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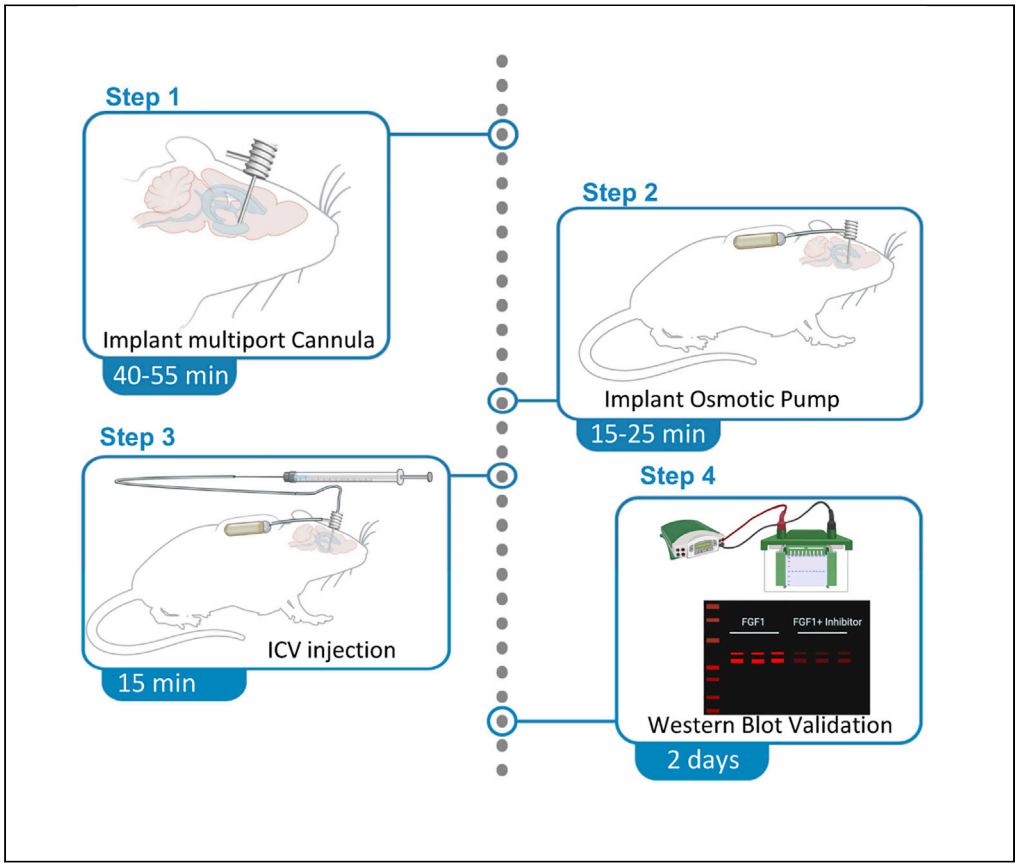
Combined micro-osmotic pump infusion and intracerebroventricular injection to study FGF1 signaling pathways in the mouse brain



Intracerebroventricular (icv) injection of fibroblast growth factor 1 (FGF1) elicits remission of diabetic hyperglycemia in rodent models of type 2 diabetes. Here, we present an optimized protocol to study the intracellular signaling pathways underlying the FGF1-induced sustained glucose lowering in the mouse brain. This protocol combines icv injection of FGF1 and osmotic mini-pump infusion of U0126, an inhibitor of MAPK/ERK signaling. We describe the surgical procedure and verification of U0126 inhibition of FGF1-stimulated hypothalamic MAPK/ERK signaling via western blot.

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Highlights

Protocol for investigating cellular mechanisms underlying FGF1-induced diabetes remission

Simultaneous icv injection and osmotic pump infusion into the third ventricle of mice

Near-infrared western blot to quantify MAPK/ERK signaling in hypothalamic extracts

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Combined micro-osmotic pump infusion and intracerebroventricular injection to study FGF1 signaling pathways in the mouse brain

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SUMMARY

Intracerebroventricular (icv) injection of fibroblast growth factor 1 (FGF1) elicits remission of diabetic hyperglycemia in rodent models of type 2 diabetes. Here, we present an optimized protocol to study the intracellular signaling pathways underlying the FGF1-induced sustained glucose lowering in the mouse brain. This protocol combines icv injection of FGF1 and osmotic mini-pump infusion of U0126, an inhibitor of MAPK/ERK signaling. We describe the surgical procedure and verification of U0126 inhibition of FGF1-stimulated hypothalamic MAPK/ERK signaling via western blot.

For complete details on the use and execution of this protocol, please refer to Brown et al. (2021).

BEFORE YOU BEGIN

Stereotaxic delivery of compounds into the central nervous system has transformed our understanding of the role of the brain in the regulation of energy and glucose homeostasis. Ventricular cannulation with a dual-port guide cannula allows for both single, acute intracerebroventricular (icv) FGF1 injections and continuous infusion of bioactive compounds as a tool with which to assess the role of specific intracellular signaling pathways in the brain response to FGF1 (Bentsen et al., 2020; Brown et al., 2019; Scarlett et al., 2016, 2019). The protocol below describes the implantation of a dual-port guide cannula targeting the third ventricle in mice, thus enabling icv FGF1 injection followed immediately by continuous icv infusion of the MAP/ERK inhibitor U0126 for 24 h. This protocol can be optimized for infusion into virtually any brain region, potentially with any combination of 2 compounds. For detailed modifications to the protocol refer to (Bentsen et al., 2020).

