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Transduction of PACAP38 protects primary cortical neurons from neurotoxic injury

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Abstract

Neurotrophic factors such as pituitary adenylate cyclase activating polypeptide (PACAP38) are promising therapeutics for neurodegenerative diseases. However, delivery of trophic factors into brain neurons remains a challenge. The objective of this study is to determine whether adeno-associated virus (AAV) can mediate PACAP38 gene delivery into neurons *in vitro* and if transduction of AAV/PACAP38 into cortical neurons protects cells against neurotoxic insult. Primary cortical neuronal cultures are transduced with rAAV/PACAP38/GFP and cell survival against the nitric oxide releasing neurotoxin sodium nitroprusside (SNP) determined. GFP expression, a surrogate marker for successful transduction, is detected using fluorescent microscopy. The results show expression of GFP transgene and AAV capsid proteins in neurons. PACAP38 transduction significantly increases cell survival of neurons exposed to SNP. These results support the feasibility of using AAV-mediated delivery of PACAP38 to enhance neuronal survival and suggest that AAV-delivered PACAP38 maybe a therapeutic strategy for neurodegenerative diseases.

Keywords

neurotrophic; viral vector; neuronal cell death; PACAP38; AAV

Introduction

Neurotrophic factors support neuronal survival and function in the CNS and are promising therapeutics for neurodegenerative diseases. The protective effects of neurotrophic factors have been demonstrated in many neuropathological conditions [7,17]. Pituitary adenylate cyclase-activating polypeptide 38 (PACAP38) is a pleiotropic neuropeptide that belongs to the secretin/glucagon/vasoactive intestinal peptide family [4,28]. In the adult brain, PACAP38 inhibits apoptotic cell death, modulates neurotransmitter release, and is strongly upregulated in several models of nerve injury [2,23,24,32,36]. PACAP38 protects cerebellar granule cells from injury induced by ethanol and oxidative stress [37,38]. In the neuronal-like PC12 cells, PACAP38 protects against the cytotoxicity of A β [20]. In rats, intracerebroventricular administration of

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low doses of PACAP38 improves memory [27]. These data suggest that PACAP38 is a promising therapeutic for the treatment of neurodegenerative diseases.

A barrier to the use of neurotrophic therapies is the difficulty of delivering peptides into brain neurons. Viral vectors such as adeno-associated virus (AAV) could be used to deliver genes for neurotrophic proteins. AAV is non-pathogenic in humans and successful transduction into the brain evokes no significant toxicity or inflammatory response [3,33]. The AAV vector has been successfully utilized to deliver a lysosomal enzyme that is deficient in a mouse model of Niemann-Pick's disease. Direct injection of an AAV-acid sphingomyelinase construct into the hippocampus significantly decreases brain pathology [21]. By injecting AAV constructs into the brain, AAV-mediated expression of galactocerebrosidase in brain results in attenuated symptoms and extends life span in a murine model of globoid cell leukodystrophy [25].

The objective of this study is to determine whether AAV-mediated gene delivery of PACAP38 can effectively transduce primary neurons *in vitro* and whether AAV/PACAP38 transduction improves neuronal survival against nitric oxide and oxidative injury.

Materials and Methods

The PACAP38 transcript was generated by RT-PCR amplification of mouse brain RNA using the primer pair PCP-1F 5' atccttaacgaggcctaccg and PCP-3R 5' acacacgagcgatgactgtt. The resulting 360 bp amplicon was used to construct the AAV/PACAP38/GFP genome as described for other AAV vectors [1,18,19].

The rAAV/PACAP38/GFP virus stock was made by transfecting HEK293 cells using FuGENE6 (Roche Diagnostics, Indianapolis, IN) [6,41]. The cell line is preferentially transformed by adenoviruses and has neuronal-like properties [29,42]. More than 95% of cells showed GFP expression at 3 days post-infection, indicating successful transduction while parallel cultures of HEK293 cells mock-infected with 1X PBS show no GFP fluorescence (data not shown). The transduced cells were then processed to collect the virus [19,42]. The titer of the virus stocks was determined by real-time PCR (iCycler, BioRad, Hercules, CA) using the PCP1F/PCP3R primers [1,39] and calculated to be 10^7 encapsulated genomes (EG)/ml.

