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Intramuscular AAV delivery of NT-3 alters synaptic transmission to motoneurons in adult rats

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Abstract

We examined whether elevating levels of neurotrophin-3 (NT-3) in the spinal cord and dorsal root ganglion (DRG) would alter connections made by muscle spindle afferent fibers on motoneurons. Adeno-associated virus (AAV) serotypes AAV1, AAV2 and AAV5, selected for their tropism profile, were engineered with the NT-3 gene and administered to the medial gastrocnemius muscle in adult rats. ELISA studies in muscle, DRG and spinal cord revealed that NT-3 concentration in all tissues peaked about 3 months after a single viral injection; after 6 months NT-3 concentration returned to normal values. Intracellular recording in triceps surae motoneurons revealed complex electrophysiological changes. Moderate elevation in cord NT-3 resulted in diminished segmental excitatory postsynaptic potential (EPSP) amplitude, perhaps as a result of the observed decrease in motoneuron input resistance. With further elevation in NT-3 expression, the decline in EPSP amplitude was reversed indicating that NT-3 at higher concentration could increase EPSP amplitude. No correlation was observed between EPSP amplitude and NT-3 concentration in the DRG. Treatment with control viruses could elevate NT-3 levels minimally resulting in measurable electrophysiological effects, perhaps as a result of inflammation associated with injection. EPSPs elicited by stimulation of the ventrolateral funiculus underwent a consistent decline in amplitude independent of NT-3 level. These novel correlations between modified NT-3 expression and single-cell electrophysiological parameters indicate that intramuscular administration of AAV(NT-3) can exert long lasting effects on synaptic transmission to motoneurons. This approach to neurotrophin delivery could be useful in modifying spinal function after injury.

Keywords

spinal cord; viral vector; gene therapy; electrophysiology

Introduction

Studies of synaptic plasticity in the spinal cord have been undertaken by many investigators because numerous disorders, notably spinal cord injury, have been shown to involve

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changes in cellular properties and synaptic relationships in spinal neurons (Mendell, 1984; Petruska *et al.*, 2007b). But even in cases where the spinal cord itself is not manipulated, there can be plastic changes, for example during aging (Boxer *et al.*, 1988), after exercise (Beaumont & Gardiner, 2002) and during chronic pain (Woolf, 1983). In many of these conditions, it would be useful to understand the mechanisms at work, in some cases to promote them and in other cases to prevent them.

Neurotrophins have emerged as important players in these conditions. After spinal cord injury expression of tropomyosin related kinase (trk) receptors is modified (King *et al.*, 2000; Liebl *et al.*, 2001). Neurotrophins and their receptors are also known to be modulated during aging (Johnson *et al.*, 1999), exercise (Vaynman & Gomez-Pinilla, 2005) and during chronic pain (Bennett, 2001). For these reasons it is important to understand the functional effects of neurotrophins in the spinal cord.

Numerous studies of neurotrophins have demonstrated their ability to affect synaptic transmission when administered acutely to the spinal cord *in vitro* (Arvanov *et al.*, 2000; Arvanian & Mendell, 2001; Garraway *et al.*, 2003) or when neurotrophin levels are manipulated during neonatal development (Seebach *et al.*, 1999; Arvanian *et al.*, 2003; Chen *et al.*, 2003; Shneider *et al.*, 2009). Because these previous studies were designed to approach acute effects or developmental questions in the immediate postnatal period, exposure to neurotrophins was either by bath application or limited to relatively brief chronic exposure *in vivo*. In order to study their long term effects in the adult spinal cord, a delivery system that permits maintenance of elevated concentrations in the spinal cord is required. Repeated application by injection is imprecise and impractical for long-term use. Osmotic minipumps are impractical for extended application, and do not deliver trophic factor to the interior of tissues, which may be necessary for some effects.

For these reasons we have undertaken studies using adeno-associated virus (AAV) to deliver the NT-3 gene. This vector can travel retrogradely from a peripheral injection site (Kaspar *et al.*, 2002), and can label spinal structures (DRG, spinal neurons) following peripheral administration to the muscle (Xu *et al.*, 2003; Hollis *et al.*, 2008; Fortun *et al.*, 2009). This technique has already demonstrated promise for reversing the progress of motoneuron diseases in a mouse model of ALS (Wang *et al.*, 2002; Kaspar *et al.*, 2003) and in improving motor function after spinal contusion injuries in rats (Fortun *et al.*, 2009). Here we have tested the effect of NT-3 delivered in this way on the function of the neural circuit underlying the monosynaptic stretch reflex previously identified as being subject to modulation by NT-3 (see above).

