Title

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Genetically Encoding Unnatural Amino Acids in Neurons in vitro and in the Embryonic Mouse Brain for Optical Control of Neuronal Proteins

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Abstract

Deciphering neuronal networks governing specific brain functions is a longstanding mission in neuroscience, yet global manipulation of protein functions pharmacologically or genetically lacks sufficient specificity to reveal a neuronal protein's function in a particular neuron or a circuitry. Photostimulation presents a great venue for researchers to control neuronal proteins with high temporal and spatial resolution. Recently, an approach to optically control the function of a neuronal protein directly in neurons has been demonstrated using genetically encoded light-sensitive Unnatural amino acids (Uaas). Here we describe procedures for genetically incorporating Uaas into target neuronal proteins in neurons *in vitro* and in embryonic mouse brain. As an example, a photocaged Uaa was incorporated into an inwardly rectifying potassium channel Kir2.1 to render Kir2.1 photo-activatable. This method has the potential to be generally applied to many neuronal proteins to achieve optical regulation of different processes in brains. Uaas with other properties can be similarly incorporated into neuronal proteins in neurons for various applications.

Keywords

Unnatural amino acid; genetic code; amber suppression; ion channel; optogenetics; light-activation; optical control; photocage; neuronal activity; brain

1. Introduction

Optical control of neuronal activity has helped answer many challenging questions in neuroscience that are not easily addressable with conventional methods. Photocaged agonists have been utilized to stimulate a local brain network [1,2]. Ion channel gating was controlled with light by attaching a photoisomerizable azobenze group to the target protein [3,4]. Light-sensitive opsin channels and pumps are widely adopted to modulate neuronal excitability in

different neuronal circuitries [5–7]. These different approaches are ingenious ways to exploit the high-specificity of photostimulation to manipulate neuronal function. However, new methods to photo-regulate various endogenous proteins in their native environment remain desired, so that photo-control specificity can be achieved at the molecular level for general proteins.

Genetically encoding Unnatural amino acids (Uaas) is a uniquely attractive tool to engineer light-responsiveness into general proteins. An orthogonal tRNA/synthetase pair incorporates a Uaa in response to the amber stop codon during endogenous translation, generating a novel protein with the intervening Uaa at the selected site [8–11]. This method imposes no restrictions on target protein type or cellular location, nor on Uaa modification site. Such great flexibility makes it available to target a wide variety of endogenous proteins. Manipulation at the translational level intrinsically gives it genetic specificity. Using this approach, we initially incorporated Uaas with different physical properties into ion channels in primary neuron culture and in neural stem cells followed by differentiation into neurons [12,13]. Recently, we succeeded in incorporating a photoresponsive Uaa into an ion channel *in vivo* in the mouse brain, which paves the way for photo-control of specific neuronal proteins in mammalian brain *in vivo* [14].

In the study by Kang *et al.* [14], we incorporated a photo-responsive Uaa, 4,5-dimethoxy-2-nitrobenzyl-cysteine (Cmn), into an inwardly rectifying potassium channel Kir2.1 (Fig. 1). The side chains of Cmn incorporated in the Kir2.1 pore sterically hinder the passage of potassium ions, shutting off the channel function of Kir2.1. A brief illumination of UV-light instantly and specifically cleaves off the Cmn side chains, turning on the normal Kir2.1 function. These *p*hoto-inducible *i*nwardly *r*ectifying potassium (PIRK) channels were expressed in rat hippocampal primary neurons as well as in the embryonic mouse neocortex *in vivo*, demonstrating the optical control of Kir2.1 function in different research settings.

The current protocol entails an easy-to-follow procedure to incorporate Uaas in neurons *in vitro* and *in vivo* to achieve optical control over neuronal proteins' activity. With the availability of numerous photoreactive Uaas, a wide application of the current technology would be sought for. We expect this protocol to be a clear guide for future studies indulging in photo control over neuronal proteins and optogenetic biological studies in general.

2. Materials

2.1. Culture rat hippocampal primary neurons

1. Sprague Dawley rat pups (postnatal day 1 – 4). One pup produces 10–12 coverslips (*see* Note 1).
2. Glass coverslips (circles, 12 mm in diameter)
3. 24-well tissue culture plates

[1]Rat embryos are also routinely used to prepare neuron culture [16].

