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High-efficiency transduction and specific expression of ChR2opt for optogenetic manipulation of primary cortical neurons mediated by recombinant adeno-associated viruses

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ABSTRACT

In recent years, optogenetic approaches have significantly advanced the experimental repertoire of cellular and functional neuroscience. Yet, precise and reliable methods for specific expression of optogenetic tools remain challenging. In this work, we studied the transduction efficiency of seven different adeno-associated virus (AAV) serotypes in primary cortical neurons and revealed recombinant (r) AAV6 to be the most efficient for constructs under control of the cytomegalovirus (CMV) promoter. To further specify expression of the transgene, we exchanged the CMV promoter for the human synapsin (hSyn) promoter. In primary cortical-glial mixed cultures transduced with hSyn promoter-containing rAAVs, expression of ChR2opt (a Channelrhodopsin-2 variant) was limited to neurons. In these neurons action potentials could be reliably elicited upon laser stimulation (473 nm). The use of rAAV serotype alone to restrict expression to neurons results in a lower transduction efficiency than the use of a broader transducing serotype with specificity conferred via a restrictive promoter. Cells transduced with the hSyn driven gene expression were able to elicit action potentials with more spatially and temporally accurate illumination than neurons electrofected with the CMV driven construct. The hSyn promoter is particularly suited to use in AAVs due to its small size. These results demonstrate that rAAVs are versatile tools to mediate specific and efficient transduction as well as functional and stable expression of transgenes in primary cortical neurons.

1. Introduction

Optogenetic approaches have promoted *in vitro* and *in vivo* experiments investigating cellular signaling and neuronal network activity with high temporal and spatial resolution (Arenkiel et al., 2007; Gradinaru et al., 2007; Madisen et al., 2012; Avermann et al., 2012; Huber et al., 2008; Desai et al., 2011; Lin et al., 2013; Dawydow et al., 2014; Renault et al., 2015). Beyond modulating and monitoring neuronal network properties, optogenetic methods have been applied to control signaling in sperm (Jansen et al., 2015) and to stimulate gene expression (Folcher et al., 2014; Konermann et al., 2013; Ye et al., 2011). Optogenetic experiments, however, require efficient introduction and expression of light-sensitive proteins into cells or tissue.

The blue light-gated cation channel Channelrhodopsin-2 (ChR2) is an intensely studied optogenetic tool (Nagel et al., 2003, 2005b; Boyden et al., 2005; Li et al., 2005; Petreanu et al., 2007; Ishizuka et al., 2006; Dawydow et al., 2014). The heterologous expression of ChR2 in electrogenic cells such as neurons can be utilized to depolarize the cell's membrane potential, thereby evoking action potentials (Zhang et al., 2006; Wang et al., 2007). Since its discovery, numerous ChR2 variants have been engineered to improve properties, such as expression level, closing dynamics (Dawydow et al., 2014; Gunaydin et al., 2010), and stable photo-switching (Berndt et al., 2009). In addition to triggering action potentials, ChR2 can be employed to silence neuronal activity by changing its ion selectivity to anions (Wietek et al., 2014; Berndt et al., 2014). Fluorescent tags such as mKate and YFP fused to ChR2 have been used to identify cells expressing ChR2 and to assess its subcellular distribution

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(Shcherbo et al., 2007; Nagel et al., 2005a), as light-induced manipulation of neuronal electrical activity depends on the expression level of ChR2 in the cell membrane (Lin et al., 2013).

Furthermore, powerful gene delivery approaches are required for effective optical control of neuronal activity. However, standard transfection strategies can be problematic due to low cell viability and low specificity, especially for primary post-mitotic neurons (Watanabe et al., 1999; Martinez and Hollenbeck 2003; Hamm et al., 2002; Zeitelhofer et al., 2009; Karra and Dahm 2010). Therefore, we assessed recombinant adeno-associated viruses (rAAVs) as a tool for gene transfer. AAVs are non-pathogenic, have a low immunogenicity, and are known to transduce dividing and non-dividing cells (Blacklow et al., 1968; During 1997; Hernandez et al., 1999). There are numerous serotypes with different tissue tropisms, permitting specific transduction of a wide range of cell types (Howard et al., 2008); reviewed in Asokan et al. (2012).

In this work, we identified rAAV6 as the most efficient serotype for transduction of cortical-glial mixed cultures. We were first concerned which serotype of rAAV was most efficient at transducing cortical neurons. Therefore, we used the constitutively active cytomegalovirus (CMV) promoter (Wilkinson and Akrigg, 1992) to drive ChR2opt gene expression. The protein was detected to varying degrees in neurons as well as in glia cells from each of the applied serotypes. After exchanging the CMV promoter for the human synapsin (hSyn) promoter, ChR2opt expression was restricted to neurons, without loss of transduction efficiency. Other restrictive promoters might be considered as well, *e.g.* FNPY or CamKIIa, but the compact size of hSyn (only 485 bp, *vs.* approx. 2600 bp for FNPY (Nathanson et al., 2009) and 1290 bp for CamKIIa (Yizhar et al., 2011)) is favorable for packaging, even large gene constructs into AAV virions. Here, we succeeded in controlling electrical activity in primary cortical neurons expressing ChR2opt and in triggering action potentials by blue light stimulation of ChR2opt. Using this strategy, we were able to trigger responses at the single cell level with high temporal accuracy.

