Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein–protein interaction module

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Nanoparticle-mediated delivery of functional macromolecules is a promising method for treating a variety of human diseases. Among nanoparticles, cell-derived exosomes have recently been highlighted as a new therapeutic strategy for the \textit{in vivo} delivery of nucleotides and chemical drugs. Here we describe a new tool for intracellular delivery of target proteins, named ‘exosomes for protein loading via optically reversible protein–protein interactions’ (EXPLORs). By integrating a reversible protein–protein interaction module controlled by blue light with the endogenous process of exosome biogenesis, we are able to successfully load cargo proteins into newly generated exosomes. Treatment with protein-loaded EXPLORs is shown to significantly increase intracellular levels of cargo proteins and their function in recipient cells \textit{in vitro} and \textit{in vivo}. These results clearly indicate the potential of EXPLORs as a mechanism for the efficient intracellular transfer of protein-based therapeutics into recipient cells and tissues.
Despite the long list of therapeutic proteins available for treating various human diseases, the vast majority of clinically available protein-based drugs, such as cytokines, hormones and monoclonal antibodies, have been limited to extracellular mechanisms of action. Intracellular proteins have been identified for their potential as biopharmaceutical drugs; however, many of the challenges associated with intracellular protein delivery have yet to be solved1. Protein transduction methods have been proposed to deliver recombinant proteins into target cells in vitro and in vivo2–4. Although the techniques are promising, exposure of the protein surface in solution and the low refolding rate of recombinant proteins in target cells remain important obstacles for the intracellular delivery of therapeutic proteins5. Several lipid nanoparticle-mediated protein delivery methods have been postulated, as has protein encapsulation for protecting the proteins6,7. However, the absence of a separation mechanism between cargo proteins and lipid nanoparticles not only limits the efficiency of cytosolic delivery, preparation of these particles often involves complicated protein purification steps.

To address these limitations, we developed an optogenetically engineered exosome system EXPLORs (exosomes for protein loading via optically reversible protein–protein interactions) that can deliver soluble proteins into the cytosol via controlled, reversible protein–protein interactions (PPIs). Exosomes are natural cell-derived extracellular vesicles that originate from internal endocytic compartments and multi-vesicular bodies and participate in intercellular communication8. Recent studies have sought to use exosomes as a new method for the in vivo delivery of siRNA or miRNA to specific target tissues by systemic injection9–11. These methods were based on the passive loading of siRNAs or miRNAs into isolated exosomes by electrophoresis, a method poorly suited for loading of macromolecular proteins. Here we propose a novel protein-loading method in which cargo proteins can be actively loaded into exosomes through endogenous biogenesis processes, allowing for efficient delivery into the cytosol of target cells through controllable, reversible detachment from the exosomes.

To achieve the controllable, reversible loading and delivery of target proteins into the exosome, we selected a photoreceptor cryptochrome 2 (CRY2), and CRY-interacting basic-helix-loop-helix 1 (CIB1) protein module, originally identified in Arabidopsis thaliana, which regulates floral initiation via blue light-dependent phosphorylation12–14. We induced a transient docking of CIBN (a truncated version of CIB1)14 conjugated CRY2-conjugated cargo proteins to the exosomes by phosphorylation12–14. We induced a transient docking of CIBN-conjugated enhanced green fluorescent protein (EGFP)-tagged CD9 (CIBN-EGFP-CD9) and mCherry-tagged CRY2 (mCherry-CRY2). A single pulse irradiation of a 488-nm laser induced rapid movement of mCherry-CRY2 from the cytosolic compartment to the plasma membrane and intracellular compartments where the CIBN-EGFP-CD9 proteins were co-localized (Fig. 1a and Supplementary Fig. 1a). Similar results were obtained by the introduction of another photoreceptor module GIGANTEA, and LOV (light-, oxygen- and voltage-sensitive domain) proteins15 and other exosome-enriched tetraspanin proteins, such as CD63, CD81 and CD82 (Supplementary Fig. 1b,c). We could also observe light-induced co-localization of mCherry-CRY2 and CIBN-EGFP-CD9 by a cryo-immunogold electron microscopy analysis (Supplementary Fig. 2). Introduction of a mutant CRY2, mutated at an amino acid important in the photolyase function (D387A)16, abrogated such light-induced co-localization of CIBN-EGFP-CD9 and mCherry-CRY2 (Supplementary Fig. 3). On termination of light stimulation, the association between CIBN-EGFP-CD9 and mCherry-CRY2 dissociated gradually within 10 min, as described previously14 (Fig. 1d,e).