Prepare surgical instruments and equipment

Timing: 1–3 h

1. Sterilize surgical tools, cannula, obturator, screws, gauze, and cotton swabs.
2. Set up stereotaxic system and connect controller unit.
3. Fill isoflurane unit and connect oxygen and vacuum lines.
4. Turn on bead sterilizer.

5. Put on appropriate Personal Protective Equipment for surgery per institutional guidelines.
6. Create sterile field.
7. Place gauze, scalpel, and surgical supplies onto sterile field.
8. Open sterilized tool kit and lay tools into the sterile field.
9. Prepare buprenorphine (working solution: 0.005 mg/mL; Dose = 0.01 mL/g body weight) for post-operative analgesia.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-p-p44/42 MAPK Erk1/2 (1:1000)	Cell Signaling Technology	Cat#4370
Mouse anti-p44/42 MAPK Erk1/2 (1:2000)	Cell Signaling Technology	Cat#9107
IRDye 680RD (1: 15,000)	LI-COR Biosciences	Cat#926-68073
IRDye 800CW (1: 15,000)	LI-COR Biosciences	Cat#926-32212
Chemicals, peptides, and recombinant proteins		
U0126	MilliporeSigma	SKU:662005
Dimethyl sulfoxide (DMSO)*	Fisher Scientific	Cas#67-68-5
Bovine Serum Albumin	Sigma-Aldrich	Cat#A9647
Recombinant FGF1	Prospec Protein Specialists	Cat#CYT-528
Tissue Protein Extraction Reagent (T-PER)	Thermo Fisher Scientific	Cat#78501
Halt ™ Protease and Phosphatase Inhibitor Cocktail (100×)	Thermo Fisher Scientific	Cat#78440
Phenylmethylsulfonyl fluoride (PMSF)*	RPI Research Products International	SKU:P20270-5.0
Saline (0.9% Sodium Chloride)	Hospira	NDC:0409-4888-02
Sterile Water	Hospira	NDC:0409-4887-24
Intercept Blocking Buffer	LI-COR Biosciences	Cat#927-60001
4× protein loading buffer	LI-COR Biosciences	Cat#928-40004
B-mercaptoethanol*	Sigma-Aldrich	Cas#60-24-2
Chameleon Duo Pre-Stained Ladder	LI-COR Biosciences	Cat#928-60000
Methanol*	Fisher Scientific	Cat#A412SK
Tris	Fisher Scientific	Cat#BP152-1
Glycine	Fisher Scientific	Cat#BP381-1
Isoflurane*	Piramal Critical Care	NDC 66794-017-10
Buprenorphine*	Par Pharmaceuticals	NDC 42023-179-05
Tween-20	Sigma-Aldrich	Cat#P1379
Bolt™ Running Buffer	Thermo Fisher Scientific	Cat# B0001
Critical commercial assays		
BCA Protein Assay Kit	Thermo Scientific	Cat#23225
Revert 700 Total protein Stain Kit	LI-COR Biosciences	P/N: 926-11010
Experimental models: Organisms/strains		
C57BL/6J mouse	The Jackson Laboratory	Stock No. 000664
B6.V-Lepob/J homozygous mouse	The Jackson Laboratory	Stock No. 000632
Other		
Hair net~	Fisher Scientific	Cat#19-170-900
Surgical mask~	VWR	Cat#41004-670
Sterile gown~	Fisher Scientific	USMS1050CF
Sterile gloves~	Kimberly-Clark Professional	Product#56844
Eye lubrication~	Major Pharmaceutical	NDC 0904-6488-38
Scalpel blade~	Aspen Bard Parker	Cat#371150
Betadine swabs~	Fisher Scientific	Cat#S41125
Ethanol wipes~	Fisher Scientific	Cat#22-363-750
Cotton swabs~	Puritan	SKU:867WC
Gauze (4 × 4)~	Covidien	Product#441215
Small plastic cups~	Fisher Scientific	Cat#FB012919
3 mL syringe ~	BD	SKU:309656

