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**Title**

A clinical-grade gene therapy vector for pharmacoresistant epilepsy successfully overexpresses NPY in a human neuronal cell line.

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**Abstract**

**Purpose** Epilepsy is a common neurological condition characterised by recurrent unprovoked seizures and often treatable with appropriate medication. However, almost 30% of cases are pharmacoresistant and while a proportion of these may be amenable to respective surgery, a gene therapy approach could be an attractive alternative option. Neuropeptide Y (NPY) has anticonvulsant and anti-epileptogenic properties in animal

models of temporal lobe epilepsy when delivered by an adeno-associated viral (AAV) vector. Here we sought to demonstrate successful secretion of NPY from AAV-transduced human neuronal cells, which would be essential in planning any clinical trial.

Methods A human neuroblastoma cell line (SH-SY5Y) was used to assess *in vitro* whether an AAV vector manufactured to clinical-grade protocols would be effective at transducing these cells to express NPY. Optimal transduction efficiency was first achieved with retinoic acid and tetradecanoylphosphol-13-acetate (TPA) treatment, prior to exposure to AAV1-green fluorescent protein (GFP) reporter vector, AAV1-NPY therapeutic vector or sham treated with no vector. Levels of NPY in cell supernatants were determined using two antibody-based methods.

Results We found that the levels of NPY released into the cell culture media supernatant, and protein extracts of the cell pellet, were significantly higher following exposure to AAV1-NPY than when compared to either a control GFP reporter vector (AAV1-GFP) or sham treated controls.

Conclusion This first demonstration that an AAV-NPY construct can successfully transduce human neuronal cells supports the pre-clinical development of a clinical trial using AAV-based NPY for pharmacoresistant epilepsy.

Keywords (3-6): epilepsy; AAV gene therapy; neuropeptide Y; SH-SY5Y cell line.

## Introduction

Epilepsy is a chronic central nervous system disorder characterised by recurrent spontaneous seizures, which can be disabling, result in injury and may occasionally be fatal. Although current pharmacotherapies are effective in 60-70% of individuals [1], there

remains a pressing need to develop alternative treatment strategies for the cases in which the pharmacological armamentarium fails. Non-pharmacological approaches, if beneficial, may also reduce the burden of anti-epileptic medication, thereby minimising the side effects associated with drug therapy.

Surgical resection of epileptogenic tissue, for example hippocampal sclerosis or focal cortical dysplasia, may be suitable for a proportion of drug-resistant patients. However, surgical intervention is, at best, effective in 70% of cases [2] and may also be associated with complications. Novel, safe therapeutic approaches that not only control the symptoms of seizures, but also aim to modify the course of the disease would therefore be of additional benefit.

In recent years, gene therapy has emerged as an attractive and viable treatment strategy for HS. Pre-clinical gene transfer studies in epilepsy have explored multiple different vectors and products. Robust data support the potential of the endogenous anticonvulsant neuropeptide Y (NPY) with an adeno-associated viral (AAV) vector to target the epileptogenic area [3–5]. NPY is critically involved in the regulation of neuronal network excitability while evidence from animal models of HS shows that transduction of NPY is safe and effective in

reducing seizure frequency [4]. Perhaps most importantly, trials in Parkinson's disease [6] and choroideremia [7] have shown that AAV transduction can be beneficial and safe in humans.

The aim of this study was to generate proof-of-concept data that an AAV1 vector with an optimized expression cassette coding for NPY is capable of transducing human neuronal cells *in vitro* to express NPY.

## Methods

*Plasmid construction and vector production* Human prepro-NPY (ppNPY) cDNA was subcloned into an optimised expression cassette [3] (Figure 1). The same expression cassette containing GFP was used as a control. Both constructs were packaged into recombinant AAV serotype 1 (AAV1-NPY and AAV1-GFP) and purified according to Good Manufacturing Practice (GMP) standards suitable for use in clinical trials in humans, as described in detail elsewhere [8].

