both left and right coordinates, as this is a bilateral injection.
    4. Bring the needles to the desired coordinates. Mark the places of where the needles are going to be inserted and with a drill, drill the marked areas.
    5. With a cotton tip applicator wipe away any excess blood or bone fragment.
    6. Bring the needles to the skull where the tips of the needles are touching the surface of the brain. Then lower the needles to the desired coordinates slowly lowering them.
    7. Once the needles are at the desired coordinates, slowly inject the contents of the syringe by twisting the plunger of the needle 0.1 µl per minute over the course of 5 min (in total 0.5 µl).
    8. Once the injection has been made leave the syringe in cortex for another 5 min.

3. **Closing Up/Care**
    1. Remove the needles from the mouse cortex steadily and slowly. Then remove the mouse from the stereotaxic frame.
    2. Apply cyanoacrylate (dermal adhesive) to the base flaps of skin from the incision and then with forceps grab the flaps of skin and place them together.

3. Allow the cyanoacrylate to dry. Place the mouse in cage over a heating pad (heating pad is optional if the surgical suite is kept under RT of 37 °C - otherwise not necessary). Be sure to place the mouse on a paper towel to make sure that bedding does not adhere to surgical site.
4. Depending on the length of the surgical procedure, ensure that the mice is out of anesthesia within 30 - 60 min after procedure. Following surgery, the animal is placed in its own cage and monitored until it becomes conscious before returning to its room to recover. No analgesics are used post-operatively, because they may alter the results of our experiments by modifying some of the brain biochemical pathways. However, animals are monitored until they recover from anesthesia on the day of surgery, and daily post-operation for signs of infection and evaluation of pain/distress.

3. Head Twitch Response Experiment

1. **Set-up**
   1. Carry out all behavioral testing between 10:00 AM and 2:00 PM, 2 - 3 days after stereotactic injection of viral particles.
   2. Dissolve (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI) into a 0.9% saline solution to 2.0 mg/kg. Also prepare a 0.9% saline solution.
   3. Prepare a home cage (28 x 18 x 15 cm) without any bedding and using a tri-pod, adjust a camera so that the view of the video camera is directly above the home cage.
   4. Habituate the mice to the room for at least 4 hr prior to the beginning of the experiment.
   5. Set up a camcorder to record the head twitch.

2. **Experiment**
   1. Position the camera so that it is directly over a home cage. Calibrate the camera so that the entire home cage is in the field of view.
   2. Weigh mouse and inject mouse intraperitoneally with appropriate dose of either 0.9% saline or DOI (0.01 ml/g). NOTE: If a mouse weighs 25 grams, administer dose to a total volume of 0.25 ml.
   3. Place each mouse back in their home cage for 10 min. After 10 min, place mouse into the center of the empty home cage and validate that there are no blind spots in the field of view of the camera. Press record on the camcorder. Leave the room.
      NOTE: Mouse movements and various behavioral responses therein (head twitch, ear scratch, *etc*. Please refer to supplemental data table 1 of Gonzalez-Maeso *et al*. 2007 for full list of behavioral responses induced by DOI.)[3], will be recorded for 30 min. Therefore, it is important there are no blind spots in the field of view recorded.
   4. After 30 min stop recording on the camcorder and place mouse back in original home cage. Repeat this process for each mouse.

3. **Review**
   1. Have each referee review the tapes blind to the experimental conditions of mouse (*i.e*., drugs used during head twitch experiment or virus used during intracranial injection)). Manually record every head twitch throughout the video.
      NOTE: Head-twitch is defined as a rapid shaking head movement conducted by a mouse (supplemental video).
   2. For each mouse, average the final HTR response from the three totals of the blind referees. Then group these values by experimental condition and carry out statistical analysis (*i.e*., t-test or ANOVA).