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dental acrylic~	Lang Dental Mfg.Co	Product#1306CLR
Ortho-Jet ™ Powder~	Lang Dental Mfg.Co	Product#1350
.5 cc insulin syringe~	Fisher Scientific	Cat#14-841-32
Electric Shaver~	Wahl	SKU:1590Q
Stereotaxic Instrument~	Kopf	Model#940
Bead sterilizer~	Fine Science Tools	Cat#NC9779311
Screwdriver~	Moody	Part#51-1524
Drill with small bit~	CellPoint Scientific	Product#67-1200
Heating pads~	American Scientific	Mfr#322702
Gel~	Bio-Serv	Product#PWGP-8
Thick forceps ~	Roboz	Product#RS-8102
Dog-tooth forceps~	Roboz	Product#RS-8164
Scalpel~	Swann-Morton	Part#0501
Fine forceps~	Roboz	Product#RS-5130
Spatula~	VWR	Cat#82027-518
Tissue punch (2.0)	Harris Uni-Core	SKU:WHAWB100029
15 mL Falcon Tube	Falcon	Product#352196
Hamilton Syringe	Hamilton	Part#7653-01
96 well plate	Falcon	Product#353075
PVDF membrane	EMD Millipore	Cat#IPFL00005
Icv injection tubing 0.020″ ID × 0.060″ OD Tygon® ND 100-80 Microbore Tubing	United States Plastic Corp.	Item#56515
Vinyl catheter tubing	Alzet	Order#0007760
Sutures – Vicryl, 6-0, C-3 needle 45 cm purple filament	Ethicon	Article#J384H
Third ventricle osmotic pump guide cannula~	P1 Technologies-Plastics One	Product info. 3260PGA/SPC cut 5.3 mm below pedestal
Third ventricle injector/internal guide	P1 Technologies-Plastics One	Product info. C315IA/SPC fits 5.3 mm 3260PGA/SPC with 0.4 mm projection
Short dummy cannula for 326OPGA/SPC~	P1 Technologies-Plastics One	Product info. C315DCOP/SPC
Long dummy cannula obturator for 326OPGA/SPC	P1 Technologies-Plastics One	Product info. C315DC/SPC fits 5.3 mm 3260PGA/SPC with 0.4 mm projection
Dummy cannula #	P1 Technologies-Plastics One	Product info. 8IC315DCSXXC
Third Ventricle Guide Cannula #	P1 Technologies-Plastics One	Product info. 8IC315GAS5C C315GAS-5/SPC cut 6.3 mm below pedestal
Third Ventricle injector #	P1 Technologies-Plastics One	Product info. 8IC315IAS5SC C315IAS-5/SPC 33GA
Bone Screws~	P1 Technologies-Plastics One	Product info. 8L0X3905201F
Micro-osmotic pump	Alzet	Order#Model 1003D
Sonicator	Virsonic	Item# 346411
10% Bis-Tris criterion XT gel	Bio-Rad	Cat#3450112
Criterion Cell	Bio-Rad	Cat#1656001
Criterion Blotter	Bio-Rad	Cat #1704070
PowerPac™ Basic Power Supply	Bio-Rad	Cat#1645050
Odyssey FC	LI-COR Biosciences	Model #2800

Note: Items marked with ~ are used during surgery to implant dual port cannula.

MATERIALS AND EQUIPMENT

U0126 Osmotic pump infusion (Fills n=15 osmotic pumps)

Reagent	Final concentration	Amount
U0126	30 mM	30 mg
DMSO (50% DMSO:50% saline)	n/a	2,478 µL
Total	**30 mM**	**2,478 µL**

Prepare solution immediately before filling the osmotic pump.

U0126 acute injection		
Reagent	Final concentration	Amount
U0126	2.5 μg/μL	100 μg
DMSO (50% DMSO:50% saline)	n/a	50 μL
Total	**2.5 μg/μL**	**50 μL**
Prepare solution immediately prior to inject and keep at room temperature 68 degree F between injections.		

Fibroblast growth factor 1 (FGF1)		
Reagent	Final concentration	Amount
FGF1	1.5 μg/μL	50 μg
Sterile Water	n/a	33.3 μL
Total	**1.5 μg/μL**	**50 μL**
Prepare solution immediately prior to inject and keep on wet ice between injections.		

Protein Lysis Buffer		
Reagent	Final concentration	Amount
T-Per	N/A	200 μL /sample
PMSF	1 mM	10 μL /mL
Phosphatase and Protease Inhibitor	1×	10 μL /mL
Total	**N/A**	**220 μL**
Prepare solution immediately prior to protein isolation.		

Protein Loader Buffer		
Reagent	Final concentration	Amount
4× protein loading buffer	2×	475 μL
Sterile Water	N/A	475 μL
B-mercaptoethanol	715 mM (5%)	50 μL
Total	**N/A**	**1,000 μL**
Prepare solution immediately prior adding to protein samples.		

Transfer Buffer		
Reagent	Final concentration	Amount
Tris	25 mM	3.03 g/L
Glycine	192 mM	14.4 g/L
Methanol	20%	200 mL
Sterile Water	N/A	Quantity sufficient
Total	**N/A**	**1,000 mL**
Transfer buffer can be stored at 4°C for up to a month.		

Wash Buffer		
Reagent	Final concentration	Amount
NaCl	150 mM	8 g/L
KCl	2 mM	0.2 g/L
Tris Base	25 mM	3 g/L
Tween-20	0.1%	1 mL
Sterile Water	N/A	Quantity sufficient
Total	**N/A**	**1,000 mL**
Wash Buffer Store at Room Temperature 68 degree F and used up to 6 months.		

⚠ CRITICAL: Chemicals marked with * in the key resources table are hazardous or toxic. Caution should be taken while using these reagents and Chemical Safety Datasheets should be followed.

Alternatives: Although this protocol employs a dual-port guide cannula, a single port guide cannula and an osmotic pump connected to the icv injector can also be used. In this case, the icv injections are performed first, followed by connecting the injector to the catheter and cementing the injector with acrylic for continuous infusion as outlined in Figure 3. Products for this alternative are marked with #. Bone screws and acrylic are used to secure the guide cannula in this protocol although dental cement is a suitable alternative (G-BOND, GC America 002277 and G-ænialTM Universal Flo). Additionally, this protocol uses near-infrared fluorescent western blot to quantify levels of both phospho-ERK and ERK proteins in tissue extracts in the same assay. However, western blot using traditional chemifluorescence is a suitable alternative.