Light-dependent loading of proteins in EXPLORs. For production of mCherry:EXPLORs, we exposed transiently transfected HEK293T cells with continuous blue light illumination by installing a 460-nm light-emitting diode (LED; maximum power; 380 μW cm⁻²) in a CO₂ incubator. After 48-h incubation with blue light illumination, EXPLORs were isolated from the supernatants by several different methods and confirmed for the hydrodynamic size by dynamic light scattering (Supplementary Fig. 4 and Supplementary Table 1). Immunoblotting results demonstrated that EXPLORs derived from optogenetically engineered HEK293T cells grown under blue light illumination contained significantly higher amounts of mCherry-CRY2 protein (84 kDa) than those obtained from cells grown in the dark (Fig. 2a and Supplementary Fig. 5). In particular, the highest efficiency in protein loading was seen in the power range from 20 to 50 μW cm⁻² (Fig. 2b). The threshold power of the light-induced PPI was measured at ~15 μW cm⁻² by quantified laser stimulation (Supplementary Fig. 6) and at ~20 μW cm⁻² by the LED light (Supplementary Fig. 7). Continuous irradiation with blue light for up to 48 h did not induce significant cell death (Supplementary Fig. 8), and we further confirmed that EXPLORs did not contain detectable DNA fragments typical of apoptotic bodies (Supplementary Fig. 9a). Because genetically modified cells may release exosomes packed with not only the encoded protein but also genetic material (pDNA and mRNA) encoding this protein17, we further examined EXPLORs containing undetectable amounts of plasmid DNA (data not shown) and mCherry-CRY2 mRNA (Supplementary Fig. 9b). In addition, we tested the loading efficiency of mCherry proteins in EXPLORs in response to cycles of blue light illumination and darkness. Using a variety of cycle combinations, we identified a cycle consisting of 1 min of blue light illumination followed by 1 min of darkness as the optimal condition to produce mCherry:EXPLORs (Supplementary Fig. 10).

We next examined the loading capacity of EXPLOR technology by producing firefly luciferase-containing EXPLORs. For the production of luciferase:EXPLOR, we cloned vectors for firefly luciferase-conjugated mCherry-CRY2 and confirmed light-induced PPI with CIBN-EGFP-CD9 by blue light stimulation with confocal microscopy (Supplementary Fig. 11). First, we compared luciferase:EXPLORs with a commercialized method for exosome protein loading, XPACK (System Biosciences, Mountain View, CA, USA), which is based on the principle that highly oligomeric, cytoplasmic proteins can be targeted to exosomes by plasma membrane anchors18,19. We also included exosomes produced from cells transfected with the luciferase-conjugated mCherry-CRY2 vector, but not with the CIBN-EGFP-CD9 expression plasmid, to exclude the possibility of nonspecific loading of overexpressed cargo proteins into exosomes. Transfection efficiency and expression profile of luciferase-mCherry fusion proteins were comparable between the different methods (Fig. 3a,b and Supplementary Fig. 12). For the quantitative analysis of cargo protein loading into the...
exosomes, we measured luciferase activity using the same numbers of isolated exosomes (Fig. 3c,d). The results showed that the loading capacity of EXPLORs was significantly higher than those of the overexpression model of luciferase-mCherry-CRY2 and a commercialized model for exosome protein targeting. In our experiment, it was estimated that, on average, 1.4 molecules of luciferase proteins were included in each exosome obtained by EXPLOR technology (Supplementary Fig. 13). We also tested another method: ex vitro protein loading in naive exosomes loaded with recombinant luciferases. In total, 50 μg of recombinant luciferases (61 kDa) dissolved in phosphate-buffered saline (PBS) was added to 2 × 10^10 exosomes derived from HEK293T cells and loaded into exosomes ex vitro with an extrusion method. Only 4.4 pg of recombinant protein
was finally included in the exosomes; thus, the loading capacity of the ex vitro protein-loading method was estimated to be 40-fold lower than that of the EXPLOR technology (Supplementary Fig. 14).