2. Materials and methods

2.1. Cortical cultures

Primary cortical cultures were prepared as described previously (Brewer et al., 1993). Briefly, cortices from embryonic day 18 (E18) Wistar rat (Charles River, Germany) brains were dissected and mechanically dissociated by trituration with a fire-polished, silanized pasteur pipette in 1 ml Hank's balanced salt solution without calcium or magnesium (HBSS-) (0.035% sodium bicarbonate, 1 mM pyruvate, 10 mM HEPES, 20 mM glucose, pH 7.4). The cell suspension was diluted 1:2 in HBSS+ (with calcium and magnesium) and non-dispersed tissue was allowed to settle for 3 min. The supernatant was centrifuged for 2 min at 200*g*. The pellet was resuspended in 1 ml Neurobasal medium (Gibco, Paisley, UK) with 1% B-27 (Gibco, Grand Island, NY, USA) and 0.5 mM L-glutamine (Gibco, Paisley, UK) and 50 μg/ml gentamicin (Sigma, Steinheim, Germany) (NB) per hemisphere. The cells were counted in a Neubauer counting chamber and plated in a concentration of 50,000 cells per well (24-well-plate) on poly-D-lysine (PDL, 10 μg/ml) coated 12 mm coverslips. Cells were kept at 37 °C, 5% CO_2 and 100% humidity in 500 μl NB. After 4 h, the medium was exchanged for 500 μl NB/well. Every third day, half of the medium was exchanged or, for rAAV transduced cultures, 100 μl NB medium was added in order to keep the neurons maximally exposed to the initially added rAAV particles.

This work was carried out with the approval of the Landesumweltamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Recklinghausen, Germany, in accordance with §6 TierschG., §4 TSchG i.V. and §2 TierSchVerV.

2.2. Plasmids and cloning

Codon optimization for expression in mammalian cells was performed on the H134R variant of Channelrhodopsin-2 from *C. reinhardtii* using the software package JCat (Grote et al., 2005). In order to facilitate identification of transfected cells without significant spectral overlap with Channelrhodopsin-2 absorbtion, the fluorescent protein mKATE was fused to the C-terminus of the channel. A linker of 15 nucleotides encoding the amino acid residues AVATI was inserted to separate the channel from the fluorophore mKATE. The 3′ end of mKATE was extended by 21 nucleotides coding for a FCYENEV motif that has been shown to serve as an ER export signal in a vertebrate potassium channel (Gradinaru et al., 2010). The final construct, which we deem ChR2opt, was synthesized by GeneArt AG (Regensburg, Germany) and subcloned into the TRex system's TetON plasmid backbone (Invitrogen, Darmstadt, Germany). This construct was validated in electrofection experiments using Lonza's Rat Nucleofector Kit and patch clamp electrophysiology in both HEK293 cells and primary cortical neurons. The gene encoding portion of the construct was then further subcloned to allow for packaging into AAV vectors.

The rAAV vector pscAGFPFG2 was kindly provided by Dr. Hildegard Büning (Labor für AAV-Vektorentwicklung, CMMC, Cologne, Germany). The pAAV-hSyn-RFP construct was obtained from Addgene (Addgene plasmid #22907). ChR2opt is available in Addgene plasmid #67958. The rAAV vectors psc-CMV-ChR2opt and psc-hSyn-ChR2opt were generated using restriction digest-based strategies. For the generation of the psc-CMV-ChR2opt vector, the ChR2opt-encoding fragment was inserted into the pscAGFPFG2 vector after excision of GFP. Based on this plasmid, the psc-hSyn-ChR2opt vector was generated by exchanging the CMV promoter for the hSyn promoter fragment.

2.3. Viral vector generation

Recombinant adeno associated viral (rAAV) vectors were generated by transient transfection of HEK293 cells (obtained from ATCC). HEK293 cells were cultivated in DH10 medium (DMEM + Glutamax (Invitrogen), 10% (v/v) FBS (Gibco), 1% (v/v) antibiotics/antimycotics (Invitrogen)) at 37 °C, 5% CO_2, and 95% relative humidity. Briefly, 24 h prior to transfection 10^7 cells were seeded on Ø 14.5 cm dishes (10 dishes/rAAV construct). Cells were triple-transfected with the vector plasmid providing the transgenic viral genome, as well as the helper plasmids pRC (Girod et al., 1999) and pXX6-80 (Xiao et al., 1998), using the calcium phosphate method modified from (Chen and Okayama, 1987). Plasmid DNA was diluted in $CaCl_2$ (250 mM) and HEPES-buffered saline (50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 7.12), incubated for 3 min at RT, and added to HEK293 cells. After 24 h incubation at 37 °C, 5% CO_2, and 95% relative humidity, the medium was exchanged for DH10 with reduced FBS (DMEM + Glutamax, 2% (v/v) FBS, 1% (v/v) antibiotics/antimycotics). Cells were harvested in PBS-M/K (in mM: NaCl 130, KCl 2.5, $MgCl_2$ 1, Na_2HPO_4 70, NaH_2PO_4 30, pH 7.4) after 24 h and collected by centrifugation.

Cells were lyzed in lysis buffer (in mM: NaCl 150, Tris/HCl 50, pH 8.5) by four freeze/thaw-cycles in liquid nitrogen and at 37 °C. Nucleic acids were digested with benzonase (50 U/ml; Merck) for 30 min at 37 °C. After removal of cell debris by centrifugation rAAV particles were enriched by density gradient centrifugation. The rAAV suspension was sub-layered with iodixanol (Sigma-Aldrich, Taufkirchen, Germany) solutions (15%, 25%, 40%, and 60% iodixanol) and centrifuged (264,000*g*, 4 °C, 2 h). The 40% iodixanol phase containing the virus particles