

## LETTER

# Probing interneuronal cell communication via optogenetic stimulation

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## Abstract

This study uses an all-optical approach to probe interneuronal communication between spiral ganglion neurons (SGNs) and neurons of other functional units, in this case cortex neurons (CNs) and hippocampus neurons (HNs), for the first time. We combined a channelrhodopsin variant (CheRiff) with a red genetically encoded calcium indicator (jRCaMP1a), enabling simultaneous optical stimulation and recording from spatially separated small neuronal populations. Stimulation of SGNs was possible with both optogenetic manipulated HNs and CNs, respectively. Furthermore, a dependency on the pulse duration of the stimulating light in regard to the evoked calcium response in the SGNs was also observed. Our results pave the way to enable innovative technologies based on “biohybrid” systems utilizing the functional interaction between different biological (eg, neural) systems. This can enable improved treatment of neurological and sensorineural disorders such as hearing loss.

## KEYWORDS

cell communication, light, neuroprosthesis, optogenetics

## 1 | INTRODUCTION

To understand how multiple neurons of different functional neuronal networks communicate with each other, it is crucial to have a culture system enabling both stimulation and recording from spatially separated populations of neurons with single-cell readout. Optogenetics, in

combination with calcium imaging, is a powerful tool enabling manipulation and monitoring of defined neural circuits and thus cell-cell communication [1–3]. With the upcoming of new spectrally separated channelrhodopsin variants [4] and genetically encoded calcium indicators (GECIs) [5] it is now possible to stimulate a sub-section of a neuronal network and record the calcium response in both stimulated and nonstimulated neurons at the same time [6]. Optogenetics can also be used to investigate the communication between cell types of different

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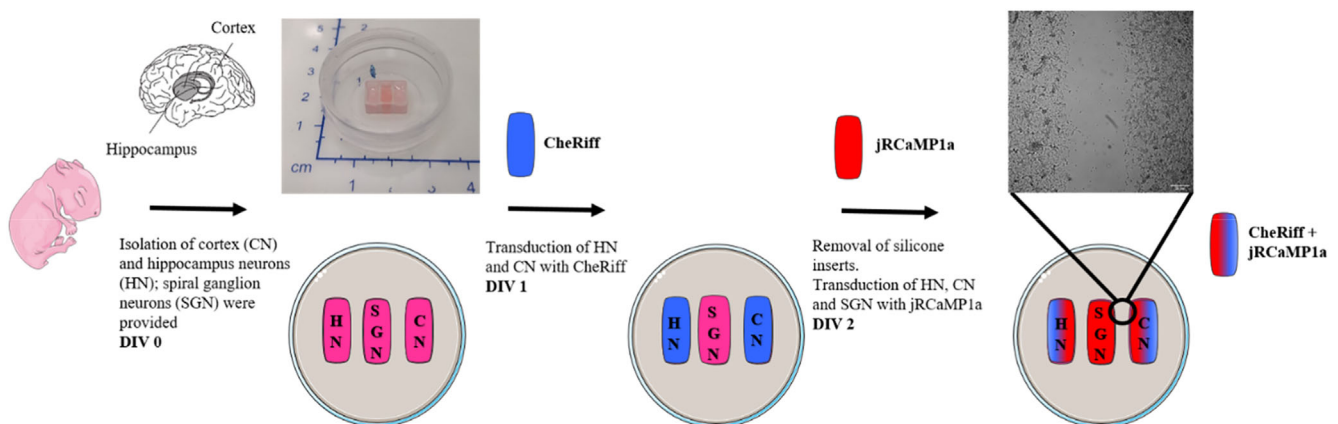
functional units. First attempts were made in cardiac optogenetics by coupling optogenetic active Human Embryonic Kidney cells via gap junctions to excitable cardiomyocytes, thus generating a sort of a light-driven cardiac pacemaker [7]. But, one could think about other applications like a light-driven cochlea implant by coupling optogenetically activated neuronal cells to spiral ganglion neurons (SGNs), the first action potential generating neurons of the auditory portion. SGNs receive afferent input of inner hair cells (IHCs) and extensive evidence is growing that glutamate (Glu) mediates excitatory neurotransmission between those two [8]. Consequently, glutaminergic neurons of other compartments like the hippocampus or cortex should also be able to contact SGNs via their dendrites and relay auditory information. This way, a new sort of cochlea implant could be engineered without direct manipulation of the auditory nerve.

