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Enduring high-efficiency *in vivo* transfection of neurons with non-viral magnetoparticles in the rat visual cortex for optogenetic applications

C. Soto-Sánchez[a,b,*], G. Martínez-Navarrete[a], L. Humphreys[a,b], G. Puras[b,c], J. Zarate[b,c], J.L. Pedraz[b,c], E. Fernández[a,b]

[a]*Bioengineering Institute, Miguel Hernández University (UMH), Spain*
[b]*Biomedical Research Networking center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain*
[c]*NanoBioCel Group, University of País Vasco (UPV), Spain*

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Abstract

This work demonstrates the successful long-term transfection *in vivo* of a DNA plasmid vector in rat visual cortex neurons using the magnetofection technique. The transfection rates reached values of up to 97% of the neurons after 30 days, comparable to those achieved by viral vectors. Immunohistochemical treatment with anti-EGFP antibodies enhanced the detection of the EYFP-channelrhodopsin expression throughout the dendritic trees and cell bodies. These results show that magnetic nanoparticles offer highly efficient and enduring *in vivo* high-rate transfection in identified neurons of an adult mammalian brain and suggest that the magnetotechnique facilitates the introduction of large functional genetic material like channelrhodopsin with safe non-viral vectors using minimally invasive approaches.

Key words: Gene therapy; Transfection; Non-viral vectors; *In vivo*; Brain; Optogenetics

Background

Gene therapy has been hailed and positioned as a promising strategy to restoring brain dysfunctions and to the treatment of incurable brain diseases. From the point of view of medical and basic neuroscience the nervous system transfection represents a promising field in the treatment of neurological diseases,[1] tumor therapies[2,3] and biotechnology applications in trending fields like optogenetics. The expression of ion channels like channelrhodopsin[4,5] or halorhodopsin[6,7] enables to artificially control the excitability of the cells that express these ion channels at the cell membrane. The optogenetic approach has been implemented successfully in research applications like the control of the epileptiform activity,[8,9] the control of parkinsonian motor behaviors[10,11] and the restoration of visual responses in photoreceptors,[12] among others. In order to be able to express the proteins of interest, the genetic material has to be carried into the cell, however numerous obstacles remain making neurons a challenging type of cell to transfect. The small size of the DNA plasmid, its negative charge and vulnerability to the degradation by DNases reduce the capacity of the naked DNA to enter into the neurons. Therefore there is a need for carriers to transport, protect and deliver the DNA inside the neurons. In spite of the numerous reagents already available, currently, non-viral methods exhibit low transfection efficiencies of neurons, especially for *in vivo* studies.[13-15] On the other side, viral vectors have been able to obtain high rates of gene expression[16] however, restrictions to the size limit of the genetic material able to be packed[17] together with reported safety concerns such as toxicity, inflammatory reaction, immunogenicity or generalized infection[18,19] are the basis to search for new alternatives.

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*Corresponding author at: Av. Universidad s/n, Ed. Vinalopó, Elche, Alicante, 03202, Spain.

E-mail addresses: csoto@umh.es (C. Soto-Sánchez), gema.martinezn@umh.es (G. Martínez-Navarrete), lhumphreys@umh.es (L. Humphreys), gustavo.puras@ehu.es (G. Puras), jon.zarate@ehu.es (J. Zarate), joseluis.pedraz@ehu.es (J.L. Pedraz), e.fernandez@umh.es (E. Fernández).

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Keeping these concerns in mind and based on the safety of the magnetotechnique, its high transfection rate in primary cultures and cell lines together with a minimum invasive neurosurgery, we have evaluated the efficiency of our magnetoplexes as an alternative vehicle for transporting and expressing optogenetic plasmids in neurons of the visual cortex of rats *in vivo*. Our objective was to find an affordable and easy-executable technique, without any special need for safety and security requirements, to be used on a regular basis to introduce large genetic constructs like EYFP-channelrhodopsin and obtain high transfection rates in the mammalian brain. We also studied the expression rates after 72 hours and 30 days in order to track the expression levels in the short and long term.

Methods

Plasmid DNA

The pLenti-Synapsin-hChR2(H134R)-EYFP-WPRE plasmid (9000 bp) (Addgene plasmid 20945, Karl Deissertoh laboratory)[20] coding for the enhanced yellow fluorescent protein (EYFP) was acquired and amplified in our laboratory.

