SHORT COMMUNICATION

Caspase-3 and GFAP as early markers for apoptosis and astrogliosis in shRNA-induced hippocampal cytotoxicity

Anne Günther1,*, Vince Luczak2, Ted Abel2 and Arnd Baumann1,‡

ABSTRACT

Genetic manipulation of cells and tissue by RNA interference has significantly contributed to the functional characterization of individual proteins and their role in physiological processes. Despite its versatility, RNA interference can have detrimental side effects, including reduced cell viability. We applied recombinant adeno-associated viruses by stereotaxic injection into the murine hippocampus to express different short hairpin RNA (shRNA) constructs along with eGFP. Tissue responses were assessed immunohistochemically for up to 8 weeks post-infection. Strong hippocampal degeneration and tissue atrophy was observed, most likely induced by high shRNA expression. The effect was entirely absent in mice injected with vectors driving only expression of eGFP. Active caspase-3 (Casp-3) and glial fibrillary acidic protein (GFAP) were identified as molecular markers and early indicators of adverse tissue responses. Our findings also demonstrate that detrimental effects of high shRNA expression in hippocampal tissue can be monitored even before the onset of tissue degeneration.

KEY WORDS: RNAi, Neurotoxicity, Hippocampus, Adeno-associated virus, Tissue atrophy

INTRODUCTION

RNA interference (RNAi) is a popular technique for uncovering the function of individual proteins in cells or organisms (Shan, 2010). As short hairpin RNAs (shRNAs) can be designed specifically for any structural gene, they provide an extensive toolbox for RNAi-based manipulation of gene expression. However, some adverse effects of shRNA expression, including reduced cell viability, have been observed (Ehloet et al., 2010; Grimm et al., 2006).

Certain aspects of shRNA-induced cytotoxicity have been described in previous studies (Börner et al., 2013; Bridge et al., 2003; Grimm et al., 2010; Slez et al., 2003; Yi et al., 2005). Notably, shRNA-induced neurotoxicity is affected by various factors, such as shRNA sequence, shRNA expression, including reduced cell viability, have been observed (Ehloet et al., 2010; Grimm et al., 2006).

Animals and stereotaxic injection

A total of 10 male C57BL/6j mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used in this study for viral injection and subsequent immunohistochemical analysis. Animals were housed in groups of 4–5 under standard conditions with access to food and water ad libitum on a 12 h:12 h light:dark cycle. Experiments were carried out in accordance with National Institutes of Health guidelines and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Mice received bilateral intra-hippocampal injections of rAAV9 vectors encoding enhanced green fluorescent protein (eGFP) and shRNA constructs at 8–10 weeks of age. Stereotaxic injection of 1 µl viral suspension (0.2 µl min−1) per hippocampus was performed at antero-posterior −1.9, medio-lateral ±1.5 and dorso-ventral −1.4 from Bregma using...
a 33 gauge beveled NanoFil needle, a NanoFil syringe and a MicroSyringe Pump Controller (World Precision Instruments, Sarasota, FL, USA). After surgery, mice were single-housed and given 5 days to recover before pair-housing. Injection and tissue collection were performed during the light phase.

**Primary hippocampal neurons**

Hippocampal tissue from 1–3-day-old mice (C57BL/6 strain from an in-house animal breeding facility) was prepared in ice-cold HBSS (Hanks’ balanced salt solution). Hippocampi were incubated in papain solution [DMEM (Invitrogen), 25 U ml\(^{-1}\) papain, 1.6 mmol l\(^{-1}\) cysteine, 1 mmol l\(^{-1}\) CaCl\(_2\), 0.5 mmol l\(^{-1}\) EDTA] at 37°C for 20 min and subsequently in inactivating solution [2.5% (w/v) trypsin inhibitor, 2.5% (w/v) albumin, in FCS solution] at 37°C for 5 min. Cells were triturated in FCS solution [DMEM, 100 U ml\(^{-1}\) streptomycin per ml, 10% FCS, 0.1% (v/v) MITO+ serum extender]. Primary hippocampal neurons (PHNs) were plated on µ-Slide 4 Well slides (Ibidi, Martinsried, Germany) pre-coated with poly-D-lysine (0.2 mg ml\(^{-1}\) poly-D-lysine, 50 mmol l\(^{-1}\) H\(_3\)BO\(_3\), 25 mmol l\(^{-1}\) Na\(_2\)B\(_4\)O\(_7\), pH 8.5). PHNs were maintained in NBA medium [Neurobasal A Medium (Invitrogen), 100 U ml\(^{-1}\) streptomycin per ml, 2% (v/v) B27-supplement (Invitrogen) and 1% (v/v) Glutamax] at 37°C, 5% CO\(_2\) and 95% relative humidity for 14 days.