Representative Results

Previous findings demonstrate that the head-twitch murine behavioral response is reliably and robustly elicited by hallucinogens, and it is absent in 5-HT_{2A}-KO mice[3]. Furthermore, it has been shown that the head-twitch response elicited by the hallucinogenic 5-HT_{2A} agonists DOI and LSD was significantly decreased in mGlu2-KO mice[5]. However, although previous findings convincingly demonstrate that 5-HT_{2A} and mGlu2 are assembled as a heteromeric complex *in vitro* in transfected cells[1,2,15], whether this structural arrangement behaves as such in living mice remained unsolved. To fully understand the role of the 5-HT_{2A}-mGlu2 receptor heterocomplex in the psychoactive-like effects induced by hallucinogenic 5-HT_{2A} receptor agonists, expression of either mGlu2 or mGlu2ΔTM4N in frontal cortex of mGlu2-KO mice to examine whether this manipulation regulates behavior.

Mice received intra-frontal cortical injections of bicistronic HSV expressing green fluorescent protein (GFP) and either mGlu2 or mGlu2ΔTM4N, or GFP alone. First, it was confirmed that the virus over-expresses mGlu2 or mGlu2ΔTM4N in mouse frontal cortex (**Figures 1A and 1B**). As previously demonstrated[5], head-twitch behavior induced by DOI was absent in mGlu2-KO mice injected with the empty vector HSV-GFP. Notably, the head-twitch response induced by the hallucinogenic 5-HT_{2A} agonist DOI was rescued in mGlu2-KO mice over-expressing mGlu2, but not mGlu2ΔTM4N, in frontal cortex as compared to that seen in animals expressing GFP (**Figure 1C**). Together, these findings suggest that the 5-HT_{2A}-mGlu2 receptor complex in frontal cortex is critical for regulating psychosis-like states.