*Cell culture* SH-SY5Y human neuronal cells (ECACC #94030304) were cultured and maintained according to an established method [9]. Differentiation was induced 24 hours after plating by changing the media to that containing retinoic acid (RA, 1E-05M) and tetradecanoylphorbol-13-acetate (TPA, 1.6E-08M), which were maintained in the media throughout. After 48 hours media was renewed and virus added at a suitable multiplicity of infection (MOI). Cell supernatants were collected over time, and correspondent cell lysates harvest in supplemented lysis buffer for protein quantification.

*Antibody-based assays* NPY protein levels in cell supernatants were determined by an Enzyme Immunoassay (EIA) (RAB0387, Sigma-Aldrich) and a sandwich ELISA (EZHNPY-25K, Millipore) according to the manufacturer's instructions. Serial dilution curves for standard controls were freshly made and run on each assay. Optical density was recorded at 450 nm (and 570 nm for reference) using a plate reader.

## Results and Discussion

During the past few years, several groups have explored gene therapy as a treatment strategy for epilepsy by manipulating endogenous genes with promising results [10]. Among the most favoured candidates is NPY, where local delivery to the epileptogenic zone offers the possibility of modifying the neuronal network and treating both seizures and associated co-morbidities without the side-effects associated with using systemic drugs. As patients with pharmacoresistant epilepsy do not necessarily have a genetic deficit in the production of NPY, a gene therapy approach would be to provide neuromodulation via gene augmentation. This is a different strategy to that employed in gene replacement therapy as has been used for inherited retinal disease and is more similar to the approach used in clinical trials for Parkinson's disease, where an analogous vector has been used. Here the construct used contains an optimised expression cassette driving human prepro-NPY cDNA [3,8]. A similar construct was designed to express GFP and serve as a reporter vector (Figure 1A).

**(A)** The expression cassette includes the human prepro-NPY (ppNPY) cDNA driven by a hybrid of the human cytomegalovirus (CMV) upstream enhancer with the chicken  $\beta$  actin promoter (CAG), the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and a bovine growth hormone polyadenylation signal (bGH pA), all flanked by the AAV serotype 2 inverted terminal repeats (ITR) and packed into recombinant AAV serotype 1 (AAV1-NPY). NPY was replaced by GFP to generate a reporter vector (AAV1-GFP). **(B-G)** GFP expression levels after 6 days in cells transduced with AAV1-GFP at MOI 1000 (B), 5000 (C) and 10000 (D), and their corresponding bright field images (E-G). The boxed MOI was selected for further investigations. Scale bar = 100  $\mu$ m.

First we assessed the ability of the AAV1-packaged vector to successfully transduce a human neuronal cell line. In order to replicate the clinical state of transducing fully differentiated adult neuronal cell types, the SH-SY5Y were first cultured in RA and TPA, thereby developing a phenotype and morphology which more closely resembled neurons [9,11]. After 48 hours, cells were transduced with AAV1-GFP at a range of MOI to study the onset of transgene

expression and its peak. Cells were imaged at day 2, 4 and 6 post-transduction using identical acquisition settings for all MOI tested (data not shown). We found that, by day 6, MOI 5000 showed readily detectable expression of GFP (Figure 1C). These data showed that the viral construct was able to transduce human neuronal cells *in vitro*, and hence the MOI of 5000 was selected for further AAV1-NPY investigations.

Next, cells were transduced at 5000 MOI with AAV1-GFP reported vector, AAV1-NPY therapeutic vector, or sham-treated with no vector (in triplicate). Cell supernatants were collected at baseline and day 2, 4 and 6 post-transduction. A time course of NPY protein levels in cell supernatants was determined by an EIA and a sandwich ELISA (Figure 2A and 2B).

**(A) (B)** NPY levels were determined by EIA **(A)** and ELISA **(B)** at day 2, 4 and 6 post-transduction with AAV1-GFP or AAV1-NPY at a MOI of 5000. The grey bar refers to the baseline levels of NPY prior to transduction. Symbols/lines are mean value of 3 replicates  $\pm$  SEM. Data was analysed by a two-way ANOVA with Bonferroni's multiple comparisons test. **(C) (D)** NPY levels at day 4 were normalised to protein content (pg/mL/ $\mu$ g) for both EIA **(C)** and ELISA **(D)** quantifications. Levels of NPY in AAV1-transduced cells were compared to sham-treated cells by one-way ANOVA with Bonferroni's multiple comparisons test (N=3). \* $p$ <0.05, \*\* $p$ <0.01; \*\*\* $p$ <0.001; \*\*\*\* $p$ <0.0001.