Following this idea, we established an easy, cost effective and reliable optogenetic test system to probe inter-neuronal communication. We demonstrate all-optical sensing and actuation in a group of interconnected neurons of different functional units for example, the brain (HN, CN) and the cochlea (SGN). By using a combination of a blue shifted channelrhodopsin variant called CheRiff and a GECI with red-shifted excitation and emission spectra, namely jRCaMP1a, we have the possibility to stimulate and image spectrally independent separated populations of neurons in vitro. Thus, we designed a system to study spread of excitation in interneuronal networks (Figure 1). Refer to Figure S1 for a high resolution brightfield and fluorescence image of the separated cell populations.

## 2 | MATERIALS AND METHODS

The experiments were in accordance with the German Animal Welfare Legislation and approved by the local Institutional Animal Care and Research Advisory Committee and permitted by the Lower Saxony State Office for Consumer Protection and Food Safety (reference number 42500/1H).

Rat pups postnatal day three (D 3) were euthanized by decapitation. The hippocampus and cortex regions were removed and treated with Papain/DNase to receive single neurons. Single cells were purified with a cell strainer with a pore size of 40  $\mu\text{m}$  and the concentration was set to approximately 1 million cells per milliliter. The dissection of the cochleae and the isolation of the SGN were conducted as described previously [9]. For enzymatic dissociation, spiral ganglion explants were incubated at 37°C for 20 minutes in a solution containing 0.1% trypsin (Biochrom, Berlin, Germany) and 0.01% DNase I (Roche, Mannheim, Germany). Thereafter, mechanical dissociation was performed via trituration. After dissociation, the yield of viable cells was counted in a Neubauer cytometry chamber using the trypan blue (Sigma Aldrich, Taufkirchen, Germany) exclusion test. The neurons were cultured in a poly-D-lysine coated 35 mm imaging dish with a polymer coverslip. To keep the three neuronal populations separated a 3 well silicone insert (ibidi) was used during the first days of cultivation. On DIV 1 lentiviral transduction of HN and CN populations was performed to deliver the plasmid DRH313: FCK-CheRiff-eGFP (kindly provided by Adam Cohen; Addgene #51693) to the neurons. The promoter was chosen for transgene expression to obtain



**FIGURE 1** Schematic representation of the experimental set-up. Cortex neurons (CN) and hippocampus Neurons (HN) were isolated and, together with spiral ganglion neurons (SGN), cultivated in separated silicone chambers (inserts) for 24 hours. The following day (DIV 1) the HNs and CNs were loaded with lentivirus carrying the channelrhodopsin variant CheRiff. On DIV 2 the silicone inserts were removed and all three neuronal populations were transduced with the calcium sensor jRCaMP1a via an adeno-associated vector AAV. The stimulation and imaging experiments took place at DIV 12 to 14

preferential gene expression in the excitatory neuron populations. After 24 hours of incubation, the virus containing solution was removed and after a washing step, to remove residual lentivirus, replaced with fresh media. Further, the silicone inserts were removed and the three populations were infected with ready-to-use AAV9 particles produced from pAAV.Syn.NES.jRCaMP1a.WPRE.SV40 (kindly provided by Douglas Kim & GENIE Project; Addgene #100848). The synapsin promoter confers highly neuron-specific long-term transgene expression. Growth media (BrainPhys Neuronal Medium supplemented with NeuroCult SM1 Neuronal Supplement, Stemcell) were supplemented with *All-trans* retinal to ensure activation of the chromophore on DIV 10. The stimulation and imaging took place on DIV 12 to 14. The medium was replaced with Tyrode's Solution to improve signal strength during calcium imaging. A high resolution brightfield and fluorescence image of the CheRiff and jRCaMP expression is shown in Figure S1. The populations are separated by a gap of ~1 mm due to the before used silicone inserts but formed connections during incubation. The jRCaMP expression is very dominant in all types of neurons, even though there is a very limited number of positive SGNs. This is due to the nature of isolation process. Most of the cells visible in the middle line (brightfield) are glia cells like Schwann cells. The CheRiff expression is more dominant in HN in comparison to CN. This is probably due to a higher preference of the CaMKII promoter for excitatory neurons in primary hippocampal cultures. The SGN isolation also led to clusters which exhibit autofluorescence. These have formed during the incubation period.