**Administration of EXPLORs in vitro and in vivo.** HeLa cells were incubated in the absence or presence of mCherry-loaded exosomes for 24 h, and tested for analysis with EXPLOR-mediated protein delivery to recipient cells (Fig. 4a). Consistent with previous results, EXPLOR-treated cells showed significantly higher fluorescence signals (Fig. 4b). Cryo-immunogold electron microscopy analysis (Supplementary Fig. 15a) and confocal microscopy (Supplementary Fig. 15b) revealed that mCherry proteins were clearly transferred into recipient cells after incubation with mCherry:EXPLORs derived under the light ON condition. Immunoblot analysis demonstrated significant increases in the amounts of intracellular mCherry proteins in recipient cells after treatment with mCherry:EXPLORs in both a time- and dose-dependent manner (Supplementary Fig. 15c,d).

To further confirm whether EXPLOR technology can be useful for the delivery of functional proteins, we cloned vectors for mCherry-CRY2 proteins fused with Bax and super-repressor IkB (srIkB) proteins (Supplementary Fig. 16). In the normal condition, Bax proteins are mainly distributed in the cytosol; however, on initiation of the apoptotic process, they undergo a conformation change and move to the mitochondrial membrane, leading to the release of cytochrome c that subsequently triggers caspase-dependent apoptosis. As expected, incubation with Bax-mCherry:EXPLORs induced a rapid release of cytochrome c from the mitochondria in HeLa cells (Fig. 4c,d). Similarly, the endogenous nuclear factor-κB (NF-κB) inhibitor IkB proteins bind to the NF-κB complex in the cytoplasm; however, on relevant stimulation, IkB is phosphorylated by IkB kinases and degraded by the ubiquitin–proteasome pathway. Then, the NF-κB complexes translocate to the nuclei and induce the expression of survival- and inflammation-related genes. Super-repressor IkB, a S32A and S36A mutant form of IkB, is not phosphorylated by IkB kinases, so that srIkB suppresses the translocation of NF-κB complex even in the presence of pro-inflammatory stimulation. As expected, treatment with srIkB-mCherry:EXPLORs significantly reduced tumour necrosis factor-α-induced translocation of the p65 subunit of NF-κB in HeLa cells (Fig. 4e,f).

