Section - Protocol

2. Resistance to Dieldrin (RDL) and Metabotropic Glutamate Receptor 1 (mGlutR1) Protein Expression

2.1. To test the expression of RDL (R-D-L) and mGlutR1 (M-glute-R-one) by immunocytochemical analysis after CRISPR-Cas9 (crisper-cass-nine) injection **[1]**, first use an online CRISPR-Cas9 tool to design the guides using the guide DNA sequence of Apis mellifera RDL and mGlutR1 **[2]**.

~~2.1.1.~~ WIDE: Talent at computer, designing guide, with monitor visible in frame **TEXT: RDL: resistant to dieldrin; mGlutR1: metabotropic glutamate receptor 1**

2.1.2. SCREEN: To be provided by Authors: Shot of AmRdl and mGlutR1 sequences

Commented [IS2]: Will be prepared for the day of filming

2.2. To prepare guide RNA complex formations for each guide RNA, label one test tube for each guide with the name of the guide RNA **[1]** and add 92 microliters of nucleotide free buffer **[2]**, 4 microliters of 100 micromolar fluorophore-labeled CRISPR-Cas9 [redacted] RNA **[3]**, and 4 microliters of the appropriate guide RNA solution to each tube **[4]**.

~~2.2.1.~~ Talent labeling tube(s)

~~2.2.2.~~ Buffer being added to tube, with buffer container and all guide tubes visible in frame

~~2.2.3.~~ Tracr RNA being added to tube, with tracr RNA container and all guide tubes visible in frame

~~2.2.4.~~ guide RNA being added to tube, with guide RNA container and all guide tubes visible in frame

2.3. When all of the materials have been added, mix the solutions gently **[1]** and spin the tube contents in a benchtop centrifuge for 5 seconds to sediment the solution **[2]**.

~~2.3.1.~~ Tube being mixed

~~2.3.2.~~ Talent placing tube(s) into bench-top centrifuge

2.4. Then heat the solutions to 95 degrees Celsius for 5 minutes to create a guide RNA complex **[1]** followed by a 10-minute cooldown at room temperature **[2]**.

~~2.4.1.~~ Talent placing tube(s) at 95 °C

~~2.4.2.~~ Talent placing tube(s) at RT

2.5. To prepare the ribonucleoprotein complex formations, add 6 microliters of each guide RNA solution **[1]** and 6 microliters of 0.5 micrograms/microliter S.p. Cas9 Nuclease V3 to the appropriately labeled tubes **[2]**.

~~2.5.1.~~ Shot of tube labels, then gRNA being added to tube, with gRNA solution container visible in frame

~~2.5.2.~~ Nuclease being added to tube(s), with nuclease container visible in frame

2.6. After gentle mixing, incubate the solutions at 37 degrees Celsius for 10 minutes **[1]**.

~~2.6.1~~. Talent placing tube at 37 °C

2.7. To make RNP-ribonucleoprotein mixtures for injection, at the end of the incubation, add 4 microliters of each ribonucleoprotein to the appropriate RDL **[1-TXT]**.

~~2.7.1~~. Mixture(s) being added to tube, with all appropriate tube labels visible in frame
TEXT: *e.g.*, RNP/RDL mix = 4 microliters RRDL1 + 4 microliters RRDL2 + 4 microliters RRDL3

2.8. To make a control no-guide RNA solution, mix 4 microliters of tracr RNA **[1]** with 92 microliters of buffer **[2]** and 4 microliters of water **[3]**.

~~2.8.~~1. TracrRNA being added to tube, with tracr RNA and tube label visible in frame
~~2.8.~~2. Buffer being added to tube, with buffer container visible in frame
~~2.8.3~~ Water being added to tube

2.9. Then mix 6 microliters of the no-guide RNA solution **[1]** with 6 microliters of 0.5 micrograms/microliter Cas9 Nuclease V3 **[2]**.

~~2.9.1~~. No guide RNA being added to tube, with no guide RNA container visible in frame
~~2.9.2~~. Nuclease being added to tube, with nuclease container visible in frame

3. Injection Procedure

3.1. For ribonucleoprotein mixture injection, after capturing individual bees in vials with a small hole in the caps **[1]**, immobilize the bees for no more than 3 minutes on ice **[2]** and secure each bee into previously prepared metal holders with duct tape **[3]** with the back thorax, wings, and head exposed **[4]**.