4. 100 mM Borate buffer: weigh 1.24 g boric acid and 1.90 g sodium tetraborate in ~350 mL of deionized distilled H_2O (ddH_2O), and adjust pH to 8.5 with dilute HCl. Bring the volume up to 400 mL.
5. 0.5 mg/mL poly-D-lysine: dissolve 20 mg poly-D-lysine in 40 mL of 100 mM borate buffer. With a serological pipette, mix the solution thoroughly by pipetting up and down (*see* Note 2).
6. Isoflurane
7. Dissection surgery tools: scissors, forceps, blades, and spatulas
8. Saline: 10 mM HEPES and 20 mM D-glucose added in Hank's balanced salt solution
9. 2.5% Trypsin
10. Glass pipettes
11. Growth media: in 46 mL Minimum Essential Medium (MEM), add 425 μL of 2.5 M sterile glucose solution, 2.5 mL Fetal Bovine Serum (FBS), 50 μL serum extender, 1 mL B27, and 0.5 mL of 200 mM L-glutamine. Ideally, the Growth media should be made fresh for each experiment.
12. Hemocytometer
13. 40 μm Nylon mesh

2.2. Calcium-phosphate (Ca-P) transfection

1. A set of recombinant DNA to incorporate Uaa into Kir2.1 (Fig. 2): (1) recombinant Kir2.1 gene with an amber stop codon TAG introduced at the desired site for Uaa incorporation, Cys169 in the current study. A fluorescent protein, mCitrine in the study, can be fused at the C-terminus of Kir2.1 to visualize successful amber suppression [14]. (2) tRNA/synthetase gene to decode the introduced amber stop codon and incorporate a specific Uaa, Cmn in this study, to the TAG-specified site (*see* Note 3, 4).
2. Cmn
3. Stock solutions: make up 0.5 M BES (N,N-bis[2-hydroxy-ethyl]-2-aminoethanesulfonic acid) buffer, 150 mM Na_2HPO_4, and 2.8 M NaCl, sterile filter and store at 4 °C.

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[2] In our hands, Poly-D-Lysine showed much better results than Poly-L-Lysine in terms of the quality of neuron culture. Neurons and some cell types were reported to release proteases which break down Poly-L-Lysine, but not Poly-D-Lysine [17].

[3] To make a protein light-inducible, the foremost step is to determine the most suitable amino acid site in the target protein sequence for Uaa incorporation. Structural and functional understanding of the target protein is crucial. Make UAG amber mutation at the target site for Uaa incorporation. Moreover, one can select a few candidate sites and test them in a mammalian cell line such as Human Embryonic Kidney (HEK) cells, to gauge the ease and efficiency of amber suppression as well as light activation at each candidate site [14].

[4] There are a number of photoresponsive Uaas with orthogonal tRNA/synthetase available, including Uaas that can be photo-switched reversibly [18–21], so researchers may choose an optimal Uaa for individual purpose of experiments. The orthogonal tRNA for a specific Uaa is transcribed under a special type-3 polymerase III promoter, such as the H1 promoter or U6 promoter, together with a 3′-flanking sequence as described previously [10,12]. Uaa incorporation efficiency is optimized by varying the copy number of tRNA expression cassette [14,22].

4. 2.5 M $CaCl_2$ solution: make it fresh with ddH_2O for every experiment, and sterile filter.
5. 2X BES-buffered Saline (BBS) buffer: add 1 mL of 0.5 M BES, 0.1 mL of 150 mL Na_2HPO_4, and 1 mL of 2.8 M NaCl to 7 mL ddH_2O. Adjust pH to 7.00 with dilute NaOH, bring the volume up to 10 mL, and sterile filter (*see* Note 5). Make fresh 2X BBS buffer for every experiment.
6. Transfection media: in 50 mL MEM, add 425 μL of 2.5M sterile glucose solution. Ideally, the Transfection media should be made fresh for each experiment.
7. Washing buffer: mix 500 mL ddH_2O with 135 mM NaCl, 20 mM HEPES, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 10 mM glucose at pH 7.4 (adjusted with dilute NaOH), and sterile filter to store at 4°C.

2.3. Whole cell recording with light activation from primary neurons

1. Extracellular solution: mix 1 L ddH_2O with 150 mM NaCl, 3 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES, at pH 7.4 (adjusted with dilute NaOH), and sterile filter.
2. Intracellular solution: mix 20 mL ddH_2O with 135 mM potassium gluconate, 10 mM NaCl, 2 mM $MgCl_2$, 10 mM HEPES, 1 mM EGTA, 2.56 mM K_2ATP, and 0.3 mM Li_2GTP at pH 7.4 (adjusted with dilute KOH), and sterile filter. Aliquot 300–400 μL per tube, and freeze for long-term storage.
3. Electrophysiology rig for whole-cell patch clamping: microscope fitted with 4x and 20x objectives, differential interference contrast (DIC), and mCitrine filter (excitation: 495/10 nm, emission: 525/25 nm), manipulator, patch-clamp amplifier, digitizer, and data acquisition and analysis software
4. Light-emitting diode (LED) with emission of 385 nm, cable light guide installed (*see* Note 6).
5. Light power meter
6. Glass patch pipettes
7. 0.5 mM $BaCl_2$ Extracellular solution: Add 25 μL of 1 M $BaCl_2$ in 50 mL Extracellular solution.