STEP-BY-STEP METHOD DETAILS

Implant dual-port guide cannula

Prepare mouse for surgery

Timing: 10–15 min

1. Prepare 8-week-old C57BL/6J male mice.

   Note: Animals are housed individually under specific pathogen-free conditions in a temperature controlled environment with either a 12 h: 12 h or 14 h:10 h light: dark cycle.

   a. All animal procedures are performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Washington.
2. Record body weight and calculate buprenorphine dosage.
3. Set induction chamber to 2% isoflurane.
4. Place mouse inside the induction chamber for a duration of 2–5 min.
5. Once anesthetized, with an electric shaver, remove hair from the incision area on top of the head.
6. Apply eye lubrication to keep eyes moist during the surgery.
7. Set surgical station isoflurane flow to 1%–2%.
8. Place mouse in stereotaxic apparatus by opening the mouth with non-sterile thick forceps moving the tongue out of the way, and placing the top teeth in the hole on the mouth plate. (Figure 1A).
9. Pull the nose cone over the mouth.
10. Place the animal in the ear bars.
11. Tighten the nose cone and the ear bars to ensure the head is firmly in place.
12. Ensure the depth of anesthesia by performing a toe pinch. The mouse should not react.

Skin preparation

Timing: 2 min

13. Sterilize the shaved incision area with 3 alternating scrubs of betadine and alcohol wipes.
14. Begin sterilizing scrubs along what will be the incision line in a circular motion and extend outward.

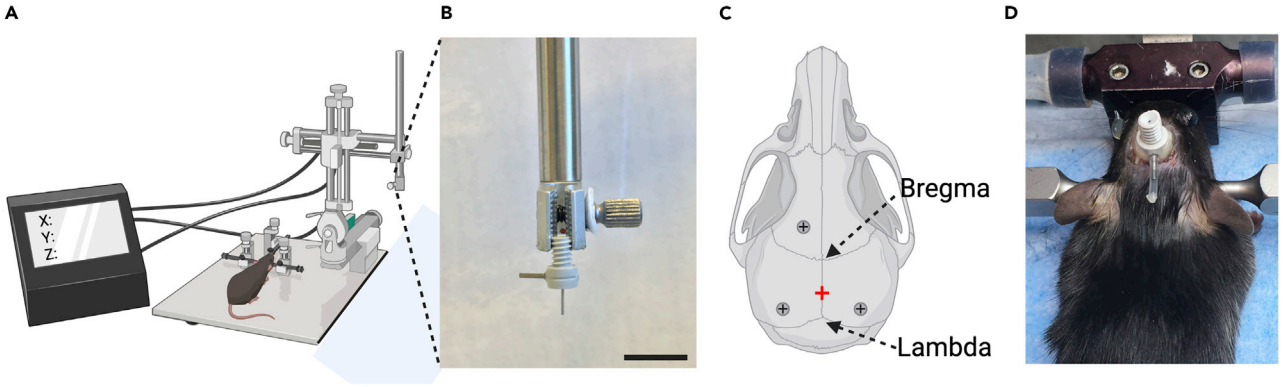

Figure 1. Implant dual-port guide cannula

(A) Stereotaxic apparatus and controller unit set-up for mouse to implant a guide cannula.

(B) Stereotaxic holder containing a multiport guide cannula. Scale bar 1 cm.

(C) Mouse skull with reference points bregma, lambda, bone screw placement, and red x denoting the cannula placement.

(D) Mouse fixed in ear bar with guide cannula implanted targeting the third ventricle. Created with BioRender.com.

Implant third ventricle cannula

Timing: 20 min

15. Replace gloves with sterile surgical gloves for surgery.
16. Using sterile forceps, place the guide cannula in the holder on the stereotaxic device to ensure the cannula tip is perpendicular, straight down by viewing it from multiple angles. (Figure 1B).
17. Connect a 1 cm vinyl catheter to the osmotic pump port.
18. Seal the end of the catheter by melting the tube close with forceps just removed from the hot bead sterilizer.
19. Using dog-tooth forceps in one hand, grasp the skin between the eyes. On the other hand, use a scalpel to make a small incision along the midline in the center of the head.
20. Use cotton swabs to clean the skull and, by opening using lateral traction, the incision of the skin will stay open.
21. Use the guide cannula to level the skull.
    a. Move the tip of the guide cannula to lambda (Figure 1C), touch the skull and zero all the coordinates on the controller by pressing reset.
    b. Move the guide cannula to bregma and touch the skull. Look at the Z coordinate to determine if the distance is within 0.1 mm, indicating the skull is level.
22. Once level, touch the tip of the guide cannula at bregma and zero all the coordinates.
23. Move the guide cannula to the 3V coordinates -1.8 mm posterior to bregma and midline zero.
    a. Mark the coordinate on the skull for the guide cannula (Figure 1C).
24. Move the guide arm out of the way.
25. Drill three holes for the screws ensuring that the holes are from the sutures on the skull and screws will not block placement of the guide cannula (Figure 1C).
    a. It is important to use light pressure to drill through the skull to ensure drill bit doesn't subsequently damage the brain by going too deep.
26. Implant the screws, in one hand hold the curved forceps with a small bone screw. In the other hand, use the screwdriver to screw in the screws.

⚠ CRITICAL: Ensure the head is secure in ear bars to prevent any movement of the head while placing the screws.

27. Once the screws are in place, move the cannula back into place, touch the skull and ensure the guide cannula lines up with the mark made.

28. Drill a hole for the guide cannula, then lower the guide cannula to -5 mm. The base of the cannula should be 0.3 mm off the skull surface.
    a. Coordinates for dual-port cannula.
        i. A/P: -1.8.
        ii. M/L: 0.
        iii D/V: -5.00.
    b. If bleeding occurs during guide cannula placement, use a sterile cotton tip applicator to apply light pressure until bleeding has stopped.