First, we made a preliminary study of tropism of AAV serotypes 1 to 5 after injection into either muscle or skin. Next we injected 3 serotypes selected for their favorable tropism profile (AAV1, AAV2 and AAV5) carrying the NT-3 gene into the MG (MG) muscle in intact adult rats. In a terminal experiment 21 to 540 days later, motoneuron cellular and synaptic properties were determined and evaluated with respect to changes in NT-3 protein levels measured with ELISA in DRG and spinal cord. NT-3 levels were found to rise to a maximum level by about 3 months after administration and fall back to normal levels by about 6 months. Although cellular and synaptic changes related to NT-3 level were observed, the interpretation was complicated by the finding that administration of control AAV virus without NT-3 also exerted some electrophysiological effects – an important consideration when evaluating results using engineered vectors.

Preliminary results have been reported in abstract form (Petruska *et al.*, 2007b; Petruska *et al.*, 2009).

Methods

General

These experiments were carried out in a total of 39 adult female Sprague Dawley rats. Procedures were approved by the SUNY-SB and Salk Institute IACUC.

Production and purification of recombinant AAV

AAV was produced by the triple transfection method (Kaspar *et al.*, 2003). HEK 293 cells were expanded to allow transfection on approximately 50 Corning 15cm tissue culture plates for each serotype. For each 15cm plate, 1×10^7 cells were transfected using the calcium phosphate transfection method. Plasmids used were AAV- β -galactosidase (β Gal), farnesylated-green fluorescent protein (GFP), or human NT-3 subcloned into an AAV vector cassette under the control of the Cytomegalovirus immediate early promoter (CMV) and with poly-adenylation signal from human B-globin gene. AAV serotypes 1-5 were produced from various preparations using Rep2 and capsid-specific serotype pAd Helper (Stratagene) was used for adenoviral functions. Preparations were purified by two rounds of cesium chloride isopycnic gradient centrifugation, dialyzed against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and titrated using quantitative PCR for the CMV promoter following DNase treatment of the virus to obviate partially packaged particles. All viruses were titrated together on the same plate to ensure equal viral titers between varying serotypes when comparisons of serotype were being made. Additionally, all preparations were evaluated for contaminating proteins by silver staining. Some AAV preparations were produced by Virapur (San Diego, CA).

RNA isolation and Quantitative RT-PCR

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) with DNase I treatment, following the manufacturer's instructions. Two μ g of total RNA was reverse transcribed to cDNA using SuperScript and Oligo-dT primers. For quantitative RT-PCR, 0.5 μ l of cDNA (10ng) was used in a 25 μ l reaction volume using SYBR GreenER qPCR SuperMix (Invitrogen). Primers used for qRT-PCR for the fGFP were: Forward (TATATCATGGCCGACAAGCA) and Reverse (GTTGTGGGCGGATCTTGAAGT). Primers used for qRT-PCR for the β Gal were: Forward (GCATGGTCAGGTCATGGATG) and Reverse (GATCATCGGTCAGACGATTC). Both assays used an Applied Biosystems Prism 7000 sequence detection system. Experiments were performed in triplicate. Values were calculated using the 2-CT method.

ELISAs

Tissue samples were homogenized in NT-3 ELISA homogenization buffer (137mM NaCl, 20mM Tris-HCl (pH 8.0), 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail tablet (Roche, IN) as follows: pooled DRGs or spinal cord samples each at the L4/L5 segmental level were homogenized in 250 μ l or 500 μ l buffer respectively with a pellet pestle on ice before sonication for 45 seconds. Whole muscle samples were crushed into a fine powder in a mortar and pestle under liquid nitrogen before homogenization with 6mg/ml buffer using a polytron PT1200 (Brinkmann Instruments, NY). Homogenates were left to incubate on ice before clearing by centrifugation at 14,000g for 30 minutes at 4°C. Supernatant protein concentrations were estimated using the DC Protein Assay (Bio-Rad, CA). The NT-3 enzyme linked immunosorbant assay was performed using the Immunoassay System (Promega, WI) following the manufacturer's protocol. All samples were assayed for NT-3 in triplicate.

Injection of viruses for molecular biology

A total of 19 female Sprague-Dawley rats were used (Taconic). For the 2 week post-injection survival experiments, each serotype (1-5) had 2 rats. For the 8-9 week post-injection survival experiments, each serotype (1, 2, 5) had 3 rats. Animals weighed 160-250g when injected. The left MG muscle was exposed by an incision in the overlying left calf skin after which it was freed from the overlying hamstring muscles and the fascia. This allowed access to both the medial/caudal and lateral/anterior portions of the muscle. Virus was injected into the MG muscle with a Hamilton microliter syringe and 33gauge needle. A total volume of 8-18 μ l was injected into 6-10 locations, delivering a total of $(1 - 5 \times 10^{10})$ viral particles.

Injection of viruses for electrophysiology

A total of 18 female Sprague-Dawley rats (Taconic) were used (AAV1(NT-3), n=4; AAV2(NT-3), n=7; AAV2(GFP), n=1; AAV5(NT-3), n=4; AAV5(B-gal), n=2). Two of the AAV2(NT-3) rats did not undergo electrophysiological studies, but were used for molecular assessments. Animals weighed 150-260g when injected. Only the left MG muscle was injected. These injections were performed as described above.