2.4. *In utero* electroporation and Uaa microinjection

1. A set of recombinant DNA to incorporate Uaa into Kir2.1 (Fig. 3) (*see* Note 7).
2. Cmn-Ala (*see* Note 8).

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[5]Preparation of 2X BBS buffer is a critical step. Calibrate the optimal pH for 2X BBS buffer between pH 6.90 – 7.15, for every new DNA construct or prep. The buffer pH could be adjusted with precision to the 0.01 digits if it helps to achieve consistent results.

[6]Cmn absorbs long and medium wave UV lights (280 – 400 nm). To avoid exposure to cytotoxic shortwave UV as well as unnecessary heat from far-red light, an LED with a single-wavelength emission is most suitable for Cmn activation.

[7]For *in vivo* expression, use a strong promoter such as CAG (chicken beta-actin promoter with CMV enhancer). Also, three copies of the tRNA expression cassette increased Cmn incorporation efficiency, as tested previously [14].

3. A timed pregnant mouse at the embryonic day 14.5 (E14.5)
4. Dissection surgery tools: scissors, forceps, and ring forceps
5. Glass capillaries
6. Electroporator
7. Sodium pentobarbital or isoflurane
8. Fast Green
9. Dulbecco's Phosphate-Buffered Saline (PBS)

2.5. Whole cell recording with light activation from acute brain slices

1. Artificial cerebrospinal fluid (ACSF) solution: mix 1 L ddH_2O with 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 11 mM glucose. Bubble ACSF with 5% CO_2: 95% O_2 gas while stirring. Subsequently, adjust pH to 7.3 with dilute HCl. Continue bubbling the solution for the rest of the experimental day. ACSF solution is made fresh every day.
2. Dissection surgery tools: scissors, forceps, blades, and spatulas
3. Microwave
4. 4% Low melting point agarose solution: weigh 4 g low melting point agarose into a flask and add 100 mL ddH_2O. Dissolve the agarose by heating up the flask in a microwave. Cool down the solution below 50 °C, and use it before it solidifies. Do not re-heat up agarose blocks.
5. Vibratome equipment
6. Tissue adhesive
7. Incubation ACSF: Mix 250 mL ACSF with 3 mM *myo*-inositol, 0.4 mM ascorbic acid, and 2 mM sodium pyruvate.
8. Water bath for tissue culture
9. Glass pipettes
10. Intracellular solution: mix 20 mL ddH_2O with 130 mM potassium gluconate, 4 mM $MgCl_2$, 5 mM HEPES, 1.1 mM EGTA, 3.4 mM Na_2ATP, 10 mM sodium creatine phosphate, and 0.1 mM Na_3GTP at pH 7.3 (adjusted with dilute KOH), and sterile filter. Aliquot 300–400 μL per tube, and freeze for long-term storage.
11. Slice electrophysiology rig: Electrophysiology rig for whole-cell patch clamping equipped with perfusion pump, perfusion chamber, water immersion objectives,

[8]In order to increase the bioavailability of Cmn, use the dipeptide Cmn-Ala (Cmn-alanine) to deliver Cmn *in vivo* [23]. Oligopeptide transporter PEPT2 is highly expressed in rodent brains, which helps transport dipeptides into neurons [24]. Inside the neurons, the dipeptides are hydrolyzed by cellular peptidases to yield single amino acid, Cmn. Sometimes, it helps to inject Cmn-Ala in the ventricle on both the electroporated and the opposite hemisphere, to increase Cmn bioavailability through diffusion.

a temperature controller, GFP filter (excitation: 480/30 nm, emission: 535/40 nm) and mCherry filter (excitation: 580/20 nm emission: 675/130 nm).

12. 0.5 mM $BaCl_2$ ACSF: Add 25 μL of 1 M $BaCl_2$ in 50 mL ACSF.

3. Methods

3.1. Culture rat hippocampal primary neurons

1. One day before the experiment, place glass coverslips in 24-well plates, one coverslip in each well. Prepare around 10–12 coverslips per pup.
2. Add ~500 μL of 70 % EtOH in each well and incubate for 15–20 min to sterilize the coverslips.
3. Rinse each well with ddH_2O 3–5 times.
4. Add ~250 μL of 0.5 mg/mL poly-D-lysine in each well, seal the culture plate, and incubate for overnight at room temperature.
5. On the day of experiment, rinse each well with ddH_2O 3–5 times.
6. Add ~500 μL of ddH_2O in each well and incubate during the dissection.
7. Warm up Saline and Growth media in a water bath at 37 °C.
8. Bring 1–4 postnatal day-old rat pups to the laboratory.
9. Anesthetize the pups in a chamber containing isoflurane (2–4%), and wait until they lose consciousness and fail to respond to tactile stimuli and to pinching of the paws.
10. Decapitate pups and dissect out neonatal brains.
11. Using the standard technique [15], dissect hippocampi from the brains in warm Saline, and collect them in a conical tube filled with 4.5 mL warm Saline (*see* Note 9).
12. Add 500 μL of 2.5% trypsin to the hippocampi (0.25% final trypsin concentration), and incubate for 10 min in a water bath at 37 °C.
13. Thoroughly rinse the tissue with Saline (3 × 10 mL) and triturate with a glass pipette.
14. Reconstitute the dissociated neurons in 1 mL of warm Growth media, and filter through a 40 μm nylon mesh.
15. Count the number of neurons with a hemocytometer.
16. In the meantime, remove ddH_2O from coverslips and dry them for a short period.
17. Plate neurons onto coverslips in 24-well plates at $1.0 – 1.5 \times 10^5$ cells/well density with 500 μL of Growth media.

