Non-invasive detection of functional integration of optically-controlled stem cells by (f)MRI *in vivo*

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Adult stem cells transplantation is a promising strategy in the therapy of both acute and chronic degenerative diseases of the central nervous system (CNS). The therapy approach - restoration of degenerated neural structures versus stimulation of endogenous repair mechanisms – still remains controversial. We established a method to non-invasively monitor stem cells in the living CNS using magnetic resonance imaging (MRI), taking advantage of high-resolution and non-limited depth penetration at the same time. It is a non-genetic, but efficient and stable targeting with very small superparamagnetic iron oxide particles (VSOPs).

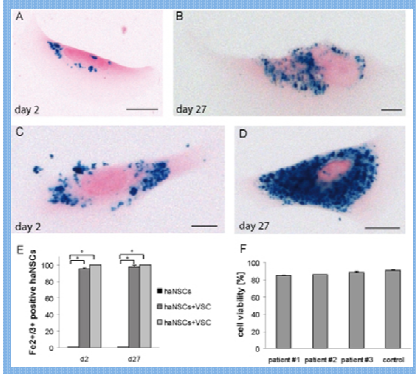
To allow for specific stimulation of magnetically labeled adult stem cells we aim at combining this technique with optogenetics. Therefore, we introduced the genetically encoded optogenetic construct Channelrhodopsin-2 (ChR2) into labeled stem cells. Using a lentiviral (pEF1a-ChR2-EYFP) or adenoviral-associated (AAV-DIO-ChR2-mCherry) gene transfer we transduced both embryonic stem cells as well as adult SEZ neurosphere-derived cells. Retroviral co-transduction of the latter with neurogenin2 (pCAG-Ngn2-IRES-Dsred) leads to neuronal differentiation.

Following illumination with blue light (470nm), the membrane protein ChR2 leads to a high-speed depolarization, fast enough to drive precisely timed light-evoked spikes in adult SEZ neurosphere-derived cells.

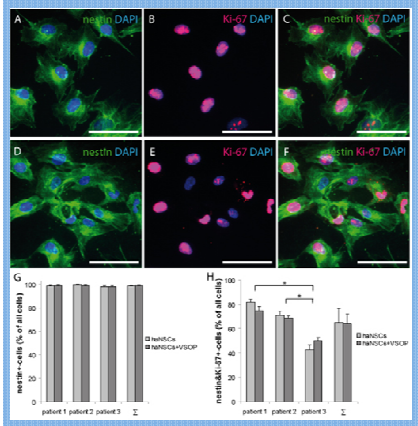
STEM CELL CULTURING

Embryonic stem cells (ESCs, CRL-1934, ATCC, Manassas, USA), were co-cultured as described in [1]. Neuronal differentiation of ESCs was induced via embryoid body formation and LIF removal. Nestin-positive cells were selected and expanded. After 10 days of expansion, progenitors were differentiated into neurons by removing N2 supplement and bFGF, and adding NGF and B-27 supplement for 14 days. Neural precursor cells from the adult mouse subependymal zone (SEZ), were isolated and cultured as neurospheres. Retroviral transduction and withdrawal of EGF and FGF lead to neuronal differentiation within 10 days.
Human adult hippocampal progenitor cells (haSCs) were obtained from a patient with temporal lobe epilepsy submitted to epilepsy surgery [2]. For neuronal differentiation, cells were cultured in differentiation media containing SHH, FGF-8, and ascorbic acid. Cells were differentiated for 15 days.

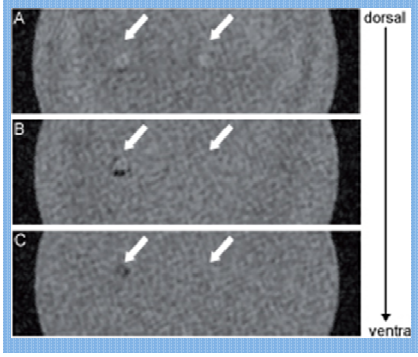
MAGNETIC LABELING



Histological analysis of magnetically labeled haNSCs. Intracytoplasmatic VSOP uptake (C200, Ferropharm, Teltow, Germany) following incubation with 0.5 mM. VSOPs consist of a 5 nm iron oxide core coated by monomer citrate yielding a diameter of 9 nm. The uptake of VSOPs was evaluated using Prussian blue staining [3] on day 2 (A, C) and on day 27 (B, D) after labeling. (A+B) Fixed and stained cells after incubation with 0.5 mM VSOPs, (C+D) lipofection with 0.5 mM VSOPs. Scale bar is 10µm. (E) Intracytoplasmatic VSOP uptake into 96-100 out of 100 haNSCs. (F) Trypan blue exclusion test directly after labeling showed no decrease in cell viability due to the labeling procedure. Data presented as mean ± s.e.m. Differences were considered significant at $P < 0.01$.