A two-way ANOVA analysis with treatment and time as factors found that treatment is a highly significant factor for both methods (EIA,  $p$ =0.0003; ELISA,  $p$ <0.0001) while time is significant only in ELISA quantification ( $p$ =0.0002). Bonferroni's multiple comparison tests showed that NPY levels were significantly increased in AAV1-NPY treated cells compared to sham-treated cells at day 4 and 6 for EIA (day 4,  $p$ =0.034; day 6,  $p$ =0.0053), and highly significantly increased at all time points for ELISA (day 2,  $p$ =0.0002; day 4 and 6,  $p$ <0.0001). Moreover, NPY levels in supernatant showed no significant pairwise difference between sham-treated and AAV1-GFP treated cells for any time point analysed.

With the quantitative methods used here (EIA and ELISA), it is not possible to differentiate between NPY that comes from the AAV transgene and that which may be produced endogenously. SH-SY5Y cells do express NPY in culture and it is known that neuronal differentiation, as used here, causes cells to reduce endogenous production [12]. This is also seen in our data; in AAV1-GFP or sham treated groups the levels of NPY in cell culture supernatant at day 2 were lower than the baseline values just prior to vector exposure (day 0) and as the experiment and neuronal differentiation continued, NPY levels in supernatant remained stable or continued to fall. Furthermore, the levels of NPY in AAV1-GFP and sham treated groups behaved identically, indicating there was not a non-specific change in the endogenous levels of NPY in reaction to AAV exposure. This is in contrast to the situation in the AAV1-NPY treated group where NPY levels in the supernatant dropped from baseline initially but then increased consistently over time (EIA dataset) or remained at or above the baseline level for the duration of the experiment (ELISA data). Thus, the control groups would have revealed any changes in endogenously produced NPY that are due to the differentiation process (i.e. a reduction) or non-specific reactions to AAV exposure (there are none). Hence we conclude that the increase in NPY seen with AAV1-NPY treatment is driven by the transgene.

We then repeated the transduction experiment to rule out the influence of cell numbers on NPY levels in the supernatant. Total protein contents were quantified and NPY levels in the supernatant normalised to the corresponding protein content at day 4 post-transduction (Figure 2C and 2D). This confirmed that AAV1-NPY leads to an increase in NPY expression, with one-way ANOVA analysis with a Bonferroni's multiple comparisons test showing significant pairwise difference in NPY levels between sham-treated and AAV1-NPY treated groups by both EIA ( $p=0.023$ ) and ELISA ( $p=0.0053$ ). As before, there was no significant

difference in NPY levels between sham-treated and AAV1-GFP treated cells for any of the methods used. These results showed that the AAV1-NPY vector tested led to a significant overexpression of human NPY protein in a human neuronal cell line.

## Conclusion

We describe how a well-characterised human neuronal cell line can be differentiated using a reproducible technique and manipulated in order to facilitate AAV1 transduction *in vitro*. We further validate this by using a potentially therapeutic vector that leads to a significant overexpression of human NPY protein following transduction. Moreover, this transgene expression was associated with NPY release from the transduced cells. As the first demonstration of transduction of human cells with clinical-grade NPY, this work brings the concept of using AAV-based NPY gene therapy for pharmacoresistant epilepsy a step closer to being tested in the clinic.

## Author contributions

Conceptualization: MIP, ARB, ALG, AS, REM; Methodology: MIP, ARB, MJD; Formal Analysis: MIP, ARB; Investigation: MIP, ARB; Writing – Original draft: MIP, ARB; Writing – Review & Editing: MIP, ARB, ALG, MJD, AS, REM; Supervision: AS, REM; Funding acquisition: REM.

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**Figure 1. Treatment of SH-SY5Y with AAV1-GFP reporter vector results in GFP expression.**

**Figure 2 – Successful NPY overexpression in SH-SY5Y cells transduced with AAV1-NPY.**

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