[9]Removing meningeal tissue as much as possible without damaging the hippocampus is the key to achieve healthy neuron culture with minimum microglia population.

18. Incubate the neuron culture at 35 °C in a 5% CO_2: 95% air humidified incubator for 2 – 3 weeks. For the best result, avoid disturbing the culture as much as possible during incubation (*see* Note 10).

3.2. Ca-P transfection

1. Perform Ca-P transfection as early as 4 days *in vitro* (DIV) after the neuron culture preparation (*see* Note 11).
2. On the day of experiment, obtain high quality plasmid DNAs using miniprep or maxiprep commercial kits according to manufacturer's protocol (*see* Note 12).
3. Warm up Transfection media, Washing buffer, and Growth media in a water bath at 37 °C.
4. Replace the culture Growth media with 500 μL of Transfection media in each well. *** Do not discard the original Growth media** (*see* Note 13).
5. Make Master Mix Transfection solution (33 μL per each coverslip X number of coverslips). For each coverslip, add 1.65 μL of 2.5M $CaCl_2$, 0.7 μg of DNA, 16.5 μL of 2X BBS buffer, and add ddH_2O to bring the volume up to 33 μL.
6. Inside the tissue culture hood, prepare Transfection solution by first combining 2.5 M $CaCl_2$ and ddH_2O in a tube while slowly agitating.
7. Continue agitating and slowly add DNA into the solution.
8. Add 2X BBS buffer drop-wise while agitating.
9. **Immediately** add 30 μL of Transfection solution to each coverslip of neuron culture inside the culture hood.
10. Rock the culture dish a few times to mix and incubate at 35 °C in a 5% CO_2: 95% air humidified incubator for 45 min – 1 hr. After neurons are incubated with the Transfection solution, very fine DNA/Ca-P co-precipitates would form a layer covering neurons.
11. Replace the Transfection media with 500 μL of Washing buffer and incubate in the incubator for 15 – 20 min. The precipitates would disappear after the Wash step.
12. Replace Washing buffer with 500 μL of fresh Growth media.

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[10] Successful Uaa incorporation in neurons depends strongly on the preparation of healthy neuron culture. Every step in the protocol should be performed in a precise and meticulous manner. After culture preparation, it is best to maintain the culture in the incubator without disturbance. Opening and closing the incubator door, as well as moving the culture dish in and out of the incubator, can add up to undermining the quality of culture. Lower incubator temperature (35 °C instead of 37 °C) helps reduce excitotoxicity.

[11] Younger neurons are transfected at higher efficiency, i.e., Ca-P transfection efficiency goes down as the neuron culture gets older. At the same time, Ca-P transfection of young neurons might not be suitable for some experiments, as they are still immature. Generally, 7 DIV is the earliest time to perform Ca-P transfection to record action potentials.

[12] Around 1.9 of the 260/280 ratio is optimal for the purified DNA. When necessary, perform an agarose gel electrophoresis to check purity of the prepped DNA [25]. Supercoiled plasmid DNA with high purity is best for neuronal transfection.

[13] It is important to save the original Growth media during Ca-P transfection, and add back to the culture to restore the original condition. Maintaining the neuron culture in fresh media after transfection would interfere with neurons' recovery, since fresh media lack key molecules for cell proliferation such as growth factors released from neurons.

13. Again, replace the Growth media with the saved original Growth media (*see* Note 13).
14. Add the Uaa Cmn (pre-mixed in 50 μL of warm Growth media) to the culture to reach 1 mM final concentration.
15. Incubate the transfected culture in the incubator for 12 – 48 hr before assays.

3.3. Whole cell recording with light activation from primary neurons

1. Prepare Extracellular/Intracellular solutions.
2. Set up the electrophysiology rig.
3. Install an LED by the microscope at the rig to deliver light to the focal point from 1 cm away at a 45 ° angle.
4. Check the power of LED with a light power meter (*see* Note 14).
5. Pull patch pipettes from glass electrodes using a commercial micropipette puller to have 3–6 MΩ pipette resistance. Follow manufacturer's instruction to set up the micropipette puller (*see* Note 15).
6. Take out a coverslip from the neuron culture in the incubator and rinse it by placing it in a 35 mm culture dish filled with fresh Extracellular solution.
7. Put a dab of vacuum grease on the bottom of a new 35 mm culture dish and fill with fresh Extracellular solution (*see* Note 16).
8. Move the coverslip to the prepared dish and hold it down onto the grease.
9. Place the coverslip/dish on the electrophysiology microscope platform.
10. Using the standard patch clamping techniques, patch a neuron showing mCitrine fluorescence.
11. Record neuronal activity using current clamp (I-clamp) method. First, adjust the resting potential to around −72 mV by injecting a small current. Then, inject a step current (10 – 200 pA) to induce continuous firing (5 – 15 Hz) of action potentials.
12. Manually, or using the data acquisition software, flash a pulse (a single pulse of 100 ms – 1 sec duration) of LED light to the neuron while recording, and see if action potentials are affected.
13. Perfuse 0.5 mM $BaCl_2$ Extracellular solution to the bath and verify if action potentials are recovered (*see* Note 17).

