Microglia are important immune cells in the central nervous system (CNS). Mutations in microglia may cause CNS disorders. Replacement of dysfunctional microglia with allogeneic wild-type microglia can correct the gene deficiency, thus treating the neurogenic diseases. However, traditional approaches cannot efficiently replace microglia at the adulthood. Here, we introduce a potentially clinical-feasible strategy named microglia replacement by bone marrow transplantation that achieves efficient microglia replacement at the whole CNS scale, including the brain, spinal cord, and retina in adult mice.
Protocol

Microglia replacement by bone marrow transplantation (Mr BMT) in the central nervous system of adult mice

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SUMMARY

Microglia are important immune cells in the central nervous system (CNS). Mutations in microglia may cause CNS disorders. Replacement of dysfunctional microglia with allogeneic wild-type microglia can correct the gene deficiency, thus treating the neurogenic diseases. However, traditional approaches cannot efficiently replace microglia at the adulthood. Here, we introduce a potentially clinical-feasible strategy named microglia replacement by bone marrow transplantation that achieves efficient microglia replacement at the whole CNS scale, including the brain, spinal cord, and retina in adult mice.

For complete details on the use and execution of this protocol, please refer to Xu et al. (2020). The original abbreviation of this microglia replacement strategy is mrBMT. We hereby change the name to Mr BMT.

BEFORE YOU BEGIN

Prepare the PLX5622-formulated AIN-76A chow diet

Prepare the diet following the below recipe: 1.2 g PLX5622 per kg AIN-76A diet. The PLX5622 was formulated by SYSE Bio. One adult mouse (25 g) consumes about 3.5 g chow diet per day.

Stored at −20°C until use. The PLX5622-formulated diet is valid for at least one year.

Prepare CX3CR1+/GFP mice

Cross CX3CR1+/GFP with C57BL/6J to obtain the CX3CR1+/GFP mice.

Prepare acid water

Prepare 2 L drink water and adjust pH to 2 to 3 by 0.1 M HCl. Autoclave the acidified water. After the water is cooled to room temperature, add 2.2 g neomycin and thoroughly mix the solution. Store the acid water at 4°C before use. The acid water is valid for at most half a year.
The overall procedure is outlined in Figure 1.

### Microglial depletion

**Timing:** 14 days

This procedure achieves efficient microglial depletion (usually > 99% in the brain cortex and 100% in the retina) in the CNS of the adult recipient mouse with the CSF1R inhibitor PLX5622 (Huang et al., 2018a; Huang et al., 2018b; Xu et al., 2020). We usually used 1.5- to 4-month-old mice as the recipient in our experiments. Both males and females are applicable. Notably, the donor cell should come from the gender-matched animal.

1. Feed the C57BL/6J recipient mouse with PLX5622-formulated chow diet *ad libitum* from D0 to D14.
2. Refill the PLX5622-formulated chow every three days.
3. Before the lethal irradiation, treat the mouse with neomycin (1.1 g/L) containing acid water (pH 2 to 3) *ad libitum* from D11 to D28. The acid water prevents most bacterial contaminations (Duran-Struuck and Dysko, 2009).

⚠️ CRITICAL: According to our experience, at the specific pathogen free (SPF) animal facility, more than 95% of microglia in the brain cortex and 100% of retinal microglia in the outer plexiform layer (OPL) are able to be ablated at day 7 of PLX5622 administration. At day 14, more than 99% of brain microglia in the cortex and all retinal microglia are ablated (Huang et al., 2018a; Huang et al., 2018b). In contrast, microglial depletion efficiency will be dampened in a non-SFP animal facility. The efficiency of microglial depletion is critical for the replacement efficiency. We strongly recommend a 14-day microglial depletion.

⚠️ CRITICAL: The animals should be kept at a specific pathogen-free (SPF) animal facility. Otherwise, the microglial depletion efficiency is relatively low in the “dirty environment”, which will influence the replacement efficiency.
**Conditioning of the recipient mouse by X-ray lethal irradiation**

**Timing:** 4 h

4. Change the PLX5622-formulated chow with sterilized AIN-76A control diet (CD) 2–3 h before the lethal irradiation at D14.

5. Cut a piece of sterilized foam plastic pad that can fit into an open mouse cage. Put the recipient mice in an open cage. Put the foam plastic piece over the cage and press down so that the mice will only be able to crawl on the floor of the cage (Figure 2).

6. Set the irradiation dosage to 9 Gy on the X-ray irradiator (Radsource, RS-2000). Put the mouse cage in the irradiator and initiate irradiation.

**Note:** The irradiation duration depends on the power of the irradiator. Typically, the irradiation time is 5–10 min. Usually, no more than 10 mice are irradiated at one time.

**Δ CRITICAL:** Please make sure the irradiation is X-ray. We found that the replacement efficiency by Gamma-ray is quite low.

7. After the irradiation, remove the covering foam plastic piece from the cage. Feed the irradiated mouse with sterilized CD and neomycin containing acid water (1.1 g/L, pH 2 to 3) *ad libitum*.