⚠ CRITICAL: The cannula is implanted directly through the superior sagittal sinus and therefore bleeding is expected. Direct pressure with a sterile cotton-tipped applicator applied for 1–2 min, or sterile gauze pads wrapped around the cannula can be used to stop the bleed. Ensure that bleeding has completely stopped prior to cementing the cannula in place.

29. Cement cannula in place (Figure 1D).
    a. Mix the acrylic and Ortho-Jet powder in a 1:2 ratio in a small plastic cup. Remove the needle on a 3 mL twist top syringe, fill the syringe with the acrylic solution and twist on the capped needle.
    b. Uncap the needle and squeeze the acrylic on the skull covering the 3 implanted screws fully and bottom $^1/_2$ of the guide cannula.
    c. Let acrylic dry completely (approximately 10–15 min).
30. Remove the cannula holder and ensure the cannula is not moving out of place. Troubleshooting 1.
31. Place a long dummy cannula obturator in the guide cannula and screw into place.
32. Clean tools with alcohol wipes before placing into bead sterilizer if multiple animals are undergoing surgery.

Postoperative care

Timing: 5–10 min

33. Remove the mouse from the nose cone and place it on a heating pad.
34. When heart rate and breathing have increased sufficiently and before the animal is mobile, administer buprenorphine subcutaneously. Troubleshooting 2.
35. Return animal to clean cage on a heating pad. Make sure the animal has access to water or gel and food.
36. Allow the mouse to recover for a week before performing injections or implanting an osmotic pump.

⚠ CRITICAL: Standard Allentown shoebox cages are used for housing mice after surgery. Monitor mice daily and replace dummy cannula if it is removed.

Osmotic pump priming and implanting

Timing: 1–2 days

Preparation of micro-osmotic pump – prime the osmotic pump the day before surgery.

37. Cut vinyl catheter tubing into 5 cm pieces using sterilized scissors.
38. In a biosafety cabinet, create a sterile field to prepare osmotic pumps. Troubleshooting 3.
39. Drop pump flow moderator and catheter into the sterile field.
40. Adjust the metal tube in the flow moderator so that 3 mm is visible above the white cap.
41. Connect the 3 cm catheter tubing to the flow moderator.

Figure 2. Osmotic pump priming and implantation

(A) Osmotic pump priming in sterile saline. Scale bar 1 cm.

(B) Example of guide cannula connected to the osmotic pump flow modulator. Scale bar 2 cm. 1. Injector 2.Osmotic pump 3. Dual port guide cannula 4.Long dummy cannula 5. Short dummy cannula.

(C) Icv injector connected to guide cannula. Scale bar 2 cm.

(D) Anesthetized mouse with osmotic pump placed under the skin on the upper back slightly off the spine. Scale bar 1 cm.

(E) The catheter is connected to the port on the cannula.

(F) Suture were placed to close the incision. Created with BioRender.com.

42. Fill one sterile 1 mL syringe with U0126 and a second 1 mL syringe with vehicle.
43. Connect the filling needle to the 1 mL syringe.
44. Insert the filling needle to the bottom of the pump. Slowly fill the pump while withdrawing the needle until a small bubble of liquid forms outside the pump.
45. Fill the catheter tubing-flow moderator from the catheter tubing end.
46. Insert the flow moderator into the osmotic pump.
47. Prime the osmotic pump in a sterile 15 mL falcon tube fully submersed in sterile saline solution at 37°C for 24 h. (Figure 2A) Troubleshooting 3.

Note: Refer to manufacturer's protocol for additional details.

Preparation for micro-osmotic pump implantation

Timing: 20 min

48. Sterilize surgical tools, gauze for surgery, icv tubing, and injectors. (Figure 2B).
49. Set up nose cone by connecting oxygen and isoflurane.
50. Turn on the bead sterilizer.
51. Create a sterile field.
52. Drop gauze, scalpel, and surgical supplies onto a sterile field.
53. Open sterilized tool kit and lay tools into the sterile field.
54. Prepare buprenorphine for postoperative care.

Prepare mouse

Timing: 7–10 min

55. Record body weight and calculate buprenorphine dosage.
56. Set induction chamber to 2% isoflurane.
57. Place mouse inside induction chamber.
58. Once anesthetized, remove hair from the incision area on the back between the scapulae with an electric shaver.
59. Apply eye lubrication to keep eyes moist during the surgery.
60. Set surgical station isoflurane flow to 1%–2%.
61. Place mouse in the nose cone.
62. Ensure the depth of anesthesia by performing a toe pinch. The mouse should not react.
63. Clean the shaved incision area with three alternating scrubs of betadine and alcohol wipes.
64. Begin along what will be the incision line in a circular motion and extend outward.
65. Drape mouse with a sterile sheet containing a small window for the surgical area.

Implant osmotic pump

Timing: 5–7 min

66. Use small scissors to make a small incision the diameter of the osmotic pump between the scapulae.
67. Create a pocket for the pump under the skin by gently inserting thick forceps into the incision the same length as the osmotic pump. Open and close the forceps to spread the subcutaneous tissue.
68. Insert a filled pump connected to vinyl catheter tubing into the pocket with the catheter and flow moderator facing the guide cannula. (Figure 2D).
    a. The pump should rest below the incision to one side of the spine.
69. Carefully remove the tubing connected to the infusion port on the guide cannula using forceps.
70. Cut the catheter tubing from the osmotic pump to fit the length (ca. 3 cm) and connect tubing to the guide cannula. (Figure 2E).

Close incision

Timing: 2–5 min

71. Place 2–4 interrupted sutures, closing the skin around the catheter tubing. (Figure 2F).
    a. Inspect the incision site to ensure it is completely closed.