The neurons were transferred to an imaging set-up consisting of an inverse Leica microscope (DMI6000B) equipped with a scientific CMOS camera (pco.edge 4.2), a high power LED for fluorescence imaging and a fiber coupled LED (Thorlabs; 25 mW; 470 nm) for stimulation. The samples were imaged using a Leica 10× NA 0.3 air objective. An Arduino control unit ensured synchronization of the camera and the LED. The manipulation LED was fixed directly above either a HN or a CN population. Three spots per population were stimulated with four different pulse durations (10; 100; 250 and 500 ms). Each recording consisted of 10 pulses of blue light separated by an interval of 5.5 seconds. Simultaneously, calcium imaging with continuous green light illumination (550 nm, 15 mW/cm<sup>2</sup>) through a filter (long pass emission filter >590 nm) at 10 fps for a total duration of 1 minute took place in the region of interest, more precisely a section, showing the change in fluorescence from jRCaMP1, either HN or CN, as well the responding SGNs. Following the acquisition, the

recordings were processed using an in house built Matlab script. For each curve, a low order polynomial was fitted to remove any trend in the baseline. The peaks were detected based on the difference of the maximal and average fluorescence value with a customized threshold level.

### 3 | RESULTS

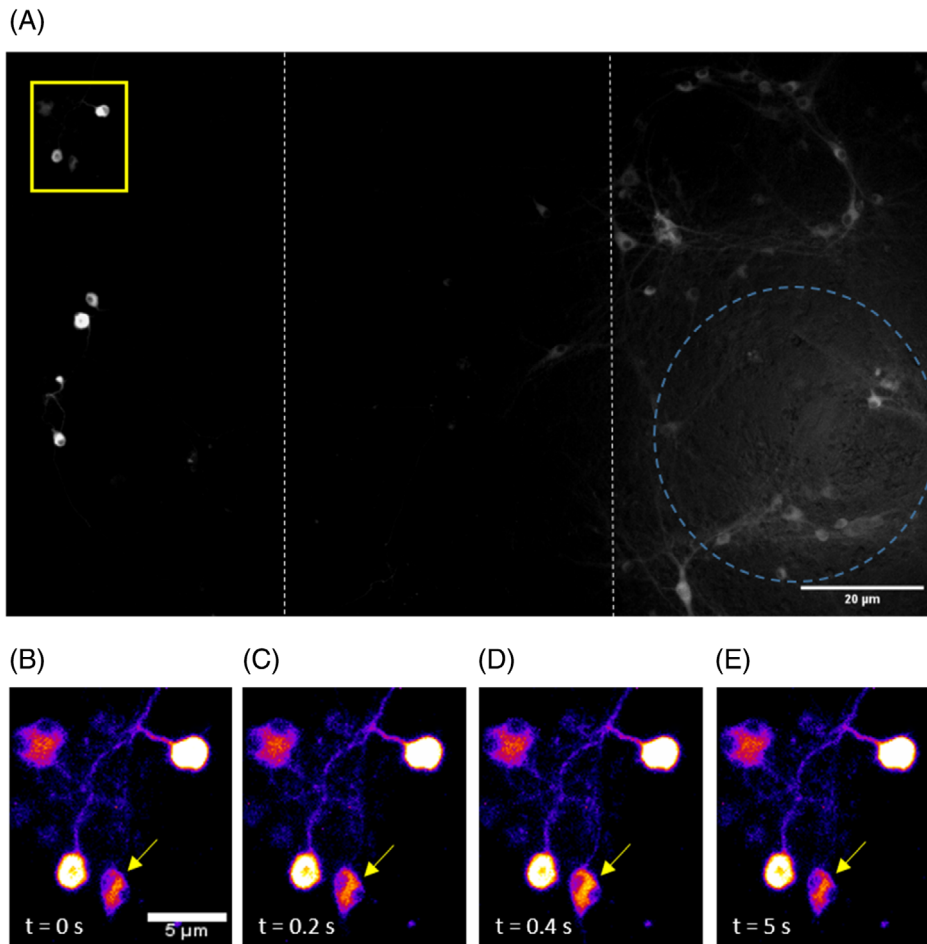
Figure 2 shows a typical fluorescence time series of a blue light activated CNs population (right) triggering a response in a single SGN (left). After a stimulation pulse (blue dotted line), the fluorescence signal in a SGN increases within 0.4 ms to its maximum and decreases to its basal level after 5 seconds, respectively (see Figure 2) and Video S1)).