**Quantification of gene expression by real-time PCR**

Total RNA was isolated from PHN cultures using the DNA/RNA/Protein AllPrep\textsuperscript{\textregistered} Kit (Qiagen, Hilden, Germany) according to the supplier’s protocol. RNA samples were split for two independent first-strand cDNA syntheses using Oligo-d(T) primers (Qiagen) and Moloney Murine Leukemia Virus reverse transcriptase (M-MLV-RT, Life Technologies) according to the supplier’s protocol. Thermocycling was performed in a LightCycler 1.5 (Roche, Mannheim, Germany) and in a LightCycler 480 (Roche, Mannheim, Germany) pre-coated with poly-D-lysine (0.2 mg ml\(^{-1}\) poly-D-lysine, 50 mmol l\(^{-1}\) H\(_3\)BO\(_3\), 25 mmol l\(^{-1}\) Na\(_2\)B\(_4\)O\(_7\), pH 8.5). PHNs were maintained in NBA medium [Neurobasal A Medium (Invitrogen), 100 U ml\(^{-1}\) streptomycin per ml, 2% (v/v) B27-supplement (Invitrogen) and 1% (v/v) Glutamax] at 37°C, 5% CO\(_2\) and 95% relative humidity for 14 days.

**Antibodies and immunohistochemistry**

For immunohistochemical analysis, brains were dissected after transcardial perfusion with ice-cold PBS followed by 4% paraformaldehyde in PBS under deep anesthesia. Tissues were cryo-protected in 30% sucrose (in PBS) for 2 days, embedded in Tissue Tek (Sakura Finetek, Zouterwoude, The Netherlands), and coronal cryo-sections (30 μm) were prepared.

For immunohistochemical staining, sections were incubated in 0.3% (v/v) H\(_2\)O\(_2\) for 30 min at room temperature before unspecific binding sites were blocked for 1 h at room temperature in blocking solution [0.75% (v/v) Triton X-100, 5% (v/v) normal goat serum (NGS), 5% (v/v) normal donkey serum (NDS), in PBS]. Subsequently, slices were incubated at 4°C for 3 days with primary antibodies (active Casp-3, 1:50, ab2302, Abcam, Milton, UK; GFAP, 1:500, MAB3402, Millipore, Hohenbrunn, Germany) diluted in incubating solution [0.75% (v/v) Triton X-100, 0.5% (v/v) NGS, 0.5% (v/v) NDS, in PBS]. After washing with PBS, samples were incubated at room temperature for 4 h with secondary antibodies (gt-α-mAb4647, 1:50, A-21235, Life Technologies, Carlsbad, CA, USA; gt-α-rbA555, 1:500, A-21428, Life Technologies) diluted in incubating solution. For DAPI staining, NucBlue Reagent (Life Technologies) was used according to the supplier’s protocol. Slices were washed, transferred onto slides (SuperFrost Plus, Menzel, Braunschweig, Germany) and, finally, a coverslip was fixed on the samples with Aqua-Poly/Mount (Polysciences, Eppelheim, Germany). Fluorescent images were obtained with an inverted confocal microscope (TCS SP8, Leica, Wetzlar, Germany).

**RESULTS AND DISCUSSION**

We applied recombinant adeno-associated virus serotype 9 (rAAV9) vectors for injection into the murine hippocampus. Viral vectors were designed to mediate expression of shRNA constructs targeting independent genes, i.e. hcn1 and luc. Because of their low immunogenicity, rAAV vectors are generally considered to be an optimal tool for genetic manipulation in vivo.