**Bone marrow transplantation**

**Timing:** 1 h (one donor and five recipients)

This procedure applies the bone marrow transplantation to the microglia-depleted mouse.

8. Isolation of bone marrow cells from the gender-matched donor CX3CR1⁺/GFP mouse. We usually used 1.5- to 4-month-old mice as the donor in our experiments.
   a. Prepare ice cold 1× PBS in three 35 mm Petri dishes. Put a 100 μm cell strainer into a 50 mL centrifuge tube. Prewet the strainer with PBS in advance.
   b. Deeply anesthetize the CX3CR1⁺/GFP donor mouse with ketamine (100 mg/kg, dissolved in saline) and xylazine (10 mg/kg, dissolved in saline) by intraperitoneal injection. Dislocate the cervical spine of the donor mouse after loss of the toe reflex.
   c. Isolate the femurs, tibias and humerus (Figure 3A). Clean up soft tissues from the bones with Kim Wipes. Put the cleaned bones in one of the dishes containing ice cold PBS. Wash the bones three times to remove attached soft tissue and furs.
   d. Cut off both ends of the bones (Figure 3B). Fill in a 5 mL syringe up with ice cold PBS with a 21 G needle. After removal of trapped bubbles, insert the needle into one end of the bone (Figure 3C). Flush out the bone marrow into the cell strainer (Figure 3D).
e. Repeat Step 8d until all bones are processed. Next, triturate the bone marrow piece with 1 mL pipette for 10 times. Pour the triturated bone marrow cells into the cell strainer.

f. Grind the remaining bone marrow against the strainer with the plunger from the syringe until no obvious pieces of bone marrow can be found (Figure 4). Wash the strainer with 2 mL ice cold PBS.

g. Centrifuge at 4°C 300 g for 5 min. Red cell pellets containing bone marrow cells and red blood cells will be at the bottom of the centrifuge tube (Figure 5A). Discard the supernatant carefully.

h. Remove the red blood cells by adding 1 mL ACK lysis buffer at room temperature (approximately 20°C–25°C) for 1 min. Then, add 9 mL ice cold PBS to the cell suspension and mix gently.

i. Centrifuge at 4°C 300 g for 5 min. White cell pellets containing bone marrow cells will appear on the bottom of the centrifuge tube (Figure 5B).
j. Discard the supernatant. Resuspend the pellets with appropriate amount of PBS (550 μL per donor mice).

k. Put the cell suspension on ice. Count the cell number using hemacytometers as follows:
   i. Add 10 μL of the cell suspension to the hemocytometer.
   ii. Count the total cell numbers in the central grid and grids of the four corners. Calculate the average cell number.
   iii. Multiply the average cell number by 10⁴ to estimate the number of cells per microliter.

**Note:** Typically, about 5 x 10⁷ bone marrow cells are able to be collected from one donor mouse. To achieve efficient replacement, at least 1 x 10⁷ cells in 100 μL PBS are recommended to be transplanted into one recipient. Thus, bone marrow cells harvested from one donor mouse can be transplanted to up to 5 recipients. If one recipient is injected with 100 μL donor cells, then the maximum amount of PBS reconstituting the donor cell pellet from one mouse is about 550 μL (50 μL for aspiration error).
9. Bone marrow transplantation
   a. Place the C57BL/6J recipient mouse into the restraining tube carefully. Insert face-mask plug into the restraining tube so that the mouse cannot move but can still breathe easily. Tighten the screw on the plug (Methods video S1).
   
   △ CRITICAL: Tightly restrain the recipient mouse avoiding suppressing its breath (Figure 6).

   b. Aspire 100 μL donor cells into a 29 G syringe without trapped bubbles.
   
   c. Straighten the tail of the mouse; locate the tail vein about 2 cm from tail tips as the injection site with the help of an orange LED light placed beneath the tail (Figure 7; Methods video S2).

   Note: There are three veins in the tail. Choose the most accessible one. We usually choose the one of the side tail veins.
   
   d. Insert half of the needle bevel into the injection site with an angle of about 30° to the tail, then parallelly insert about 1 mm of the needle into the tail vein (Methods video S2).

   Note: The tail vein is “enlarged” after insertion of the needle with the help of LED light illumination.
   
   e. Slowly inject the cells into the vein. Press the injection site and withdraw the needle. Keep pressing the injection site while releasing the mouse from the restrainer with the mouse tail still in the operator’s hand. After releasing the mice, press the injection site for another 20 s (Methods video S2).

   See Troubleshooting 3.
f. Feed the transplanted mouse with sterilized CD for recovery of 30 days ad libitum. Meanwhile, the mouse is treated by neomycin containing acid water (1.1 g/L, pH 2 to 3) for the first 2–3 weeks after transplantation. Then, the endogenous microglia are replaced by CX3CR1GFP/+ Mr BMT cells.

Note: We usually conducted the bone marrow cell transplantation within 3 hours post irradiation.