Finally, we validated the potential of EXPLOR-mediated intracellular protein delivery for in vivo application. To test the intracellular delivery of Cre recombinase containing EXPLORs...
(Cre:EXPLORS), we cloned a vector for Cre-conjugated mCherry-CRY2 (Cre-mCherry-CRY2) protein. Unlike mCherry-CRY2 protein, Cre-mCherry-CRY2 proteins were predominantly expressed in the nuclei, and blue light illumination induced limited translocation of these proteins to the plasma membrane and intracellular endosomal fractions (Supplementary Fig. 17a). To avoid unwanted nuclear localization and increase the loading efficiency of Cre-mCherry-CRY2 proteins, cells were maintained under blue light illumination immediately after transfection. As expected, continuous light illumination immediately after transfection markedly enhanced co-localization of Cre-mCherry-CRY2 proteins with CIBN-EGFP-CD9 (Supplementary Fig. 17b). The enzymatic activity of Cre recombinase was validated in the target cells previously transfected with a reporter plasmid containing a loxp-STOP-loxp sequence upstream of the ZsGreen-encoding gene. Treatment with Cre:EXPLORS clearly induced green fluorescence at levels comparable to that of transfection of pCMV-Cre vectors in various cells including rat embryonic primary neurons (Supplementary Fig. 18). Because the transient transfection of primary neurons is extremely inefficient, we next tested the effect of Cre:EXPLORS on neurosphere-derived differentiated neuronal cells isolated from transgenic mice having a loxp-P-STOP-loxp-EGFP gene. As expected, treatment with Cre:EXPLORS induced EGFP expression in over 95% of cells; while treatment with naive EXPLORS had no effect (Fig. 5a,b). Next, we injected Cre:EXPLORS into the ventrolateral part of the brain of pCAG-loxp-STOP-loxp-eNpHR3.0-EYFP (enhanced yellow fluorescence protein) transgenic mice (Fig. 5c). After 96 h, eNpHR3.0 EYFP proteins were expressed widely near the ventrolateral part in vivo, including low purification efficiency, failure to separate from nanoparticles in recipient cells and induction of immune responses against host immune cells. To address these limitations, we developed an exosome-based delivery system EXPLORS that can be readily produced using live cells as a factory via the endogenous biogenesis of extracellular vesicles (Fig. 6).

This novel method provides significant advantages for both research and clinical applications. First, EXPLORS can carry cargo proteins without isolation or purification of recombinant proteins. Because EXPLORS contain cell-produced cargo proteins in unbound soluble form, this approach eliminates many of the issues such as misfolding of recombinant proteins or separation...
of nanoparticles and cargo proteins in the target cells. Second, EXPLORs can be readily and easily produced once the appropriate EXPLOR-producing donor cells have been selected from genetically engineered cells. By simply irradiating with a blue light, we can generate EXPLORs loaded with specific cargo proteins, including those derived from other cell types. This stable production of EXPLORs from genetically engineered cells has great potential for the commercialization of standardized exosome-based protein therapeutics. Finally, EXPLORs are compatible with many personalized medicine-based therapeutic approaches. By modifying patient-derived cells to produce EXPLORs28, we would be able to manufacture patient-customized drug delivery systems with reduced risk of triggering host immune responses, opening a new paradigm for personalized protein-based therapeutics.

In the present study, we showed that exosomes from cells transiently transfected with a luciferase-mCherry-CRY2 vector rarely had luciferase molecules; however, EXPLORs that were derived from cells transfected with luciferase-mCherry-CRY2 and CIBN-EGFP-CD9 vectors under the light OFF condition had considerable numbers of luciferase molecules. The difference between the two conditions was CIBN-EGFP-CD9 expression. Recently, one research group showed that overexpressed CRY2-conjugated proteins have a weak binding activity with CIBN-conjugated proteins in the normal state29. Thus, we suggest that unintended binding of mCherry-CRY2 to CIBN-EGFP-CD9 could be partially responsible for loading of mCherry proteins into exosomes under the light OFF condition.