~~3.1.1~~. *WIDE: Talent entering lab with bees/placing vials onto bench of similar*
~~3.1.2~~. *Shot of vial(s) on ice*
~~3.1.3~~. *Bee being secured in holder*
~~3.1.4~~. *ECU: Shot of bee in holder w/ back thorax, wings, and head exposed*

3.2. Use a 5-milliliter syringe to feed the bees with a 1-molar sucrose solution until they are no longer hungry **[1]** and place the secured bees in a box with a wet paper towel for humidity **[2]**.

~~3.2.1~~. Bee being fed
~~3.2.2~~. Talent placing holder into box

3.3. Next, place wax on the inside of the lids of two 35-millimeter Petri dishes **[1]** and place the bottoms of the dishes onto the wax **[2]**.

~~3.3.1~~. Wax being placed into lid
~~3.3.2~~. Dish bottom being placed onto wax

Step 3

6 bees
6 bees

SHOT TOGETHER IN ONE SHOT
331 - 341

3.4. Inject 1-molar sucrose between the cover and bottom of each dish **[1]** and place one feeding Petri dish into a white box with one glass side **[2]** and place the other into a black box with one glass side **[3]**

~~3.4.1.~~ Dish being injected
~~3.4.2.~~ Talent placing dish into white box, with glass side visible in frame
~~3.4.3.~~ Dish being placed into black box, with glass side visible in frame

SHOT TOGETHER

3.5. Then place a small comb into each box **[1]** and load a microinjection system with the RDL-CRISPR-Cas9 mixture **[2-TXT]**.

~~3.5.1.~~ Talent placing comb into box
~~3.5.2.~~ Talent loading capillary onto microinjection system, with RPL container visible in frame **TEXT: See text for full microinjection system loading details**

DID TWO TAKES

3.6. For RDL-CRISPR-Cas9 injection, inject the median ocelli of eight bees with 345 nanoliters of ribonucleoprotein-RDL mix **[1]** followed by feeding with 1-molar sucrose **[2]** before releasing the bees into the ~~white~~ BLACK experimental box **[3]**.

~~3.6.1.~~ ECU: Bee being injected
~~3.6.2.~~ Bee being fed
~~3.6.3.~~ Talent releasing bee(s) into ~~white~~ BLACK box

3.7. Feed a second set of eight bees as controls without any injections **[1]** and release them into the ~~black~~ WHITE control box **[2]**.

~~3.7.1.~~ Bee being fed
~~3.7.2.~~ Talent releasing bee(s) into ~~black~~ WHITE box

3.8. Then place both boxes into a polystyrene container with wet paper **[1]**, observing the bees twice a day to ensure they have enough food and a good humidity **[2]**.

~~3.8.1.~~ Talent placing box(es) into container
~~3.8.2.~~ Talent checking food/humidity

4. Brain Tissue Harvest and Sectioning

4.1. Forty-eight hours after the injection, immobilize the bees on ice for 30 seconds **[1]** and use scissors to decapitate each insect **[2-TXT]**.

~~4.1.1.~~ WIDE: Talent placing bees onto ice
~~4.1.2.~~ Talent decapitating bee **TEXT: Caution: Abdomen can still sting after sacrifice**

TWO TAKES COMBINED

4.2. Place the heads in 4% paraformaldehyde in PBS under a dissecting microscope in a fume hood **[1]** and use Barraquer iris scissors to carefully but rapidly remove the antennae, compound eyes, and the top exoskeleton **[2]**.

~~4.2.1.~~ Talent placing heads into PFA under microscope in hood
~~4.2.2.~~ LAB MEDIA: To be provided by Authors: Tissues being removed

FILMED EIGHT AFTER 412

4.3. Allow the heads to sit in the fixative solution for 10 minutes **[1]** before removing the rest of the exoskeleton from the head **[2]** and all of the remaining tracheae **[3]**.

~~4.3.1.~~ Talent setting timer, with dish visible in frame
~~4.3.2.~~ LAB MEDIA: To be provided by Authors: Tissues Exoskeleton being removed
~~4.3.3.~~ LAB MEDIA: To be provided by Authors: Tissues Trachea being removed

4.4. Then place each brain in a 1.5-milliliter microcentrifuge tube containing at least 1 milliliter of 4% paraformaldehyde overnight at 4-8 degrees Celsius **[1]**.

~~4.4.1.~~ Talent placing brain(s) into tube(s)

4.5. The next morning, add 3.8 grams of agarose to 50 milliliters of distilled water in an Erlenmeyer flask **[1]** and microwave the solution until the agarose liquifies **[2]**.