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[14] Regularly check the LED performance using an optical power meter.
[15] To test pipette resistance, first fill a pipette with Intracellular solution and position it on the electrode holder. Dip the pipette in a 35 mm culture dish filled with Extracellular solution, placed on the microscope platform. Immerse a ground electrode into the dish to complete a circuit. Turn on the amplifier/digitizer and start a data acquisition software. Monitor pipette resistance with a membrane test protocol.
[16] Steps 7–9 in section 3.3 can be minimized if a coverslip-compatible chamber is equipped at the microscope.
[17] $BaCl_2$ is a Kir2.1 specific inhibitor, and its addition would re-block PIRK after photo-activation. This step verifies the specificity of light-control.

3.4. *In utero* electroporation and Uaa microinjection (Fig. 4)

1. Plasmid DNA preparation: Purify DNA with an endotoxin-free Maxiprep kit, then perform phenol-chloroform extraction followed by ethanol precipitation to prepare highly purified plasmid DNA (condensed to 2–5 μg/μl).
2. Anesthetize a timed pregnant mouse at E14.5 with pentobarbital (50 μg/gram body weight, intraperitoneal injection) or isoflurane (2–4% inhalation).
3. Make a small incision at the abdominal midline, then pull out the uterine horns with forceps, ring forceps, and fingertips onto a 37 °C pre-warmed PBS-moistened cotton gauze placed around the incision (*see* Note 18).
4. Inject ~1μl of plasmid DNA solution, mixed with 0.1 % Fast green for visualization, into the lateral ventricle of each littermate with a glass capillary inserted through the uterine wall.
5. Electroporate the embryos with an electroporator using a tweezer electrode (33–35 V, 50 msec duration, 950 msec interval, 4–8 pulses) (*see* Note 19).
6. Return the uterine horns to the abdominal cavity gently with forceps, ring forceps, and finger tips. Suture the muscle wall, then the skin with surgical suture to allow the embryos for continuing development *in utero*.
7. After 2 days, make a small incision at the abdominal midline again at E16.5, and then pull out the electroporated embryos gently with forceps, ring forceps, and fingertips onto a 37 °C pre-warmed PBS-moistened cotton gauze placed around the incision. (*see* Note 18)
8. Inject 2–5 μl Cmn-Ala (500 mM) to the electroporated side or both side of the lateral ventricle with a glass capillary inserted through the uterine wall.
9. Return the uterine horns again to the abdominal cavity gently with forceps, ring forceps, and finger tips. Suture the muscle wall, then the skin with surgical suture to allow the embryos for continuing development *in utero*.

3.5. Whole cell recording with light activation from acute brain slices

1. From 1 L of ACSF solution, take 200 mL and fast-freeze at − 80 °C for 20 – 30 min. Pre-chilled ACSF will form slush and be used at Step 13. Bubble the rest 800 mL of ACSF with 5% CO_2: 95% O_2 gas at room temperature.
2. Prepare and sterilize dissection surgery tools to harvest brains from the embryos.
3. Prepare a bucket of ice.
4. Make ~100 mL of 4% low melting point agarose solution in a flask by heating in a microwave. Cool down solution for about 5 min before it starts to solidify.

[18]Because this procedure requires two major surgeries, it is required to handle exposed uterus/embryos really gently to reduce stress and damage. Keep the embryos warm and moist during procedures with 37 °C pre-warmed PBS. After each surgery, look after the mouse throughout the recovery.

[19]This technique could be applied not only to neocortical neurons but also to neurons in other brain regions such as striatum, diencephalon, and cerebellum, when electroporation/injection site is adjusted for each region.