EXPLORs had the highest efficiency in protein encapsulation under blue light illumination in the power range of 20–50 μW cm⁻², not at the strongest power (200 μW cm⁻²). We found that incubation of cells under blue light illumination at a power of 200 μW cm⁻² did not induce significant cell death; thus, cell death was not the cause of decreased protein loading efficiency according to strong light. We presumed that aggregation of CRY2 proteins by strong blue light might disturb the light-induced PPI between mCherry-CRY2 and CIBN-EGFP-CD9 proteins16. To address this, we used CRY2 fusion proteins conjugated with Cre recombinase. Unlike other CRY2 fusion proteins conjugated with cytotoxic proteins, Cre-mCherry-CRY2 proteins were expressed mainly in the nuclei. Because aggregated forms of Cre-CRY2 proteins cannot diffuse readily through nuclear membranes, we can readily study the effect of light intensity on the aggregation of CRY2 proteins by simply analyzing the subcellular localization of Cre-mCherry-CRY2 proteins. As expected, we observed that the stronger the blue light illuminated, the more Cre-mCherry-CRY2 stayed in the cytosolic compartment, rather than inside the nucleus, confirming that CRY2-fusion proteins can form aggregates under high-intensity light conditions (Supplementary Fig. 20).
We found that one or two cargo molecules were contained in one particle of EXPLOR on average; however, this limited loading efficiency might still have room for improvement. In the present study, we used fusion proteins of fluorescence reporters with both the CIBN and CRY2 protein for better tracking of target proteins. Because we have confirmed that target proteins conjugated with CRY2 can be successfully recruited to engineered exosomes, direct conjugation of cargo proteins or tetraspanins to CRY2 or CIBN proteins without fluorescent reporter proteins can further decrease the molecular size of cargo proteins and subsequently improve loading efficiency of cargo proteins into EXPLORs. In addition, the current strategy using two vectors can further be optimized by using a one-vector system, by introducing an internal ribosome entry site. These approaches might increase the transfection efficiency, as well as the loading of cargo proteins into EXPLORs.

In the present study, we developed a novel protein carrier EXPLOR that has a higher loading capacity and delivery efficiency than previous methods for protein-loaded exosomes. Also, we demonstrated the intracellular delivery of mCherry, Bax, super-repressor IκB protein and Cre enzyme as functional proteins into the target cells in vitro and into brain parenchymal cells in vivo. Many intracellular proteins, such as transcription factors, signal transducers and enzymes can also be attractive targets for EXPLOR-based therapeutics. Using our EXPLOR technique, we might be able to design various EXPLOR-producing cells that would be equivalent to monoclonal antibody-producing hybridoma cells for protein-based therapeutics targeting intracellular events.

**Methods**

**Cell culture.** HEK293T human embryonic kidney cells (CRL-3216, ATCC, Manassas, VA, USA), HT1080 cells (CCL-121, ATCC) and HeLa cells (CCL-2, ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Welgene, Seoul, Korea) containing 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) and 1% penicillin–streptomycin (Gibco).

**Reagents.** Primary antibody sources were as follows: mouse monoclonal mCherry antibody (diluted 1:1,000 for western blotting, 1:50 for EM), rabbit monoclonal CD9 antibody (diluted 1:1000 for immunocytochemistry, 1:2000 for Western blotting), rabbit anti-GAPDH antibody (diluted 1:1000 for western blotting, 1:1000 for immunocytochemistry), goat anti-CD63 antibody (diluted 1:1000 for western blotting, 1:500 for immunochemistry), mouse secondary antibody (sc-2005) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); cytochrome c antibody (diluted 1:1000 for immunocytochemistry, 1:56432) was from BD biosciences (San Jose, CA, USA); and the GFP antibody (diluted 1:1000 for western blotting, 2555) was from Cell Signaling Technology (Beverly, MA, USA). Secondary antibody sources were as follows: anti-rabbit (sc-2004) and mouse secondary antibody (sc-2005) were obtained from Santa Cruz Biotechnology, and Alexa Fluor 488 goat anti-mouse IgG (H + L) antibody (diluted 1:1000 for ICC, A-21235) and Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibody (diluted 1:1000 for ICC, A-11088) were purchased from Thermo Fisher Scientific (Wilmington, DE, USA).