Administer icv injection

Administer icv injections

Timing: 10 min

72. Prepare icv injection Hamilton syringes for use, one for FGF1 and one for U0126.
    a. Drop the sterile icv tubing and the injectors into sterile field.
    b. Connect one end of icv tubing to the 10 ul Hamilton syringe and remove the plunger.
    c. Fill a 3 mL syringe with sterile water and attach a 25 gauge needle.
    d. Connect the syringe to the icv tubing and fill the icv tubing and Hamilton syringe with sterile water.
    e. Replace the Hamilton syringe plunger.
    f. Disconnect the syringe and connect the injector.
    g. Pull 1 ul of air into the tube to separate the sterile water and the drug.
    h. Place the injector into the drug and pull in 4–5 ul.
73. While mouse is under anesthesia place injector into the U0126 solution and pull out the plunger to fill the icv tubing.
74. Remove the long dummy cannula obturator from the guide cannula and insert the injector (Figure 2C) with the syringe filled with U0126.
75. Depress the plunger to administer U0126 (2 μL/min), remove the injector and replace the dummy cannula obturator.

⚠ CRITICAL: Watch the air bubble move in the icv tubing to ensure fluid is flowing through the cannula during the icv injection. Troubleshooting 4.

76. Wait 10 min and repeat steps 72 and 75 with the syringe filled with FGF1.

After the icv injections are complete replace with the short dummy cannula to create passage for flow through the osmotic pump.

⚠ CRITICAL: The short dummy cannula ensures the osmotic pump flow is not obstructed.

Postoperative care

Timing: 5–10 min

77. Remove the animal from nose cone and place on a heating pad.
78. When heart rate and breathing have increased sufficiently and before the animal is mobile, administer buprenorphine subcutaneously. Troubleshooting 2.
79. Return animal to the cage on a heating pad. Make sure the animal has access to gel or water and food.

Western blot validation

Isolate hypothalamus for western blot

Timing: 10–15 min

80. Sacrifice the mouse 24 h post icv injection/osmotic pump implant.
81. Use CO_2 to euthanize the animal.
82. Carefully dissect the brain from the skull.
83. Place the whole brain into an ice-cold metal mouse brain matrix. (Figure 3A).
84. Use two new razor blades to cut a 1.5 mm coronal section of the hypothalamus.
    a. Use the Circle of Willis as a marker for the locations of the razor blades. (Figure 3B).

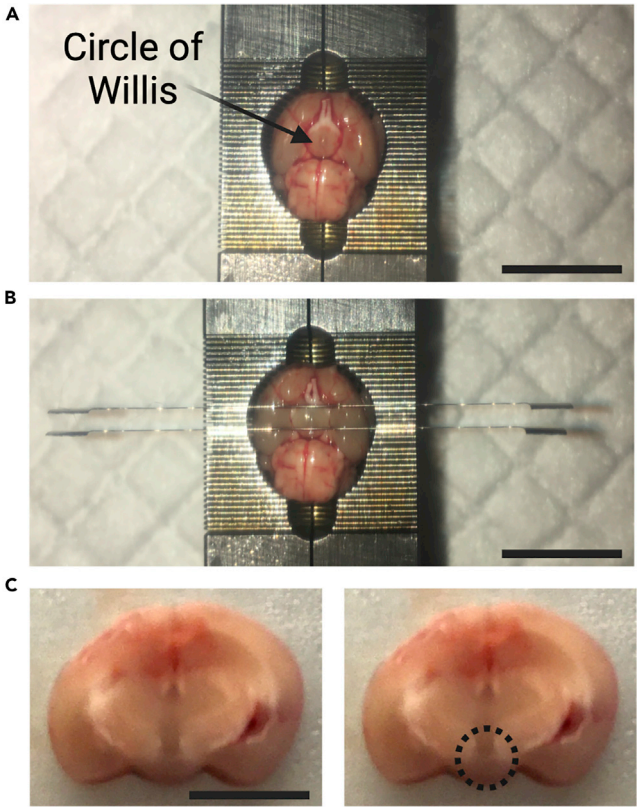

Figure 3. Hypothalamus isolation

(A) Mouse brain placed in metal brain matrix ventral side up. Arrow referencing the Circle of Willis. Scale bar 1 cm.

(B) One razor blade placed at the anterior and one at the posterior region of the Circle of Willis to generate a coronal section. Scale bar 1 cm.

(C) Coronal section place on flat metal surface on ice. Black circle representing the tissue punch of the medial basal hypothalamus. Scale bar 0.5 cm. Created with BioRender.com.

85. Place the coronal section on a metal block on ice.
86. Punch out the mediobasal hypothalamus with a 3 mm tissue punch. (Figure 3C).
87. Place the mediobasal hypothalamus into a labeled tube.
    a. Place a hole in the top of the tube to release the pressure to avoid tube rupturing.
88. Drop tube to snap freeze in liquid nitrogen.
    a. Samples can stay in liquid nitrogen until all samples are collected.
89. Store at −80°C until ready to process or process immediately.

⚠ CRITICAL: Remove osmotic pump and measure the volume of remaining liquid to ensure adequate flow of drug occurred.

Pause point: Tissue can be stored at −80°C for up to 6 months.

Isolate protein from hypothalamus

Timing: 20–30 min

90. Place each sample on ice and pipet 200 μL of cold Lysis buffer.
91. Set sonicator to Duty cycle 50% and set power to 5.