5. Euthanize the mouse 12 – 24 hr after Cmn-Ala injection by CO_2 overdose [14] (*see* Note 20).
6. Make large incisions at the abdominal area, and dissect out the electroporated/microinjected embryos from the uterus with fine scissors and forceps.
7. Harvest brains from the embryos with fine scissors and forceps.
8. Place each brain on a 10 cm culture dish placed (upside down) on the ice bucket.
9. Divide two hemispheres using a sharp blade, and place each hemisphere on the dish with the midsagittal plane touching the bottom of the dish.
10. Quickly pour the agarose solution over brains (around 500 μL per hemisphere) (*see* Note 21).
11. Using the blade, square cut the agarose around a brain to make a brain-embedded agarose block.
12. Using tissue adhesive, glue down the midsagittal plane surface of the agarose block on a mount for vibratome.
13. Place the mount in a vibratome chamber and fill with pre-chilled ACSF.
14. Cut 200 μm sagittal brain slices, and incubate in the Incubation ACSF at 33 °C for 42 min while bubbling with 5% CO_2: 95% O_2 gas [14](*see* Note 22).
15. Turn off the heat from the water bath, and continue to incubate the slices at room temp while bubbling. Start whole-cell patch recording at least 15 min after turning off the heater.
16. Prepare Intracellular solution.
17. Set up the slice electrophysiology rig, and superfuse the recording chamber with ACSF at 2 mL/min rate with a perfusion pump. Adjust the chamber temperature to around 33 °C.
18. Install an LED by the microscope at the rig to deliver light to the focal point from 1 cm away at a 45 ° angle.
19. Check the power of LED with a light power meter (*see* Note 14).
20. Pull patch pipettes from glass electrodes using a commercial micropipette puller to have 3–6 MΩ pipette resistance. Follow manufacturer's instruction to set up the micropipette puller (*see* Note 15).
21. Carefully pick up a brain slice with a glass pipette and place in the perfusion chamber. Hold down the slice with a harp.

[20]Depending on the target protein's half-life, one can wait longer or shorter after Uaa microinjection before harvesting embryos.
[21]Agarose embedding helps stabilize soft embryonic brains for vibratome cutting. Although low melting point agarose minimizes temperature shock to the brain tissue, caution is required when pouring melted agarose over cold brains. The effect of temperature shock to the tissue from agarose embedding was not reported in the previous study [14].
[22]Sagittal brain slices work best for recording/imaging neocortical Uaa incorporation. However, coronal or horizontal sections might be more suitable for other brain regions.

22. Patch a neuron showing mCherry and GFP fluorescence from the neocortical region.

23. Record PIRK activity using voltage clamp (V-clamp) method. First, hold the membrane potential at −60 mV. Then, record currents at the fixed negative membrane potential (–100 mV) or voltage ramps (–100 mV to +40 mV). Specifically, monitor Kir2.1 specific inward currents at −100 mV.

24. Manually, or using the data acquisition software, flash a pulse (a single pulse of 100 ms – 1 sec duration) of LED light to the neuron while recording, and see if PIRK proteins are activated. Once PIRK is activated, inward currents at −100 mV would increase significantly.

25. Perfuse 0.5 mM $BaCl_2$ ACSF to the bath and verify if PIRK gets inactivated again (*see* Note 17).

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References

1. Callaway EM, Katz LC. Photostimulation using caged glutamate reveals functional circuitry in living brain slices. Proc Natl Acad Sci U S A. 1993; 90:7661–7665. [PubMed: 7689225]
2. Yoshimura Y, Callaway EM. Fine-scale specificity of cortical networks depends on inhibitory cell type and connectivity. Nat Neurosci. 2005; 8:1552–1559. [PubMed: 16222228]
3. Banghart M, Borges K, Isacoff E, Trauner D, Kramer RH. Light-activated ion channels for remote control of neuronal firing. Nat Neurosci. 2004; 7:1381–1386. [PubMed: 15558062]
4. Volgraf M, Gorostiza P, Numano R, Kramer RH, Isacoff EY, Trauner D. Allosteric control of an ionotropic glutamate receptor with an optical switch. Nat Chem Biol. 2006; 2:47–52. [PubMed: 16408092]
5. Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K. Millisecond-timescale, genetically targeted optical control of neural activity. Nat Neurosci. 2005; 8:1263–1268. [PubMed: 16116447]
6. Fenno L, Yizhar O, Deisseroth K. The development and application of optogenetics. Annu Rev Neurosci. 2011; 34:389–412. [PubMed: 21692661]
7. Adamantidis A, Arber S, Bains JS, Bamberg E, Bonci A, Buzsaki G, Cardin JA, Costa RM, Dan Y, Goda Y, Graybiel AM, Hausser M, Hegemann P, Huguenard JR, Insel TR, Janak PH, Johnston D, Josselyn SA, Koch C, Kreitzer AC, Luscher C, Malenka RC, Miesenbock G, Nagel G, Roska B, Schnitzer MJ, Shenoy KV, Soltesz I, Sternson SM, Tsien RW, Tsien RY, Turrigiano GG, Tye KM, Wilson RI. Optogenetics: 10 years after ChR2 in neurons--views from the community. Nat Neurosci. 2015; 18:1202–1212. [PubMed: 26308981]
8. Wang L, Brock A, Herberich B, Schultz PG. Expanding the genetic code of Escherichia coli. Science. 2001; 292:498–500. [PubMed: 11313494]
9. Wang L, Xie J, Schultz PG. Expanding the genetic code. Annu Rev Biophys Biomol Struct. 2006; 35:225–249. [PubMed: 16689635]
10. Wang Q, Parrish AR, Wang L. Expanding the genetic code for biological studies. Chem Biol. 2009; 16:323–336. [PubMed: 19318213]
11. Liu CC, Schultz PG. Adding new chemistries to the genetic code. Annu Rev Biochem. 2010; 79:413–444. [PubMed: 20307192]
12. Wang W, Takimoto JK, Louie GV, Baiga TJ, Noel JP, Lee KF, Slesinger PA, Wang L. Genetically encoding unnatural amino acids for cellular and neuronal studies. Nat Neurosci. 2007; 10:1063–1072. [PubMed: 17603477]