On each day of measurement, three dishes were processed like in the experimental setup shown in Figures 1 and 2 with the above mentioned parameters. In total, five test series were performed ( $n = 5$ ).

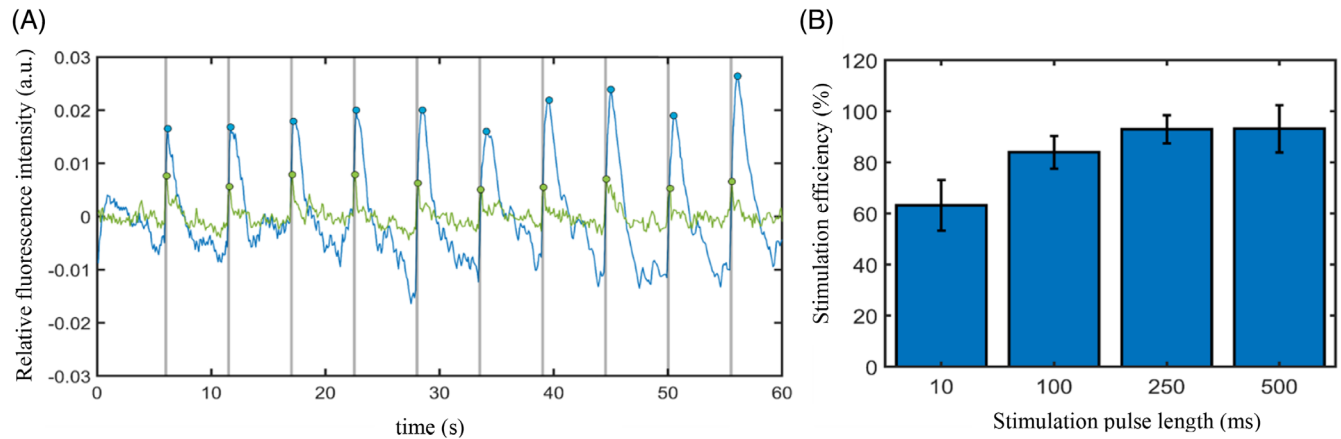
An example of the corresponding Matlab graphs is shown in Figure 3 and Figure 4, cells without CheRiff showed no induced calcium stimulations (see Figures S2 and S3). Every 5.5 seconds, a stimulation pulse (in this case 250 ms) of the LED (gray solid line) triggers either HNs (Figure 3A, blue spikes) or CNs (Figure 4A, orange spikes). This in turn stimulates connected SGNs (green spikes). Peak maxima were marked with dots to simplify counting process. Every light pulse in these two examples resulted in a stimulation of the HNs or CNs leading to an immediate response of the SGNs. The SGN peaks which occurred in correlation with the HN or CN peaks of all stimulation series were counted and plotted to get a quantitative statement of the stimulation efficiency. The rise of calcium in the illuminated region always started before the rise in the SGNs. Even with very short stimulation pulses of 10 ms, the stimulation efficiency of the SGNs reached roughly 60% for both, HNs and CNs. With increasing stimulation pulse length, the stimulation efficiency of both HNs and CNs increased rapidly and reached a plateau at 250 ms. The overall stimulation efficiency of the SGNs triggered by the HNs reached over 90% with 250 and 500 ms pulses, respectively, whereas SGNs triggered by CNs shows slightly lower efficiency (~80%) with the same parameters.

### 4 | DISCUSSION

We have presented an all-optical approach to simultaneously record the activity of spatially separated