Rat cerebral cortical cultures were prepared from cortices isolated from 18 day gestation fetuses [11,26] and transduced with the recombinant virus stock 4 days after plating at a multiplicity of infection of 10^7 EG/ml. Transduced cells were exposed to sodium nitroprusside (SNP) 3 days post-infection and cell survival measured using the MTT based Cell Titer 96 AQueous One Cell Proliferation Assay Kit (Cat. No G3581) from Promega. The amount of sample cell survival was compared to untreated controls. Comparison of survival of AAV/PACAP/GFP transfected cells to mock-infected cells was used throughout this paper, as AAV/GFP itself has little or no effect on neuronal cell survival [14].

Lipofection

Neuronal cells were lipofected at 4 days after plating using the cationic liposome-mediated transfection reagent N-[1(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) (Roche Diagnostics, Indianapolis, IN) with final PACAP38 (GeneScript, Piscataway, NJ, USA) concentration at 100 nM.

PACAP38 and AAV protein expression was measured by indirect ELISA. Primary antibodies for PACAP38 and adeno-associated virus capsid proteins were obtained from Peninsula Laboratories (San Carlos, CA, Cat. No. T-4478) and Fitzgerald Industries International, Inc. (Concord, MA Cat. No. RDI-PRO61058) respectively.

Prism version 4.0 software (GraphPad Inc., San Diego CA) was used for graphical presentation and statistical analyses. Statistical significance was defined as $p < 0.05$. Data are presented as mean $\pm$ SEM of values obtained from three separate experiments performed in triplicate.

Results

By day 4, cells demonstrated extensive neuronal processes and were well differentiated. GFP transgene expression was observed in infected neuronal cultures 72 h after infection, indicating successful transduction (Figure 1). The transduction efficiency was lower than that observed in the HEK293 cells. Exposure of neuronal cultures to SNP (1 mM) for 4 h resulted in a significant ($p < 0.001$) decrease in cell survival in untransfected cells. In contrast, SNP treated neurons transduced with AAV/PACAP38 show cell survival comparable to the untreated cells (Figure 2).

The ability of PACAP38 transduced cells to withstand SNP insult was also determined in neurons that were transduced by either AAV or lipofection (DOTAP). In neuronal cultures exposed to 0.5 mM SNP for 24 h, PACAP38 lipofection using DOTAP did not increase cell survival. Neurons transduced with rAAV/PACAP38/GFP showed a significant increase in cell survival ($p < 0.001$) compared to both untransfected cells and PACAP38 lipofected cells (Table 1). Examination of AAV-and lipofection-mediated PACAP38 delivery in SNP-treated neuronal cultures over an extended time course (0 – 72 h) showed that AAV transduced cells had higher survival than both the untransfected cells and cells lipofected with DOTAP at 24 to 72 h (Figure 3). These results indicate that AAV-mediated PACAP38 delivery produced greater protection than lipofection-mediated PACAP38 delivery.

We also determined the expression level of PACAP38 in transduced and untransduced neurons before and after exposure to 1 mM SNP for 4 h. The level of PACAP38 in untransduced cells diminished slightly with SNP treatment, compared to cells not exposed to SNP. PACAP levels in lipofected neurons were unaffected by SNP. In contrast, levels of PACAP38 and AAV capsid proteins were significantly ($p < 0.05$) higher in AAV transduced cells treated with SNP compared to cells not exposed to SNP (Figure 4).

Discussion

In our study we report expression of AAV capsid proteins and PACAP38 in AAV-transduced cells and their upregulation in response to SNP treatment. Since the PACAP38 transgene is in tandem with the AAV vector construct, the data showing up-regulation of AAV capsid proteins and PACAP38 could be interpreted to mean that increased PACAP gene expression itself is induced by SNP treatment and that AAV protein increase is a secondary effect. Alternatively, the AAV genome may replicate in response to SNP treatment and PACAP38 upregulation is secondary. Although the data support either interpretation, we believe that the increase is due to induction of PACAP gene expression based on literature reports of injury-induced expression of PACAP and other neurotrophic factors [10,22,31,35].

An efficient and effective delivery system to transport PACAP38 and other neurotrophic factors in the brain is important to the development of these factors as effective therapeutics for neurodegenerative disease. AAV has many attributes (e.g. safety, efficiency, gene expression level, and stability) that have fostered interest in it as a vector for gene therapy [12]. Genes delivered in AAV vectors have demonstrated stable, high-level expression in many animal models and the AAV virus has not been associated with any human disease. Our results show that cortical neurons are transduced by AAV and that delivery of PACAP38 to these cells via AAV enables neurons to withstand injury by SNP. The potential for AAV as a useful reagent for transport of PACAP38 to brain neurons is further highlighted by our results showing the

inability of PACAP38 lipofection to ameliorate SNP-induced injury in cortical neurons. PACAP38 is especially attractive as a therapeutic neurotrophic factor because it can stimulate its own expression (autocrine) as well as that of other growth factors (paracrine) such as BDNF [13,22,31].