13. Shen B, Xiang Z, Miller B, Louie G, Wang W, Noel JP, Gage FH, Wang L. Genetically encoding unnatural amino acids in neural stem cells and optically reporting voltage-sensitive domain changes in differentiated neurons. Stem Cells. 2011; 29:1231–1240. [PubMed: 21681861]
14. Kang JY, Kawaguchi D, Coin I, Xiang Z, O'Leary DD, Slesinger PA, Wang L. In vivo expression of a light-activatable potassium channel using unnatural amino acids. Neuron. 2013; 80:358–370. [PubMed: 24139041]
15. Beaudoin GM 3rd, Lee SH, Singh D, Yuan Y, Ng YG, Reichardt LF, Arikkath J. Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex. Nat Protoc. 2012; 7:1741–1754. [PubMed: 22936216]
16. Kaech S, Banker G. Culturing hippocampal neurons. Nat Protoc. 2006; 1:2406–2415. [PubMed: 17406484]
17. Banker, G., Goslin, K. Culturing Nerve Cells. MIT Press; Cambridge: 1998.
18. Beene DL, Dougherty DA, Lester HA. Unnatural amino acid mutagenesis in mapping ion channel function. Curr Opin Neurobiol. 2003; 13:264–270. [PubMed: 12850209]
19. Lemke EA, Summerer D, Geierstanger BH, Brittain SM, Schultz PG. Control of protein phosphorylation with a genetically encoded photocaged amino acid. Nat Chem Biol. 2007; 3:769–772. [PubMed: 17965709]
20. Hoppmann C, Lacey VK, Louie GV, Wei J, Noel JP, Wang L. Genetically encoding photoswitchable click amino acids in Escherichia coli and mammalian cells. Angew Chem Int Ed Engl. 2014; 53:3932–3936. [PubMed: 24615769]
21. Hoppmann C, Maslennikov I, Choe S, Wang L. In Situ Formation of an Azo Bridge on Proteins Controllable by Visible Light. Journal of the American Chemical Society. 2015; 137:11218–11221. [PubMed: 26301538]
22. Coin I, Katritch V, Sun T, Xiang Z, Siu FY, Beyermann M, Stevens RC, Wang L. Genetically encoded chemical probes in cells reveal the binding path of urocortin-I to CRF class B GPCR. Cell. 2013; 155:1258–1269. [PubMed: 24290358]
23. Parrish AR, She X, Xiang Z, Coin I, Shen Z, Briggs SP, Dillin A, Wang L. Expanding the Genetic Code of Caenorhabditis elegans Using Bacterial Aminoacyl-tRNA Synthetase/tRNA Pairs. ACS Chem Biol. 2012; 7:1292–1302. [PubMed: 22554080]
24. Lu H, Klaassen C. Tissue distribution and thyroid hormone regulation of Pept1 and Pept2 mRNA in rodents. Peptides. 2006; 27:850–857. [PubMed: 16202478]
25. Green, MR., Sambrook, J. Molecular Cloning: A laboratory manual. 4. Cold Spring Harbor Laboroatory Press; New York: 2012.

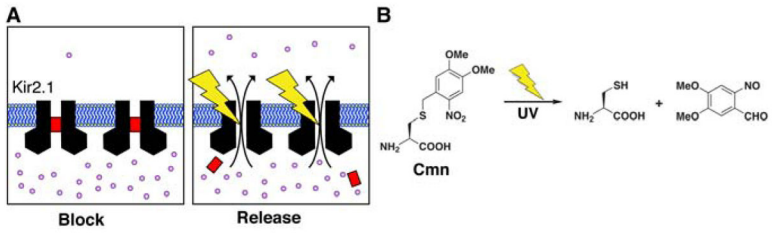

Fig. 1.

Genetically encoding a photocaged Uaa to generate photo-inducible channels in neurons. (**a**) Left: Incorporation of the Uaa Cmn (in red) in the pore of Kir2.1 channels renders the channel nonconducting; Right: UV light exposure irreversibly removes the photocaging group, dimethoxynitrobenzyl, to allow permeation through the Kir2.1 channel, restoring outward K^+ current. (**b**) Photolysis of Cmn into Cys.