Elaboration of DNA-magnetic complexes

Magnetoplexes (DNA-magnetic complexes) were elaborated by mixing 1 μg of the aforementioned plasmid with 70 μl of positively charged iron oxide magnetic nanoparticles (NeuroMag, www.ozbiosciences.com). Magnetoplexes were incubated for 30 minutes at room temperature to enhance the electrostatic interactions between cationic magnetic nanoparticles and the negatively charged plasmid.

Size and zeta potential measurements

The particle size of magnetic nanoparticles and magnetoplexes vectors was reported as hydrodynamic diameter by cumulative analysis. Particle size was determined by dynamic light scattering (DLS), and the superficial charge of both magnetic nanoparticles and magnetoplexes vectors by laser Doppler velocimetry (LDV). All measurements were taken on a Malvern Zeatsizer Nano ZS (Malvern Instruments, UK). Samples were diluted in NaCl 0.1 mM Milli-Q water. All measurements were carried out in triplicate.

Transmission electron microscopy

The morphology of magnetic nanoparticles and magnetoplexes vectors was characterized by transmission electron microscopy (TEM). Briefly, 5 μl of each sample were adhered onto glow discharged carbon coated grids for 60 seconds. Remaining liquid was removed by blotting on paper filter. Samples were visualized under the microscope, TECNAI G2 20 TWIN (FEI, Eindhoven, The Netherlands), operating at an accelerating voltage of 200 KeV in a bright-field image mode. Digital images were acquired with an Olympus SIS Morada digital camera.

Condensation, SDS-induced release of DNA and DNase I protection assays

Naked DNA or vectors samples (20 μl, containing 200 ng of the plasmid) were subjected to electrophoresis on an agarose gel (0.8%). The gel was immersed in a tris-acetate-EDTA buffer and exposed for 30 minutes to 120 V. DNA bands were stained with GelRed™ (Biotium, Hayward, CA, USA) and images were observed with a TFX-20M transilluminator (Vilber-Lourmat, Germany). To analyze the release of DNA from the magnetic nanoparticles, 20 μl of a 4% SDS (sodium dodecyl sulfate) solution was added to the samples to get a final SDS concentration of 2% in each well.[21,22] The pDNA protection capacity of the vectors against enzymatic digestion was analyzed by adding the DNase I enzyme to the vector formulations (final concentration of 1 U DNase I/2.5 μg DNA). Afterwards, the mixtures were incubated at 37 °C for 30 minutes, and finally, 20 μl of a 4% SDS solution was added to analyze the released DNA. The integrity of the DNA in each sample was compared to a control of untreated DNA.

Surgery and magnetoparticles injections

Data was obtained from 18 female Sprague-Dawley adult rats weighing 250-300 g.

Nine animals were processed 3 days after the injection and 9 animals 30 days after the injection. Three of the animals processed after 3 days corresponded to the experimental group injected with the magnetoplexes, and 6 belonged to the control conditions (3 injected only with the nude plasmid and 3 injected only with the magnetoparticles). Three animals injected with the magnetoplexes and other 6 animals under the control conditions were processed after 30 days.

Surgical analgesia was induced by buprenorphine (0.025 mg kg^{-1} s.c) and anesthesia and sedation were induced by a cocktail of ketamine HCl (40 mg kg^{-1} i.p) and diazepan (5 mg kg^{-1} i.p). Afterwards, the anesthesia was continued and maintained with a mix of oxygen and 4% of isofluorane for the rest of the surgery. The depth of the anesthesia was evaluated continuously by monitoring heart rate and blinking and toe pinch reflexes. Levels of O_2 concentration in the blood, as well as temperature and heart rate were monitored throughout the surgery. During the surgery the animals' bodies were maintained warm with a water thermal pad. Rats were pre-treated with dexamethasone (1 mg kg^{-1} i.p) 24 hours prior to surgery. A new dosage was administrated 20 minutes prior to and 24 hours after surgery. We drilled a small craniotomy to expose the dura mater and arachnoids and performed a minimally invasive incision to introduce a Hamilton 33-gauge needle. pLenti-Synapsin-hChR2(H134R)-EYFP-WPRE (0.4 μg final) were incubated with NeuroMag in Opti-MEM® (Invitrogen) for 15 minutes at room temperature before the injection. A Hamilton 5 μl micro-syringe (Hamilton Company) was held within a stereotaxic frame and lowered 1 mm inside of the primary cerebral cortex to inject a total volume of 0.8 μl of NeuroMag-pDNA solution. After the injection, the needle remained *in situ* for 5 minutes before being withdrawn slowly. According to the NeuroMag magnetotransfection protocol, we exposed the brain to a magnetic field (magnet underneath the head) during