~~4.5.1.~~ Talent adding agarose to water, with agarose container visible in frame
~~4.5.2.~~ Talent placing flask into microwave

4.6. Transfer 3-4 fixed honeybee brains into a new 35-milliliter Petri dish **[1]** and use tissue paper to remove the excess fixative **[2]**.

~~4.6.1.~~ Brains being placed into dish
~~4.6.2.~~ Fixative being removed

4.7. Carefully pour the liquid agarose over the brains **[1]** and orient the samples within the agarose so that the antenna lobes are facing up **[2]**.

~~4.7.1.~~ Agarose being poured onto brains
~~4.7.2.~~ Samples being oriented

4.8. After the agarose has solidified, use a scalpel to cut out individual blocks of agarose containing a single brain **[1]** and fill each well of a 24-well plate loaded with one basket with a hydrophobic mesh bottom and 600 microliters of PBS per well **[2]**.

~~4.8.1.~~ Shot of solidified agarose, then block being cut
~~4.8.2.~~ Talent adding basket to well, with unloaded baskets and PBS container visible in frame

4.9. Then use a vibratome to acquire 70-micrometer cross sections of the brain tissue from each agarose block **[1]**, placing the sections from a single brain onto the mesh of the same basket as they are acquired **[2]**.

~~4.9.1.~~ Section being acquired
~~4.9.2.~~ Section being placed onto basket

4.10. After labeling the sections with fluorophore-conjugated antibodies according to standard protocols **[1-TXT]**, embed one section per slide with a drop of mounting medium **[2]** and visualize the samples by fluorescence microscopy **[3]**.

~~4.10.1.~~ Talent removing plate from orbital shaker, with primary and/or secondary antibody containers visible in frame **TEXT: See text for Ab labeling details**

~~4.10.2.~~ Mounting medium being added to section, with medium container visible in frame
~~4.10.~~3. Talent at microscope, viewing sample

Section – Results

5. **Results: Representative Anti-RDL and Anti-mGlutR1 Receptor Antibody Testing in Honeybee Brain Sections**

5.1. Anti-RDL labels neuropils in the frontal section of wild-type **[1]** but not CRISPR-Cas9 RDL knockout bee brains **[2]**. A similar specificity was observed for anti-mGlutR1 antibodies **[3]**.

5.1.1. LAB MEDIA: Figures 1 D1 and 1 D2: JoVE Video Editor please emphasize green signal in Figure 1 D1
5.1.2. LAB MEDIA: Figures 1 D1 and 1 D2: JoVE Video Editor please emphasize lack of signal in Figure 1 D2
5.1.3. LAB MEDIA: Figures 2B, 2C, 2 D1, and 2 D2: JoVE Video Editor please emphasize green signal in Figure 2B and 2 D1

5.2. In these representative quantitative PCR drop-off tests **[1]**, in bee brains injected with RDL-CRISPR-Cas 9, the relative reduction of the fluorescence corresponded to the number of the modified guide DNA in the samples **[2]**.

5.2.1. LAB MEDIA: Figure 3A
5.2.2. LAB MEDIA: Figure 3A: JoVE Video Editor please emphasize salmon data bar

5.3. After mGlutR1-CRISPR-Cas 9 injection, the relative modification of guide DNA was approximately 59% in the brains of injected bees compared to the guide DNA observed in the brains of noninjected bees **[1]**.

5.3.1. LAB MEDIA: Figure 3A: JoVE Video Editor please emphasize pink data bar

5.4. In this quantitative RT-PCR in a separate group of bees **[1]**, the relative reduction of the messenger RNA RDL was approximately 59% compared to the level of RNA in non-injected bees **[2]** and the relative reduction of the messenger mGlutR1 RNA in injected bees was approximately 53% **[3]**.

5.4.1. LAB MEDIA: Figure 3B
5.4.2. LAB MEDIA: Figure 3B: JoVE Video Editor please emphasize red data bar
5.4.3. LAB MEDIA: Figure 3B: JoVE Video Editor please emphasize pink data bar

5.5. Notably, the injection of RDL-CRISPR-Cas9 through the ocelli might not always reach a large number of brain cells **[1]**.

5.5.1. LAB MEDIA: Figures 4A-4D

5.6. For example, in these preparations, only one bee out of 8 had RDL CRISP-Cas9 in a large number of brain cells compared to other bee brains **[1]**, with the RDL-CRISPR-Cas9 concentrated within the cells ofin the protocerebrum **[2]** but not the antennal lobe **[3]**.