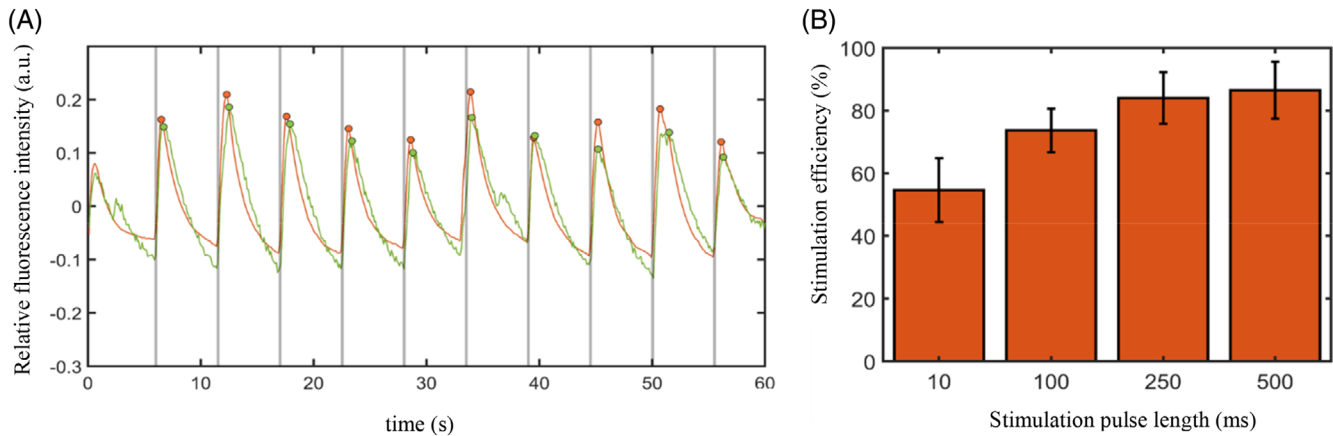
**FIGURE 2** Fluorescence series of spiral ganglion neurons activated by light triggered cortex neurons. A, A representative fluorescence image showing population of SGNs (left) transduced with the GECI jRCaMP1a and CN (right) double transduced with CheRiff and jRCaMP1a. Yellow square points out responding SGN. The neurons are separated by a gap of 1 mm due to silicone inserts. Blue circle indicates the position of the stimulation LED. B-E, Time series of a responding SGN after blue light stimulation of the CNs with a 250 ms pulse, respectively. (b) SGN of interest (yellow arrow) shows basal level of fluorescence at beginning of stimulation. B-D, Fluorescence signal increases within 0.4 seconds after the stimulation pulse to its maximum. E, Five seconds after the stimulation pulse the SGN fluorescence drops down to its basal level again



**FIGURE 3** Calcium signals of A, LED stimulated HN (blue) and responding SGNs (green) and B, bar chart of compiled peak counts of SGNs in dependence of the pulse duration

neuronal populations of different functional units (brain and cochlea) with single-neuron readout. Specifically, it allowed us to simultaneously stimulate one neuronal network while recording evoked calcium responses in both nonstimulated and stimulated

neurons. This allowed us to investigate the connectivity of different neuron types for example, hippocampal and SGNs and enabled us to make a quantitative statement about the interneuronal communication.



**FIGURE 4** Calcium signals of A, LED stimulated CN (blue) and responding SGNs (green) and B, bar chart of compiled peak counts of SGNs in dependence of the pulse duration

As this indicates that it is generally possible to trigger SGNs with optogenetic activated neurons of other functional units for example hippocampus or cortex it might pave the way to use already optogenetically activated neurons, for instance, embedded in a hydrogel, as an implant. Several studies have demonstrated high survival rates of neuronal cells in hydrogels for long-term applications [10, 11]. This implant would come along with a light guiding fiber to trigger these neurons. The neurons itself act as the stimulation unit to conduct the signal to residential SGNs. Therefore, it is possible, that in future studies also stem cell derived neurons could be applied. This would limit batch to batch variety of cell isolation and also allow autologous transplantation in later clinical settings [12]. Thus, innovative technologies based on “biohybrid” systems utilizing the functional interaction between biological (eg, neural) systems and artificial devices could emerge for improved treatment of neurological and sensorineural disorders such as hearing loss. The genetic modification of cells outside of the patients' bodies could increase safety of the treatment, especially if combined with gene insertion of the opsins in a safe-harbor locus [13].

Our study also indicates, that the stimulation efficiency of the SGNs generally increases with the stimulation length and reaches a plateau at 250 ms. This finding implicates that longer stimuli do not result in stronger calcium signals. But the plateau formation could also be due to an effect called excitotoxicity, where strong stimulus and thus  $\text{Ca}^{2+}$  activity can lead to calcium induced neuronal damage [14].