Note: Though we did not systematically test the recovery day shorter than 30 days, the replacement was not sufficient at day 21 post transplantation as we observed from one signal case.

EXPECTED OUTCOMES
At D44, the majority of IBA1-positive microglia-like cells are GFP-positive by Mr BMT of X-ray irradiation (typically > 95%) (Figure 8A). In contrast, the Gamma-ray irradiation results in a low replacement efficiency (Figure 8B). The bone marrow cell-derived microglia-like Mr BMT cells are GFP-positive while the endogenous microglia are GFP-negative.

LIMITATIONS
The replaced microglia-like cells by Mr BMT (Mr BMT cells) exhibit a macrophage-like phenotype with the less complexed morphology and macrophage-like gene profiles (Xu et al., 2020). To obtain a more microglia-like phenotype, please refer to the strategy of microglia replacement by microglia replacement (Mr MT, previous name mrMT) (Xu et al., 2020) (to editor: please also cite the Mr MT STAR Protocols paper STAR-PROTOCOLS-D-21-00164 we submitted simultaneously).

The donor cell of Mr BMT is bone marrow cells. The limited donor source availability may limit the usage in real clinical practices. To solve this problem, we also developed microglia replacement by peripheral blood (Mr PB, previous name mrPB) that achieves efficient microglial replacement at the CNS-scale by peripheral blood cells (Xu et al., 2020) (to editor: please also cite the Mr PB STAR Protocols paper STAR-PROTOCOLS-D-21-00165 we submitted simultaneously).

Mr BMT achieves efficient microglia replacement at the whole-CNS scale. It cannot replace microglia at specific brain regions. Mr MT can achieve microglia replacement at specific brain regions of interest (Xu et al., 2020) (to editor: please also cite the Mr MT STAR Protocols paper STAR-PROTOCOLS-D-21-00164 we submitted simultaneously).

The microglia replacement is largely dependent on the microglia-free niche. In our hand, if the microglial depletion is not sufficient, the replacement efficiency could be low. Since the microglia...
are immune cells and sensitive to the microenvironment. An SPF animal facility is recommended. Otherwise, the microglial depletion and subsequent replacement efficiencies could be low.

**TROUBLESHOOTING**

**Problem 1**  
Low efficiency of microglia depletion (step 1–3).

**Potential solution**  
In our experience, an SPF environment and chemically defined feeding chows are crucial for efficient depletion of microglia. Also, change and refill the PLX5622 chow every 3 days in case the drug decomposes at the room temperature. Following these rules, the efficiency of microglia depletion can achieve as high as 99% after 14 days of PLX5622 treatment.

**Problem 2**  
Low yield of bone marrow cells (step 8).

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**Figure 6.** Restraing mouse in the restraining tube for tail vein injection  
The recipient mouse must be tightly restrained (A), but can still breathe (B).

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**Figure 7.** Using a mini-LED to help locate the tail vein.
Potential solution
Keep flushing the bones with PBS till the bones' color turned from red to white. If no PBS is seen flowing from the tips of bones, change the syringe needle in case that it is clogged. After triturating bone marrow with 1 mL pipette tips, grind the large pieces of remaining bone marrow against a cell strainer till all pieces disappear.

Problem 3
Failure of intravenous injection (step 9a-e).

Potential solution
Using an injection cone equipped with LED light and a magnifier, it was relatively easy to locate the tail vein. Still, in order to achieve reliable success rate of intravenous injection via tail vein, it is inevitable to practice on at least 20 mice. Start injection from a site that is about ~2 cm from the tail tip; if the injection fails, move toward the proximal end of the tail and start a new injection. Keep in mind that the tail vein was never greater than 0.5 mm deep beneath the tail skin, so only the bevel tip of the injection needle was needed to insert into the tissue. We find that warming the tail was not necessary for successful injection.

Problem 4
High death rate of mice after irradiation (step 4–7).

Potential solution
Ensure that donor bone marrow cells were successfully injected via tail vein. Also, during the first weeks post irradiation, give the mice acidified drink water (pH 2–3) containing antibiotics.

Problem 5
Low efficiency of microglia replacement (step 9f).

Potential solution
Ensure that the irradiation source was X-rayed based. We found that Gamma-ray irradiation generated a low replacement efficiency. Also ensure that at least 10^7 donor bone marrow cells are injected.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bo Peng (bopeng@connect.hku.hk).
Materials availability
This study did not generate new unique reagents.

Data and code availability
The data that support the findings of this study are available from the corresponding author Bo Peng at Fudan University for reasonable request.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100666.

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AUTHOR CONTRIBUTIONS
B.P. and Y.R. conceived, designed, and conceptualized this study. B.P. supervised this study. Z.X., B.P., and Y.R. wrote the manuscript. Z.X. performed most of experiments. X.Z. performed Mr BMT by using gamma ray. B.P. and Y.R. contributed to the interpretation of results. All authors discussed results and commented on the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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