5.6.1. LAB MEDIA: Figures 4A-4D: JoVE Video Editor please emphasize bee-3 image

jove FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 59993
Scriptwriter Name: Bridget Colvin
Project Page Link: http://www.jove.com/files_upload.php?src=18297913

Title: Anti-RDL and Anti-mGlutR1 Receptors Antibody Testing in Honeybee Brain Sections Using CRISPR-Cas9

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Project Page Link: http://www.jove.com/files_upload.php?src=18297913

Interview Statement Summary

Videographer: Interviewee Headshots are required. Take a headshot for each interviewee.

REQUIRED Interview Statements:

~~1.1.~~ **Irina Sinakevitch**: ~~Here~~ we have established, for the first time, a modification of the genes coding for ionotropic GABAa and metabotropic GlutR1 receptors in subsets of neurons in the adult honeybee brain **[1]**.

~~1.1.1.~~ INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

1.2. **Brian Smith**: CRISPR-Cas9 gene editing can be used to modify one or multiple genes in adult bee brains to explore their roles in learning and memory under controlled laboratory conditions **[1]**.

~~1.2.1.~~ INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL Interview Statements:

1.3. ***Irina Sinakevitch***: This method can be used to study the function of specific proteins in adult bees, as well as to test the specificity of antibodies against their corresponding proteins **[1]**.

anti

~~1.3.1.~~ INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Introduction of Demonstrator statement:

1.4. **Brian Smith**: Demonstrating the procedure with Irina Sinakevitch will be Zev Kurtzman and Hyun Choi, former undergraduate students from my laboratory **[1][2]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~1.4.2~~. The named technician, post doc, student looks up from workbench or desk or microscope and acknowledges the camera

CONCLUSION Interview Statements:

6.1. **Irina Sinakevitch**: Be sure to avoid frozen and refrozen RNA, and to take care not to overcool the bees, harnessing them immediately once they stop moving in the vial **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (Step: 3.1.3.)

6.2. **Irina Sinakevitch**: This technique can be used to study the effects of the reduction of inhibitory ionotropic and metabotropic receptors in the mushroom body and central complex on honeybee behavior **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Author Questionnaire:

1. Microscopy: Does your protocol require JoVE to film through your microscope? N
2. Does your protocol demonstrate software usage? N
3. Which steps from the protocol section below are the most important for viewers to see?

n/a

4. What is the single most difficult aspect of this procedure and what do you do to ensure success?

n/a

5. Will the filming need to take place in multiple locations (greater than walking distance)? N

Section - Conclusion

6. **Conclusion Interview Statements: (Said by you on camera) - All interview statements may be edited for length and clarity.**

6.1. Irina Sinakevitch: Be sure to avoid frozen and refrozen RNA and to take care not to overcool the bees, harnessing them immediately once they stop moving in the vial **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (Step: 3.1.3.)

6.2. Irina Sinakevitch: This technique can be used to study the effects of the reduction of inhibitory ionotropic and metabotropic receptors in the mushroom body and central complex on honeybee behavior **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Section - Introduction

Commented [BC1]: Authors: Each author can give a maximum of two Introduction statements, not including the Demonstrator Introduction statement.

Videographer: Interviewee Headshots are required. Take a headshot for each interviewee.

1. **REQUIRED Interview Statements (Said by you on camera): All interview statements may be edited for length and clarity.**

    1.1. **Irina Sinakevitch**: Here we have established, for the first time, a modification of the genes coding for ionotropic GABAa and metabotropic GlutR1 receptors in subsets of neurons in the adult honeybee brain **[1]**.

    1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

    1.2. **Brian Smith**: CRISPR-Cas9 gene editing can be used to modify one or multiple genes in adult bee brains to explore their roles in learning and memory under controlled laboratory conditions **[1]**.

    1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL Interview Statements: (Said by you on camera) - All interview statements may be edited for length and clarity.

    1.3. **Irina Sinakevitch**: This method can be used to study the function of specific proteins in adult bees, as well as to test the specificity of antibodies against their corresponding proteins **[1]**.

    1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Introduction of Demonstrator (Said by you on camera):

    1.4. **Brian Smith**: Demonstrating the procedure with Irina Sinakevitch will be Zev Kurtzman and Hyun Choi, former undergraduate students from Irina Sinakevitch's laboratory **[1][2]**.

    1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

    1.4.2. The named technician, post doc, student looks up from workbench or desk or microscope and acknowledges the camera

Ethics title card: (for human subjects or animal work, does not count toward word length total)

    1.5. Protocols involving honeybees follow the guidelines of honeybee care at Arizona State University.