We designed viral constructs to express two distinct shRNA-encoding fragments as well as eGFP for the identification of infected cells (Fig. 1). Prior to stereotaxic injection, viral constructs were tested for knockdown efficiency and specificity of endogenous hcn1 and hcn2 expression in primary hippocampal neurons by qPCR. We assessed cell viability and morphology in primary cultures by immunofluorescence microscopy of neurons co-expressing shRNA and eGFP in comparison either to neurons transduced with rAAV vectors mediating only eGFP expression or to non-transduced control cultures. No detrimental effects on the health of neurons were observed in these cultures (data not shown).

**Fig. 1. Schematic representation of adeno-associated viral vector constructs.** Viral vectors were designed to encode enhanced green fluorescent protein (eGFP) under the control of the neuron-specific CaMKII\(_{\alpha}\) promoter and two short hairpin RNA (shRNA) sequences under the control of the human U6 (hU6) promoter. The shRNA expression cassettes were placed upstream of the eGFP expression cassette. The constructs were flanked by inverted terminal repeats (ITR) for viral packaging. (A) The control construct encoding only eGFP with the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). (B) The luc-targeting construct. (C) The hcn-targeting construct. Sequence segments are indicated by colored bars.
After establishing the functionality of shRNAs in vitro, rAAV9 vectors were injected into the dorsal hippocampus. Animals were killed at 3–8 weeks post-infection. We observed widespread eGFP fluorescence in hippocampal tissue after a single stereotaxic injection of rAAV9 constructs (Fig. 2). Expression of eGFP was driven by the neuron-specific CaMKIIα promoter, whereas shRNAs were expressed under the control of the constitutive hU6 promoter. Because of vector design, transduced neurons can be identified on the basis of eGFP fluorescence, whereas transduced non-neuronal cells do not show eGFP fluorescence, but could still express shRNAs.

Some animals injected with shRNA-encoding rAAVs displayed neuronal degeneration at 3 weeks post-infection (Fig. 2D). In contrast, eGFP expression alone did not induce an adverse tissue response (Fig. 2B), confirming that the degeneration was not due to eGFP expression per se. Tissue degeneration advanced with time and was even stronger in animals injected with luc-targeting control constructs than with hcn-targeting constructs.

In order to further assess the observed tissue degeneration, we examined the expression of potential cellular stress markers (Fig. 3), including Casp-3 and GFAP. As all of the applied rAAV constructs encoded eGFP as a marker, transduction of the dorsal hippocampus was monitored based on eGFP fluorescence. Strong immunoreactivity of Casp-3 and GFAP was observed in the dorsal hippocampus at 3 weeks post-infection even though tissue degeneration was not yet apparent. Neither Casp-3 nor GFAP was detected in the ventral hippocampus at this time point. At 4 weeks post-infection, degeneration in the dorsal hippocampus became apparent and immunoreactivity of Casp-3 and GFAP was also detected in more ventral regions of the hippocampal formation. Notably, detection of Casp-3 and GFAP was always in concordance with eGFP expression.

RNAi-based strategies frequently use viral vectors for specific manipulation of gene expression at defined tissue sites in the rodent brain. Previous studies have employed shRNA constructs without apparent detrimental effects on cell viability in the hippocampus (Mosser et al., 2015; Omata et al., 2011; Kim et al., 2012). In this study, we applied virus titers within the range chosen by most rAAV-based RNAi studies in the CNS, i.e. between 10^9 and 10^11 total virus particles. We examined three different eGFP-encoding rAAV9 constructs. Viral vectors mediated expression of:
(1) shRNAs targeting endogenous hcn genes, (2) shRNAs targeting
the luc gene, which is not endogenously expressed in mammals and
is used widely as a control in similar studies, and (3) a negative
control without a shRNA-encoding sequence. Surprisingly, even
though no adverse effects of the shRNA-encoding constructs were
observed in primary hippocampal neurons cultivated in vitro,
rAAV9-mediated shRNA expression induced strong cellular
degeneration in the hippocampus of mice (see Fig. 2). The
detrimental effects were independent of the encoded shRNA
sequence as they were observed for constructs targeting both
endogenous and non-endogenous transcripts (i.e. hcn and luc), but
were not apparent in animals treated with rAAVs encoding only
eGFP.