⚠ CRITICAL: Protect your ears by wearing noise-canceling earmuffs.

92. Sonicate the sample with three pulses five-second each to ensure the tissue has been fully homogenized.
93. Clean sonicator between samples with deionized water and Lysis buffer.
94. Centrifuge samples 10,000 × rcf for 15 min at 4°C.
95. Transfer supernatant, containing the proteins of interest, into a new tube on ice.
96. Store protein at −80°C.

Pause point: Protein can be stored at −80°C for up to 6 months.

97. Measure protein concentration by following the manufacturer's protocol for BCA protein assay kit.

Prepare protein samples and run gel

Timing: 10–25 min

98. Calculate total protein concentration to load on the gel for proper concentration for "linear range."
99. Prepare samples to load 15 μL with 3 μg protein per well.
100. Add correct concentration of Protein Sample loading buffer.
101. Heat samples at 100°C for 5 min.

Running and transferring the gel

Timing: 2–2.5 h

102. Place 26 well 10% criterion gel in the Criterion cell buffer tank and fill the tank with Bolt™ Running Buffer.

⚠ CRITICAL: Remove the white tap strip across the bottom of the gel.

103. Load 5 ul of the Chameleon Duo Pre-Stained Ladder into the first well.
104. Pipet 15 ul of each sample into the gel to avoid pipetting bubbles into the well.
105. Connect the Criterion cell to PowerPac and run electrophoresis at 200 V for 25 min.
106. While the gel is running, prepare for the transfer.
107. Submerge the PVDF membrane cut the exact size of the gel in methanol to saturate it completely.
108. Soak blotting papers and sponge pads in cold transfer buffer.
109. Carefully remove the gel from the plastic casing.
110. Assemble the Bio-rad submerged electrophoretic protein transfer apparatus containing both the gel and the PVDF membrane according to bio-rad Instructions.
111. Place ice pack inside the transfer chamber.
112. Run the transfer chamber at 100V for 45–60 min in a cold room.

⚠ CRITICAL: Ensure all the running and transfer apparatus are clean.

Incubate and image membrane

Timing: 1 day

113. Remove membrane from transfer apparatus.
114. Follow the Total Protein Stain manufacture protocol.
    a. Rinse the membrane in ultra-pure water.
    b. Incubate in 5 mL of REVERT Total Protein Stain solution for 5 min with gentle rocking.

c. Decant Total Protein Stain Solution completely.
d. Rinse membrane 2 × 30 s with 5 mL of Wash Solution. Briefly rinse membrane in water.
e. Image membrane immediately in 700 nm channel with Odyssey FC according to the manufacture protocol. Troubleshooting 5.
f. Incubate the membrane in 5 mL of REVERT Reversal solution for 5–10 min with gentle shaking. Do not reverse for longer than 10 min.
g. Rinse membrane briefly with water and proceed to block.

115. Block membrane in blocking Intercept Blocking Buffer for 1 h at room temperature 68 degree F (RT).
116. Wash membrane 1 × 15 min, 2 × 5 min at RT, replacing the wash buffer each time.
117. Incubate blot in pERK 1:1,000 and total ERK 1:2,000 overnight 12–14 h in cold room gentle shaking.
118. Rinse membrane with wash buffer 1 × 15 min, 2 × 5 min at RT, replacing the wash buffer each time.
119. Incubate blot with secondary antibody IRDye 800CW 1: 15,000 and IRDye 680RD 1:15,000 in wash buffer + 0.01% SDS for 1 h at RT gently rocking.
120. Wash membrane with wash buffer 1 × 15 min, 2 × 5 min at RT with wash buffer, and rinse membrane with wash buffer.
121. Detect using Odyssey FC for 700 and 800 channels for 2 min. Troubleshooting 5.

Note: Refer to manufacture protocol and online resources.

⚠ CRITICAL: The Odyssey FC is sensitive to contamination, leading to excess fluorescence on the membrane during detection. To avoid nonspecific fluorescence, ensure incubation boxes and trays used for the western blot are clean before use.

EXPECTED OUTCOMES

To investigate if FGF1 induced activation of the hypothalamic MAPK/ERK signaling pathway in male C57BL/J6 mice, we stereotaxically implanted a guide cannula targeting the third ventricle in the hypothalamus. After one week, we gave an icv injection of an initial 'loading dose' of U0126 or vehicle, followed by icv FGF1 injection and subsequent implantation an osmotic pump containing U0126 or vehicle that was and connected to the cannula. Surprisingly, FGF1 induced activation of MAPK/ERK signaling lasted for at least 24 h in mice receiving icv vehicle (rather than U0126). To block activation this response, the MAPK inhibitor U0126 was infused via osmotic minipump into the third ventricle at a rate of 1 ul per h for 24 h. Continuous icv infusion was required due to the short $^{1}/_{2}$ life of U0126 that made conventional icv injections ineffective at blocking FGF1 action. Mice were sacrificed 24 h post-injection and protein was isolated from the mediobasal hypothalamus. Using near-infrared fluorescence western blot to quantify hypothalamic levels of pERK, a biomarker of MAPK/ERK pathway activity, we confirmed that FGF1-induced MAPK/ERK signaling was blocked in the hypothalamus by continuous infusion of U0126 into the adjacent 3rd ventricle for 24 h (Figure 4A).