Although transduction efficiency appears lower in neurons than that observed in the HEK293 cells, the strategy appears to have considerable efficacy. Detectable transgene expression and 100% transduction efficacy at 5–7 days were reported using an AAV-2-hSYN promoter driven vector transduced into primary hippocampal neurons at the day of plating [30]. It is possible that the transduction efficiency could have been higher in the current study if performed immediately after plating (day 0). However, transduction of neuronal cultures at day 4 when the neurons are more mature reflects the neuronal phenotype present in the adult brain.

Although delivery of neurotrophic factors such as PACAP38 across the blood-brain-barrier remains an obstacle in future work, AAV can currently be used to deliver genes to neurons when injected directly into the brain. AAV-mediated delivery of endothelin converting enzyme 1 into the hippocampus reduces amyloid beta deposition in a transgenic mouse model of AD [5]. Similarly, injection of AAV encoding anti-A β single-chain antibody into the corticohippocampal regions induces clearance of amyloid plaques in AD mice models [9]. AAV has also successfully delivered genes for GAD65 [16] VEGF [34] and GDNF [40] in rat models of Parkinson's disease. Results from a recent Phase I clinical trial utilizing AAV-mediated delivery of aromatic L-amino acid decarboxylase gene into the putamen demonstrate sustained gene expression and modest clinical improvement [8,15]. These data show that AAV-mediated gene therapy is safe and well tolerated by patients with advanced Parkinson's disease, and suggest that *in vivo* AAV-gene therapy in the adult brain might be safe and efficacious for other neurodegenerative diseases including Alzheimer's disease.

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Figure 1.

Neurons transduced with AAV/PACAP38/GFP express the GFP. Neurons are shown at 3 days post-infection, stained with DAPI and imaged under fluorescence. A. Neurons mock infected with 1X PBS. B. Neurons infected with rAAV/PACAP38/GFP.

Figure 2.

Neurons transduced with AAV/PACAP38/GFP have increased survival. rAAV/PACAP38/GFP transduced neurons and untransfected neurons were treated with 1 mM SNP for 4 h and survival determined by MTT assay (n=3). A one-way ANOVA was performed followed by Bonferroni's multiple comparison tests.

*** p<0.001, vs. untransduced control;

* p<0.05, vs. untransduced cells treated with SNP.

Figure 3.

AAV delivery of PACAP38 improves neuronal survival compared to delivery of PACAP38 by DOTAP. Untransfected neurons and neurons transduced with PACAP38 by AAV or DOTAP were exposed to 0.5 mM SNP and survival assessed by MTT assay over a 0–72 h time course (n=3). A one-way ANOVA was performed followed by Bonferroni's multiple comparison test. ***p<0.001, vs. untransfected cells.

** p < 0.01 vs untransfected cells

Figure 4.

SNP treatment increases PACAP38 expression in transduced neurons. Untransfected neurons, lipofected, and AAV/PACAP transduced neurons were treated with 1 mM SNP for 4 h. PACAP38 and AAV capsid protein levels were measured by ELISA (n=3). Values are expressed as mean + SEM.

* $p < 0.05$ vs corresponding cells not treated with SNP

*** $p < 0.001$, vs corresponding cells not treated with SNP

$p < 0.001$, vs untransduced cells treated with SNP

Table 1

AAV-mediated PACAP38 delivery produced greater protection against SNP than lipofection-mediated delivery in primary neurons.

Delivery Method	Cell Survival
Untransduced Cells	74.76 ± 1.16
Lipofection by DOTAP	68.90 ± 0.70 ^{a, **}
AAV-mediated Delivery	110.93 ± 0.46 ^{***}

Values are mean OD490 readings ± SEM from MTT assay expressed as percent of untransduced, non-SNP treated cells. Primary neurons were treated with 0.5 mM SNP for 24 h.

^a significant difference, P < 0.001 versus AAV mediated delivery

^{**} significant difference P < 0.01 versus untransduced cells

^{***} significant difference, P < 0.001 versus untransduced cells