Cytotoxic effects of shRNAs have previously been shown to be
casused by overloading of the endogenous miRNA machinery
(Börner et al., 2013; Grimm et al., 2006, 2010; McBride et al.,
2008; Yi et al., 2005). These observations were corroborated by the
pronounced shRNA dose dependency of cytotoxicity (Grimm,
2011). However, as the total number of virus particles may vary
depending on the applied construct and the treated CNS region,
potential effects on the tissue must be assessed for each construct,
individually. As AAV9 is known to transduce neurons as well as
astrocytes (Aschauer et al., 2013), we cannot rule out that
constitutive shRNA expression in astrocytes might be an
additional factor in the observed hippocampal degeneration.

So far, reports examining shRNA-induced cytotoxicity have not
consistently described the expression of molecular markers related
to tissue degeneration (Bauer et al., 2009; Hutson et al., 2014;
Martin et al., 2011; McBride et al., 2008). To address this issue in
the context of the observed hippocampal degeneration, we assessed
expression of Casp-3, a marker of apoptosis (Ashkenazi and
Salvesen, 2014), and GFAP, a marker of astroglial activation
(Sofroniew, 2009). Expression of both molecular markers strongly
correlated with rAAV9-mediated shRNA expression.

Our results emphasize that detrimental effects of shRNA
application in vivo might not always be readily identified in cell
culture. We suggest that the design of shRNA-based studies should
involve careful consideration of several parameters, such as shRNA
dose (Grimm, 2011), promoter choice (Lebbink et al., 2011; Sun
et al., 2013), AAV serotype (Ehlert et al., 2010) and construct
backbone (Boudreau et al., 2009; Han et al., 2011; McBride et al.,
2008). These factors should subsequently be tested with respect to
titer, construct and specificity in vivo. Furthermore, RNAi-based
studies should take into account that the detrimental effects
observed here for standard C57BL/6J mice may differ depending
on the employed mouse strains. However, the identification of
reliable markers, such as increased Casp-3 and GFAP expression,
for monitoring shRNA-induced tissue stress even before the onset
of degeneration is a requirement for validating behavioral
experiments that are based on RNAi strategies.

Acknowledgements
We gratefully acknowledge the assistance of Dr H. Bünning with establishing the rAAV
cell culture. We thank Dr D. Kaschuba for her help with the identification of hcn-
targeting shRNA constructs.

Fig. 3. Markers of shRNA-induced tissue degeneration in the hippocampus. Mice were injected with rAAV encoding hcn-targeting shRNA sequences. Immunohistochemical analysis of animals was carried out at 3 and 4 weeks post-infection. Positions of sections along the antero-posterior axis of the brain are indicated schematically. DAPI staining of nuclei is depicted in gray, eGFP fluorescence in green, caspase-3 (Casp-3) staining in red and glial fibrillary acidic protein (GFAP) staining in cyan. Proteins were stained with specific antibodies and examined by confocal microscopy. Z-stacks (5×3 µm sections) were registered and average intensity projections were generated. Scale bars, 1 mm.
Competing interests
The authors declare no competing or financial interests.

Author contributions
A.G., A.B. and T.A. designed the study. A.G. planned and cloned the rAAVs. A.G. and V.L. performed AAV injection. A.G. performed the immunological analysis. A.G. wrote the manuscript. A.G., A.B. and T.A. revised the manuscript.

Funding
This work was supported by the National Institutes of Health (grant RO1 MH 099544 to PI: T.A.) and the National Science Foundation (grant 1515458 to PI: T.A.). Deposited in PMC for release after 12 months.

Supplementary information
Supplementary information available online at http://jeb.biologists.org/lookup/doi/10.1242/jeb.154583.supplemental

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