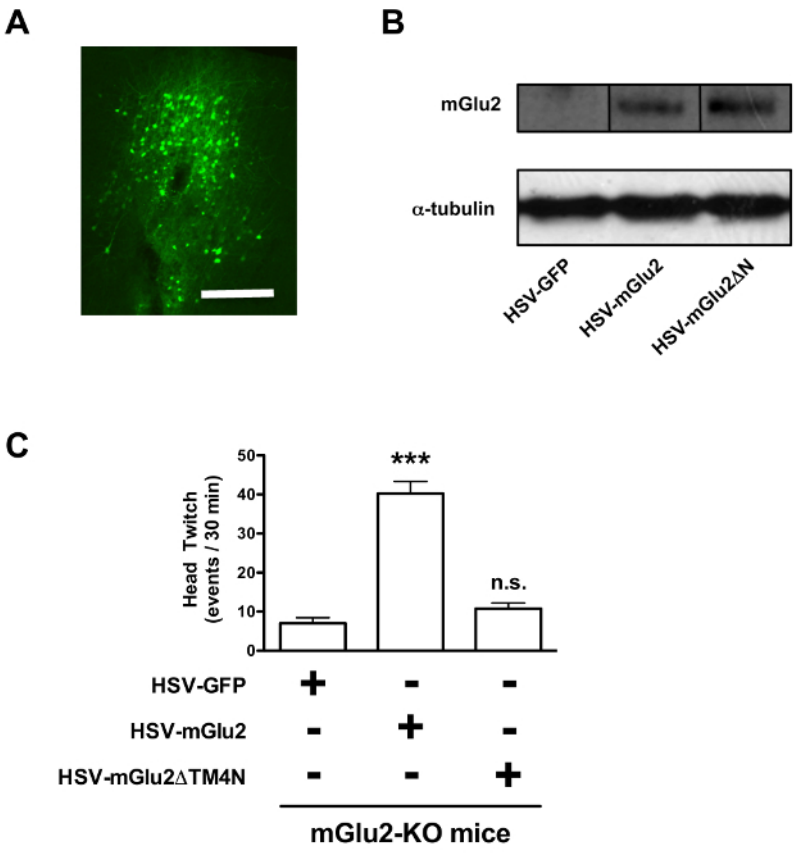

Figure 1. Expression of mGlu2 as a Receptor Heterocomplex. Expression of mGlu2 as a receptor with 5-HT_{2A} is necessary for psychosis-like behavior induced by hallucinogenic drugs. (**A**) Representative image of HSV-mediated transgene expression in frontal cortex. HSV-mGlu2, which also expresses GFP, was injected into frontal cortex, and GFP expression was revealed by immunocytochemistry, scale bar, 200-um. (**B**) HSV-mediated transgene expression in mouse frontal cortex of mGlu2-KO mice, and anti-mGlu2 reactivity was measured by Western Blotting. Specificity of the primary antibody against the mGlu2 receptor has previously been confirmed in knockout mice[6]. Metabotropic glutamate receptors are GPCRs that form covalently linked homodimers. We measured immunoreactivity of mGlu2 as a monomer (100 kDa)[6]. (**C**) Viral-mediated expression of mGlu2, but not mGlu2ΔTM4N, in frontal cortex of mGlu2- KO mice significantly rescues the head-twitch response induced by the hallucinogenic 5-HT2A agonist DOI (n = 4 per group). ***p <0.001; n.s, not significant; Bonferroni's post hoc test of one-way ANOVA. Error bars represent S.E.M. Figure was modified from Moreno *et al* (2012)[6]. Please click here to view a larger version of this figure.

Supplemental Video 1. Head Twitch Response. (Right click to download). CD-1 WT mice were injected with 2.0 mg/kg DOI and placed in a cage (wall blacked out between the two cages) to elicit head-twitch response (behavior elicited after *).

Discussion

Together with previous findings in mGlu2-KO mice[5], the results with mGlu2 and mGlu2/mGlu3 chimeric constructs that do not form the 5-HT_{2A}-mGlu2 receptor complex in cultured cells suggest that the 5-HT_{2A} -mGlu2 heteromeric receptor complex in mouse frontal cortex is needed to induce head-twitch behavior by LSD-like hallucinogenic 5-HT_{2A} receptor agonists. A limitation of this method is that it does not measure close molecular proximity at a subcellular level in native tissue. In addition, there are various critical points to be noted. Because the mice are injected with a HSV viral vector, the time frame that the experiments to be performed are 2 - 4 days after injection. The location and expression of the viral vectors should be verified with immunofluorescence staining of sectioned brain no more than 4 days after the initial injection. Mice in which the coordinates do not match or do not express the viral vector should be excluded from the experimental data as they do not express the mGlu2 or mGlu2ΔTM4N. Care after stereotactic surgery is also crucial, as improper closing of the head wound can lead to infection which can cause issues in both the *in vivo* experiments and the immunofluorescence staining. Lastly, after stereotactic injection any behavioral paradigm (open field, alternating t-test, *etc.*) can be used as long as it is within the 2 - 4 after injection of viral particles. Again, coordinates and expression should be confirmed by immunofluorescence.

The concept that GPCRs function as homo- and/or heteromers in living cells is now well established [16-18]. However, despite some progress, more studies are needed to define the precise role(s) of GPCR heteromeric complexes in whole animal models [19]. Approaches such as BRET and/or FRET imaging to investigate protein-protein physical proximity within deep tissues of small animal models may provide means to enhance the understanding of the functional role of GPCR heteromers in living subjects[20]. Using HSV-mediated expression of GPCRs either native or with modified transmembrane proteins, provide an *in vivo* model to evaluate the function and interaction of GPCRs.

Further studies in rodent models are also needed to examine the stability and life- time (formation and dissociation) of GPCR heteromers[21-23], structural rearrangements between their components[24], and potential G protein coupling after receptor internalization[25,26]. Given the fundamental role of GPCRs in cell signaling and function, it seems likely that this area might lead to interesting basic and translational studies.

Although further investigation is required to quantitatively characterize the ultrastructural co-localization of both receptors in human and mouse CNS, together with previous studies that convincingly demonstrate the electrophysiological, cellular, and behavioral responses induced by hallucinogens in mouse models are intrinsic to 5-HT_{2A} receptor-expressing cortical pyramidal neurons[3,27,28], the findings obtained using the HSV-mediated expression approach described here suggest that heteromeric formation between 5-HT_{2A} and mGlu2 receptors in mouse frontal cortex is needed for the head-twitch psychosis-like behavior induced by the hallucinogenic 5-HT_{2A} receptor agonist DOI.

HSV-mediated expression of mGlu2, but not mGlu2ΔTM4N, in frontal cortical neurons of mGlu2-KO mice rescues the head-twitch behavior induced by the hallucinogenic 5-HT_{2A} receptor agonist DOI. This translational tool might be advantageous for preclinical studies to evaluate behavioral phenotypes of GPCR heteromers.

Disclosures

The authors have nothing to disclose.

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