**Plasmids and transfection.** All plasmids and PCR primers used in this study are listed in Supplementary Table 2. pcMV-CIBN-EGFP and pcMV-mCherry-CRY2 (codon-optimized for expression in mammalian cells) were generated by gene-specific primers. The CIBN-EGFP and CIBN-mCherry vectors were ligated into an EcoRI site of XPACK CMV-XP-MCS-EFI-Puro Cloning Lentivector (System Biosciences, Mountain View, CA, USA). To assemble pcMV-Cre-mCherry-CRY2, genes encoding Cre were PCR-amplified.
from the pCMV-Cre vector (Addgene plasmid 11916) using gene-specific primers. For the construct, the first fragment (Cre-) contained an EcoRI site, a sequence encoding a 23-base length Cre stop codon, a flexible linker (SAGGPPVAT). The second PCR fragment (mCherry-CRT2) contained the flexible linker, a sequence encoding full-length mCherry-CRT2 and a XhoI site, followed by 15 base pairs with homology to the pcDNA3.1 (+) vector. A pCAG-loxP-Dloxp-ZsGreen plasmid was obtained from Addgene (plasmid 51269). The construct was amplified from the L12215 cDNA library, super-repressor iXb was obtained from Addgene (plasmid 15294) and firefly luciferase gene was obtained from the pGL4.11[luc2P] vector (Promega, Madison, WI, USA). Cells were transfected with Effectene Transfection Reagent (Quagen, Valencia, CA, USA) at a density of 50–80% confluence, according to the manufacturer’s protocol.

**Microscopy for cell imaging.** Live cells were imaged using a Zeiss LSM 510 confocal microscope with a 1.4×0 1.0 numerical aperture (NA) objective (Carl Zeiss, Thornwood, NY, USA). Laser light (488 nm) was used to induce dimerization between CRY2PHR and CIBN and to image GFP fusion proteins; a 594 nm laser was used to image mCherry-fusion proteins. Z-stack and time-lapse imaging were also performed using the confocal microscope, and the three-dimensional reconstruction and real-time images were processed using ImageJ software. An environmental chamber (Harvard Apparatus, South Natick, MA, USA) enclosing the microscope stand was used to maintain the temperature at 37 °C with 5% CO2 during live cell imaging. For fluorescence microscope imaging, cells were fixed with 4% paraformaldehyde at 30 min and imaged using a Zeiss Axiosvert 200M fluorescence imaging microscope with > 10 Plan-NeoFluar 0.3 NA objective and a > 20 × Plan-NeoFluar 0.5 NA objective (Carl Zeiss). The quantification of cell fluorescence was analysed using the CellProfiler software (Broad Institute, Cambridge, MA, USA).

**Exosome isolation.** Cells were collected and transfected to fresh PBS-free DMEM (Welage) containing 1% penicillin–streptomycin (Gibco) or DMEM containing 1% penicillin–streptomycin and 10% exosome-depleted FBS. After 48 h, medium was collected and centrifuged at 3,000g for 15 min to remove cellular debris. Exosome isolation was performed by precipitation or ultracentrifugation. For precipitation, the supernatant was transferred to a sterile vial and the appropriate volume of ExoQuick-TC Exosome Precipitation Solution (System Biosciences) was added. Samples were mixed by inverting or flicking the tube and then refrigerated overnight. The ExoQuick-TC was centrifuged (10,000g) at 4 °C. After centrifugation, the supernatant was removed and the mixture was spun down again (1,500g). All traces of fluid were removed by aspiration, and the exosome pellet was resuspended in 100 μl of PBS by passing 5–10 times through a sterile 27-gauge needle (BD Biosciences, San Jose, CA, USA). Then, the suspended exosomes were filtered through a syringe filter (0.2 μm; Sarotis, Goettingen, Germany) to prevent aggregation of exosomes and to remove ExoQuick polymers. For ultracentrifugation-based purification of exosomes, supernatants were transferred to an appropriate vial for the MF-600 ultracentrifuge (Hanbel Scientific, Industrial, Incheon, Korea) and centrifuged (120,000g, 2 h). After centrifugation, all traces of fluid were removed by aspiration, and the exosome pellet was resuspended in 100 μl of PBS by passing 5–10 times through a sterile 27-gauge needle (BD Biosciences). The suspended exosomes were then filtered through a syringe filter (0.2 μm, Sarotis). The concentration of suspended exosomes was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The number of exosomes was measured using an enhanced chemiluminescence system (Ab Frontier, Seoul, Korea). For measurement of luciferase activity, a 594-nm laser was used to image mCherry-fusion proteins. A Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was used to measure the luciferase activity.