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#### DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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#### REFERENCES

- [1] J. Akerboom, N. C. Calderón, L. Tian, S. Wabnig, M. Prigge, J. Toló, A. Gordus, M. B. Orger, K. E. Severi, J. J. Macklin, R. Patel, S. R. Pulver, T. J. Wardill, E. Fischer, C. Schüller, T. W. Chen, K. S. Sarkisyan, J. S. Marvin, C. I. Bargmann, D. S. Kim, S. Kügler, L. Lagnado, P. Hegemann, A. Gottschalk, E. R. Schreiter, L. L. Looger, *Front. Mol. Neurosci.* **2013**, *6*(FEB), 1. <https://doi.org/10.3389/fnmol.2013.00002>.
- [2] A. M. Stamatakis, M. J. Schachter, S. Gulati, K. T. Zitelli, S. Malanowski, A. Tajik, C. Fritz, M. Trulson, S. L. Otte, *Front. Neurosci.* **2018**, *12*(JUL), 1. <https://doi.org/10.3389/fnins.2018.00496>.
- [3] C. Nguyen, H. Upadhyay, M. Murphy, G. Borja, E. J. Rozsahegyi, A. Barnett, T. Brookings, O. B. McManus, C. A. Werley, *Biomed. Opt. Exp.* **2019**, *10*(2), 789. <https://doi.org/10.1364/boe.10.000789>.
- [4] N. C. Klapoetke, Y. Murata, S. S. Kim, S. R. Pulver, A. Birdsey-Benson, Y. K. Cho, T. K. Morimoto, A. S. Chuong, E. J. Carpenter, Z. Tian, J. Wang, Y. Xie, Z. Yan, Y. Zhang, B. Y. Chow, B. Surek, M. Melkonian, V. Jayaraman, M. Constantine-Paton, G. K. S. Wong, E. S. Boyden, *Nat. Methods* **2014**, *11*(3), 338. <https://doi.org/10.1038/nmeth.2836>.
- [5] H. Dana, B. Mohar, Y. Sun, S. Narayan, A. Gordus, J. P. Hasseman, G. Tsegaye, G. T. Holt, A. Hu, D. Walpita, R. Patel,

- J. J. Macklin, C. I. Bargmann, M. B. Ahrens, E. R. Schreiter, V. Jayaraman, L. L. Looger, K. Svoboda, D. S. Kim, *Elife* **2016**, 5(MARCH2016), 1. <https://doi.org/10.7554/eLife.12727>.
- [6] W. A. Saber, F. M. Gasparoli, M. G. Dirks, F. J. Gunn-Moore, M. Antkowiak, *Front. Neurosci.* **2018**, 12(JUL), 1. <https://doi.org/10.3389/fnins.2018.00451>.
- [7] Z. Jia, V. Valiunas, Z. Lu, H. Bien, H. Liu, H. Z. Wang, B. Rosati, P. R. Brink, I. S. Cohen, E. Entcheva, *Circ. Arrhythmia Electrophysiol.* **2011**, 4(5), 753. <https://doi.org/10.1161/CIRCEP.111.964247>.
- [8] D. O. J. Reijntjes, S. J. Pyott, *Hearing Res.* **2016**, 336, 1. <https://doi.org/10.1016/j.heares.2016.03.011>.
- [9] O. Kaiser, G. Paasche, T. Stöver, S. Ernst, T. Lenarz, A. Kral, A. Warnecke, *Neuropharmacology* **2013**, 75, 416. <https://doi.org/10.1016/j.neuropharm.2013.08.008>.
- [10] Y. Luo, M. S. Shoichet, *Nat. Mater.* **2004**, 3(4), 249. <https://doi.org/10.1038/nmat1092>.
- [11] S. Koutsopoulos, S. Zhang, *Acta Biomater.* **2013**, 9(2), 5162. <https://doi.org/10.1016/j.actbio.2012.09.010>.
- [12] M. Waqas, R. Chai, *Approaches to Regenerate Hair Cell and Spiral Ganglion Neuron in the Inner Ear*, Springer, Cham, **2020**.
- [13] E. P. Papapetrou, A. Schambach, *Am. Soc. Gene Cell Ther.* **2016**, 24(4), 678. <https://doi.org/10.1038/mt.2016.38>.
- [14] L. P. Mark, R. W. Prost, J. L. Ulmer, M. M. Smith, D. L. Daniels, J. M. Strottmann, W. D. Brown, and L. Hachein-Bey, *Am J Neuroradiol* **2001**, 22(10), 1813.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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