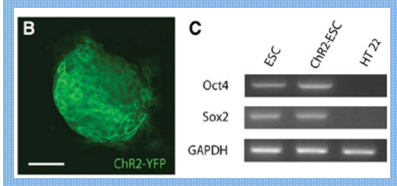


Fluorescence immunocytochemistry analysis of haNSCs. 99 % of haNSCs express the intermediate filament protein nestin, and 65 % co-express the nuclear proliferation marker Ki-67. No significant difference between control cells and VSOP labeled haNSCs could be detected. A-C shows unlabeled control cells, and D-F the VSOP labeled pendant. Scale bar is 50µm. Nestin expression of haNSCs from three different patients was compared (G), showing that there is neither a significant difference between the patients nor between control cells or labeled cells. H) 65 % of haNSCs co-express nestin and Ki-67, but significant differences between the three patients become apparent. Data presented as mean ± s.e.m. Differences were considered significant at $P < 0.01$.

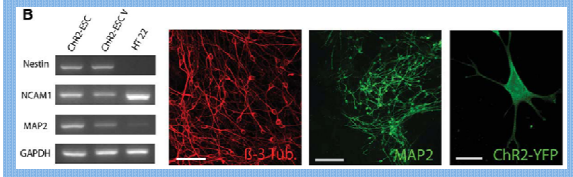


Gradient echo MR images of gel phantoms after injection of haNSCs. (A) Coronal section at gel surface showing two needle tracks as hyperintense signal change. 1x10³ VSOP labeled haNSCs were injected on the left side; on the right side 1x103 unlabeled control haNSCs were injected. (B) 4.5 mm ventral of (A): In the left injection track an area of signal loss can be visualized, due to clusters of magnetically labeled haNSCs. No signal change in the right injection track was observed. (C) 5 mm ventral of (A): The left injection track again shows an area of signal loss, no signal change in the right injection track.

ChR2-ESCs DIFFERENTIATE INTO EXCITATORY NEURONS

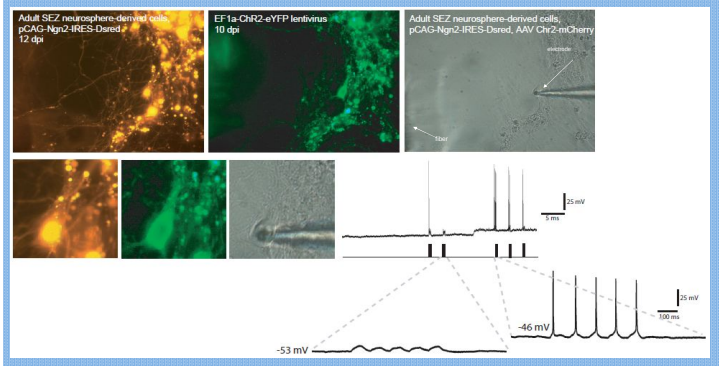


Functional expression of ChR2. (B) Confocal micrograph revealing membrane-localized expression. Vitality, morphology, and population doubling times were indistinguishable from native cells. Scale bar is 50 µm. (C) RT-PCR of pluripotency marker expression of both ChR2-ESCs and native ESCs revealed strong expression of Oct4 and Sox2, compared with mouse hippocampal cells HT22. GAPDH was used as loading control [4].



ChR2-ESCs differentiate into excitatory neurons. (B): Transcriptional analysis of neural lineage markers of differentiated ChR2-ESCs at day 22. ChR2-ESCs were compared with cells reinfected with EF1a-ChR2 lentivirus at neural stage (day 15) and mouse hippocampal HT22 cells. Transcripts of neural markers nestin were present in ChR2-ESCs and in reinfected ChR2-ESCs, in contrast to HT22 cells. NCAM1 transcript could be detected in all three cell populations, MAP2 is only strongly transcribed in both ESC populations. GAPDH was used as loading control. Immunocytochemistry for stage-specific markers (right). After 20 days, cells adapt bipolar morphology, a dense network of b3-tubulin-expressing cells can be observed indicating progression down the neuronal lineage. Scale bar is 50 µm. After 30 days, cells express the mature neuronal marker MAP2. Scale bar is 100 µm. Reinfected ChR2-ESC-derived neurons display strong expression of ChR2. Scale bar is 10 µm [4].

EXCITATORY ADULT SEZ-NEUROSPHERE DERIVED CELLS



Excitatory adult SEZ-neurosphere derived cells. Co-transduction with pCAG-Ngn2-IRES-Dsred and pEF1a-ChR2-EYFP, and withdrawal of EGF and FGF for 10 days leads to neurogenin2 and ChR2 expression. Following illumination with blue light (470nm), the membrane protein ChR2 leads to a high-speed depolarization, fast enough to drive precisely timed light-evoked spikes. Due to optogenetic stimulation (10 Hz, 50 ms duration, 5 pulses) the cells get excited.

[1] Bibel M. *et al., Nat. Neurosci.*, 2004.
[2] Siebzehnrubel FA. *et al., Exp. Brain Res.*, 2007.
[3] Stroh A. *et al., Mol. Imaging*, 2009.
[4] Stroh A. *et al., Stem Cells*, 2010.

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