**Immunoblotting.** Collected cells or isolated exosomes were washed in ice-cold PBS, and suspended in adjusted volumes of buffer provided with Reporter Lysis Buffer (Promega). A total volume of 20 μl of samples was loaded in a black 96-well plate (SPL Life Sciences, Pocheon, Korea), and 100 μl of Luciferase Assay Reagent (Promega)-reconstituted substrate was added to each reaction manually. Measurements were performed using a Berthold LB942 TriStar® multidection microplate reader (Berthold, Bad Wildbad, Germany). The integration time was set at 1 s for all measurements. To perform a quantitative luciferase assay, Quantilum Recombinant Luciferase (Promega) was used to obtain a standard curve. On the basis of the standard curve, a fitting curve and the number of luciferase molecules were calculated.

**Measurement of DNA-binding activity of NF-κB.** Nuclear extracts (5 μg) were assayed for the binding activity of p65/C-Rel (NF-κB) using the TransAM NF-κB assay kit (ActiveMotif, Carlsbad, CA, USA) according to the manufacturer’s manual.

**Culture of differentiated neurosphere-derived cells.** The cerebral cortical tissues from E12.5 Rosa-EGFP reporter mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were dissected from the brain. Isolated tissues were incubated with Accutase (Innovative Cell Technologies, San Diego, CA, USA) for 5 min at 37 °C. Cells were grown on an ultrawell attachment surface and maintained as neurospheres for 4 days in the presence of basic fibroblast growth factor (20 ng ml−1; R&D systems, Minneapolis, MN, USA) and epidermal growth factor (20 ng ml−1; Invitrogen) in DMEM/F12 (Gibco) media containing 1% N2 (Gibco), 2% B27 (Gibco) supplements, and 1% penicillin–streptomycin (Gibco). For differentiation, neurospheres were dissociated and cultured on 18-mm cover glass coated with poly-L-lysine (100 μg ml−1) and laminin (5 mg ml−1) with removal of epidermal growth factor/basic fibroblast growth factor. On the seventh day of differentiation, purified exosomes were treated to the cells and maintained the culture for an additional 3 days. For immunocytochemistry, cells were fixed with 4% paraformaldehyde, stained with anti-NFAT (diluted 1:1,000; Sigma–Aldrich, St. Louis, MO, USA, T2200), anti-GFP (diluted 1:1,000, Abcam, ab290) and counterstained with Hoechst 33342 (Invitrogen). Images were obtained using an EVOS FL cell imaging system (Invitrogen) with a EVOS AMEP 4624 (Plan Fluor × 20/0.45) objective lens.

**Animals.** Animal care and handling were performed according to the guidelines of the Animal Care and Use Committee of the Korea Advanced Institute of Science and Technology (KAIST, Daejeon, Korea). LoxP-stop-loxP-NeoH3.0-EYFP transgenic mice (The Jackson Laboratory) were generated by mating heterozygous transgenic and wild-type mice (Males, 22 weeks, C57BL/6j background) in a specific-pathogen-free environment. All mice were maintained on a 12/12-h light/dark cycle (light cycle beginning at 6:00) at a temperature of 23 °C. Food and water were supplied ad libitum.