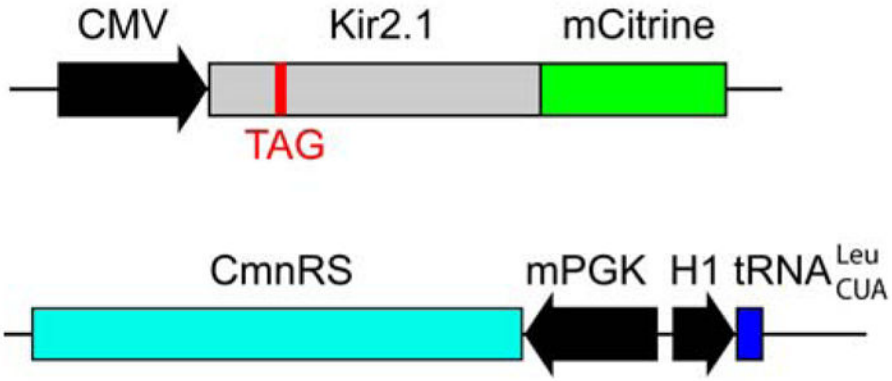

Fig. 2.

Plasmids for incorporation of Cmn into Kir2.1 in neuron culture. The top plasmid encodes Kir2.1 gene containing a TAG amber codon at Cys169 site for Uaa incorporation driven by the cytomegalovirus (CMV) promoter. Fluorescent protein mCitrine was fused to the C-terminus for easy visualization of Uaa incorporation and Kir2.1 expression. The bottom plasmid encode CmnRS (the synthetase specific for Cmn) driven by the mouse phosphoglycerate kinase-1 (mPGK) promoter and the orthogonal amber suppressing leucyl tRNA driven by the H1 promoter.

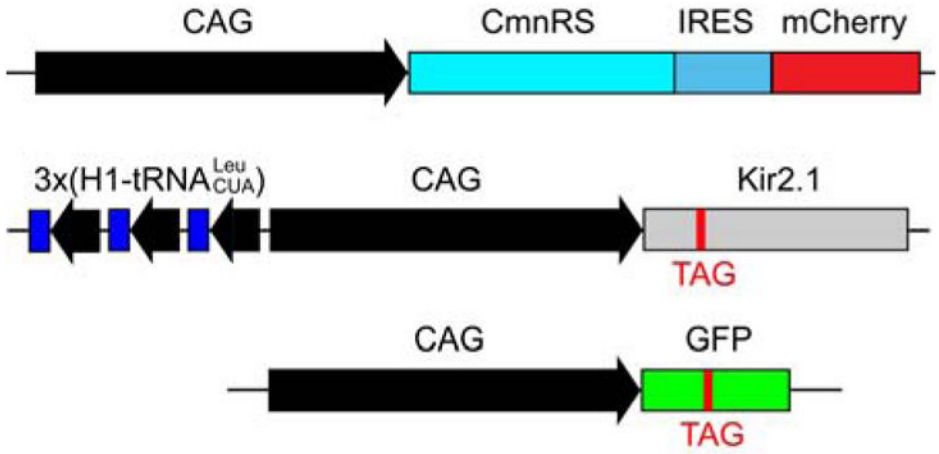

Fig. 3.

Plasmids for incorporation of Cmn into Kir2.1 in the mouse neocortex *in vivo*. The top plasmid encodes CmnRS driven by a strong chicken beta-actin (CAG) promoter and red fluorescent protein mCherry via internal ribosomal entry site (IRES). mCherry fluorescence serves to verify the expression of CmnRS. The middle plasmid encodes Kir2.1 gene containing the amber stop codon TAG at Cys169 site, together with three copies of the orthogonal tRNA expression cassette. The bottom plasmid encodes GFP gene with an amber stop codon introduced at the permissive Tyr182 site. Green fluorescence of GFP indicates Cmn incorporation at TAG sites.

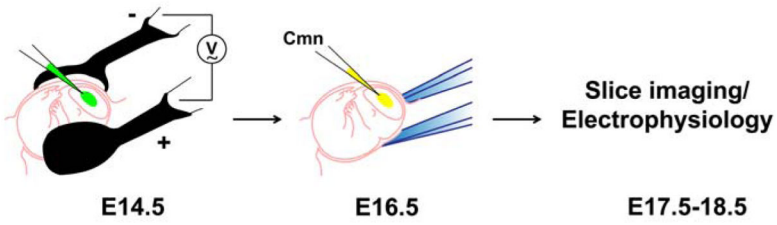

Fig. 4.

Experimental procedure for Cmn incorporation in Kir2.1 in the mouse neocortex *in vivo*. Plasmids shown in Fig. 3 are injected into the mouse neocortex (E14.5) and electroporated *in utero*. Two days later, Cmn-Ala dipeptide is injected in the ventricle in the electroporated side or both sides of cerebral hemispheres. On E17.5 to E18.5, slice imaging and electrophysiological assay can be performed.