To determine if pharmacological inhibition of MAPK/ERK signaling blocked the sustained antidiabetic action induced by icv injection of FGF1, we repeated the above study in adult male, diabetic Lep$^{ob/ob}$ mice and monitored blood glucose levels daily for 15 days (Figure 4B). To control for the potential confounding effect of DMSO (vehicle used to dilute U0126) or U0126 alone, we included additional groups with these treatments as controls. As previously reported, central injection of FGF1 induced sustained glucose-lowering in diabetic Lep$^{ob/ob}$ mice, an effect that was not impacted by co-infusion of DMSO. In contrast, 24-h icv infusion of U0126 following icv injection of FGF1 blocked the sustained glucose-lowering action of centrally administered FGF1. As expected, neither icv infusion of DMSO vehicle nor infusion of U0126 for 24 h had a significant effect on blood glucose in diabetic Lep$^{ob/ob}$ mice that did not receive an icv injection of FGF1 (Figure 4C). Taken together, these results show that hypothalamic ERK1/2 activation is required for sustained remission of diabetic hyperglycemia induced by the central action of FGF1. Refer to Brown et al. (2021) for full study.

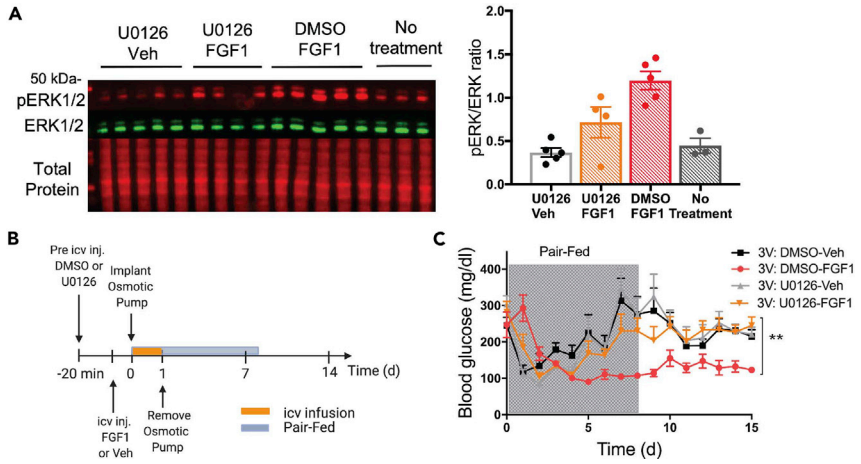

Figure 4. Pharmacological inhibition of MAPK/ERK signaling blocked the sustained antidiabetic action induced by icv injection of FGF1

(A) Representative western blot (left panel) and quantitative comparison of phosphorylated (red) and total ERK1/2 (green) and total protein (red) (right panel) from hypothalamic punches from adult male C57Bl6J mice 24 h after icv injections into the 3rd ventricle (3V) followed by continuous infusion via osmotic pump of 30 mM 1 μL/h U0126 or DMSO for 24 h.

(B) Strategy for MAPK/ERK inhibition by continuous 3V infusion of U0126 or Vehicle DMSO for 24 h followed by disconnection of osmotic pump and metabolic phenotyping image created with BioRender.com.

(C) Blood glucose measured in diabetic Lepob/ob mice (n=5–8/group repeated measures nparLD ANOVA.test statistic treatment =4.99 df=2.67 p=0.002) measured for 15 days post-treatment with icv injection of either saline vehicle or FGF1 (3 μg) followed by a 24 h continuous infusion of U0126 or DMSO vehicle into the third ventricle. Data are represented as mean ±SEM.

LIMITATIONS

A significant limitation of this protocol is that it requires multiple surgeries that collectively increase post-surgical morbidity and mortality. To minimize this risk, osmotic pump insertion and icv injection is performed following recovery from cannula implantation. Additionally, for experiment that require studying the blood glucose in animal the osmotic pump should be removed 24 h after the osmotic pump is implanted.

TROUBLESHOOTING

Problem 1

Failure to implant/secure guide cannula (step 30).

Potential solution

Ensure the mouse is fixed in the stereotactic apparatus.

Ear bars should hold the head firmly in place preventing movement from left to right.

Top incisor teeth should sit in the incisor bar and nose cone tightened around the nose.

Ensure the cannula is fixed to the skull.

The skull should be completely dry and bleeding is stopped before applying the cement.

Acrylic solution should be sufficient to cover the bone screws completely.

Ensure the acrylic is dry and hard prior to removing the stereotaxic arm.

Problem 2
Death of mouse (steps 34 and 78).

Potential solution
Monitor the mouse respiration during the procedures.

Place mouse on heating past post procedure to prevent hypothermia.

Ensure the mouse has access to food and water.

Problem 3
Failure to prepare osmotic pump (steps 38 and 47).

Potential solution
When preparing and implanting the osmotic pump everything should remain sterile.

Completely fill osmotic pumps to ensure no air bubbles.

Problem 4
Obstruction in cannula during icv injection (step 75).

Potential solution
Remove icv injector.

Replace long dummy cannula and secure fully on.

Screw long dummy cannula off and on 2–3 times.

Problem 5
Contamination on the western blot (steps 114 and 121).

Potential solution
Completely wash the running and transfer chamber along with all the trays.

Make sure to use ultra-pure water when rising the membrane.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jarrad Scarlett (Jarrad.Scarlett@seattlechildrens.org).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate datasets.

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AUTHOR CONTRIBUTIONS

J.B., B.P., and J.S. performed the experiments. J.B., B.P., N.A., G.M., M.S., and J.S. designed the experiments. J.B., B.P., N.A., M.S., and J.S. wrote and revised the manuscript. All authors read, revised, and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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