**Stereotaxic injection of EXPLORs in mouse brain.** All mice were anaesthetized with avertin (20 mg ml−1 of tribromoethanol, 20 μl g−1 intraperitoneally, Sigma–Aldrich, Milwaukee, WI, USA) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). All injections were delivered at a rate of 0.3–0.8 μl min−1 via a 1-μl Hamilton syringe (Hamilton Company, Reno, NV, USA) and an injection needle (33-gauge NanoFil Needle Assortment, blunt; WPI, Worcester, MA USA) using a syringe pump (KD Scientific, Holliston, MA, USA). For expression of eNhR3.0-EYFP, control EXPLORs and Cre:EXPLORs were prepared. In total, 500 μg of EXPLORs were injected into the ventrolateral part of the brain in each mouse (−2.8 mm anteroposterior, −2.2 mediolateral and 3.8 dorsoventral). At 5 days after transfection, mice were killed for visualization.

**Histology and immunohistochemistry of mouse brain.** Mice were deeply anaesthetized with avertin (20 mg ml−1 of tribromoethanol, 20 μl g−1 intraperitoneally, Sigma–Aldrich, Milwaukee, WI, USA) and perfused first with heparin and then with 4% paraformaldehyde diluted in PBS. Brains were removed and post-fixed overnight at 4 °C. Coronal sections (40-μm thick) were acquired using a vibratome (Leica VT1000S, Leica, Rockleigh, NJ, USA) and collected in PBS. Slices were mounted on glass slides with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI; Vector Labs, Peterborough, UK). Fluorescent images (low magnification) were acquired with an Axios Imager 2 fluorescent microscope (Carl Zeiss). Confocal images were acquired with a LSM780 confocal microscope (Carl Zeiss) equipped with a Plan-Apochromat ×20/0.8 M27 objective. Brain sections were incubated in PBS with 0.3% Triton X-100 (Sigma–Aldrich) for 30 min at room temperature. After washing in PBS for 10 min, sections were incubated in a blocking solution composed of 0.5% bovine serum albumin and 0.25% Triton X-100 for 1 h at room temperature. For NeuN/GFAP immunostaining, sections were incubated with mouse anti-NeuN primary antibody (MAB377; Millipore) and chicken anti-GFAP primary antibody (AB551; Millipore, diluted 1:1,000 in PBS containing 0.25% Triton X-100, for 15 h at room temperature. After washing twice in PBS for 5 min each, sections were incubated with fluorescein Alexa 647-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) and Alexa 555-conjugated anti-chicken IgG secondary antibody (Jackson Immunoresearch, and diluted 1:300 in PBS for 1 h at room temperature. Sections were washed twice in PBS for 5 min each and mounted on glass slides with Vectashield mounting medium containing DAPI (Vector Labs).

**Statistical analyses.** Data are presented as means ± s.e.m. The significance of the difference between two independent samples was determined using Student’s t-test. Groups were compared using one-way analysis of variance, with Tukey’s post hoc
test applied to a significant main effect. All data were normally distributed and the variances were similar between the groups being statistically compared. Sample size was based on previous experience with experimental variability, and no statistical method was used to predetermine sample or animal sizes. No samples or animals were excluded from the analysis. All experiments were not randomized. The investigators were not blinded to allocation during experiments or outcome assessment.

**Data availability.** All data supporting the findings in this study are included in the article, either in the main figures or in the Supplementary Information files.

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**Author contributions**

N.Y. and C.C. designed the EXPLOR strategies and wrote the paper; C.C. and K.C. supervised the project; N.Y. built EXPLOR-related constructs and EXPLOR-producing system; N.Y., S.-W.R. and K.C. collected and analysed the data; N.Y., S.-W.R., K.C., N.K., S.L., H.C. and M.R.S. performed in vitro experiments; J.K. performed *in vivo* experiments; W.S., J.-H.P., D.K. and W.D.H. provided reagents, analysing tools and mice; K.R.L., S.L. and H.C. gave technical support; J.H.P., D.K. and W.D.H. gave conceptual advice.

**Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** C.C. is the scientific founder and Chief Executive Officer of Cellex Life Sciences Inc. S.-W.R. and K.C. are employees of Cellex Life Sciences Inc. A patent application has been submitted by KAIST based on these results. The remaining authors declare no competing financial interests.

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