Preparation of the rat for electrophysiology

Rats were anesthetized with a mixture of ketamine (80mg/kg) and xylazine (10 mg/kg) (diluted 1:5 in lactated Ringer's solution with 5% dextrose) administered i.p and were intubated via the jugular vein for supplemental anesthesia. They also were intubated via the trachea to monitor end-tidal CO₂, and to provide externally controlled ventilation, if necessary. The lumbar enlargement, L4/5 dorsal roots, and T10/T11 spinal cord segments were exposed by dorsal laminectomy. Platinum bipolar electrodes gently held the L4/5 dorsal roots for in-continuity recordings of the afferent volley. Branches of the tibial nerve were exposed. The MG and lateral gastrocnemius/soleus (LGS) nerves were dissected free, transected, and placed on bipolar platinum hook electrodes (Figure 1A).

Electrophysiological procedures

Intracellular recording was carried out from antidromically-identified motoneurons in the L4/L5 segments of the spinal cord using micropipettes filled with 3M KCl (tip resistance 15-30 M Ω). Only records from motoneurons with resting membrane potential between -60mV and -80mV (corrected after withdrawal from the cell), and an action potential (AP) amplitude of more than 60mV were used. Input resistance (R_{in}) was determined by measuring the voltage deflection produced by a 1 nA current (Fig. 1D). Rheobase was measured by determining the minimum current (20 ms width) required to induce an AP in the motoneuron (Fig. 1E). All measures (except rheobase) were taken from traces generated from averages of multiple (4-20) trials. Not all measures were obtained from all cells.

Once a motoneuron was antidromically identified (as MG or LGS), the heteronymous nerve was stimulated (stimulating the MG nerve for LGS motoneurons, and vice-versa) to obtain a maximum monosynaptic segmental Ia-EPSP (sEPSP) (Fig. 1B). A monopolar stimulating electrode placed in the ipsilateral ventrolateral funiculus (VLF) at T10/T11 was used to elicit EPSPs from spinal white matter (cEPSP) (Fig. 1C). These stimuli were delivered at 2x threshold for the monosynaptic response in the recorded motoneuron (see Petruska *et al.*, 2007a) for more details). At the end of the experiment the rats were euthanized with an overdose of urethane (2ml of 1.5g/ml in dH₂O).

Statistical procedures

In general we calculated the mean value of each parameter from all motoneurons recorded in that preparation. Thus the number of observations was generally the number of animals, not the number of cells. In a few, clearly identified cases, when this conservative procedure missed statistical significance, we carried out the analysis on the results from individual cells to evaluate significance. Details of each test are given in the text. We used SigmaPlot 11.0 (SPSS, Cary, NC.) to carry out these tests.

Results

Characterization of viral vectors - mRNA

In initial experiments we injected AAV serotypes 1 through 5 [carrying β -Galactosidase (β Gal)] into the left MG muscle. Two weeks after injection we measured the expression of β Gal mRNA in the muscle, DRG and spinal cord using RT-PCR (Fig. 2). After MG injection, the highest expression in the DRG came from AAV1 with considerably less expression from AAV2. AAV3, AAV4 and AAV5 resulted in the smallest β Gal expression in DRG. The same injections gave a different pattern of expression in the spinal cord with the highest levels from AAV1 and AAV5 and slightly less from AAV2. AAV3 and AAV4 gave very weak expression, as in the DRG (Fig.2), and were not studied further.

For the selected serotypes (AAV1, AAV2 and AAV5) we also examined the transgene expression at longer survival times. In separate animals we injected AAV1, AAV2, or AAV5 (carrying the gene for farnesylated GFP; fGFP) and let them survive for 8-9 weeks before processing tissue for mRNA analysis. The commonly used AAV2 serotype had the weakest transgene expression in the two neural tissues housing cells that would be retrogradely infected (DRG and spinal cord), and it also resulted in the weakest expression in injected muscle (Fig. 2).

NT-3 expression is temporarily elevated in muscle, DRG and spinal cord after a single intramuscular injection of AAV(NT-3)

AAV(NT-3) was administered to the MG muscle of adult rats and the rats were prepared for ELISA measurements of NT-3 protein at delays ranging from 21 to 540 days, in most cases after a terminal electrophysiological experiment (see below). Total NT-3 levels (host plus viral vector-mediated) were measured in the treated left MG muscle, untreated left LGS and untreated right MG muscles as well as the left and right L4/L5 spinal cord segments and dorsal root ganglia which supply the hindlimbs (see Methods). Plots of NT-3 concentration vs. time of incubation with AAV are shown in Fig. 3 for each of the 3 serotypes (AAV1(NT-3), AAV2(NT-3) and AAV5(NT-3)) as well as for AAV-control (AAV2(GFP) and AAV5(β Gal)). The clearest time course was observed for muscle NT-3 expression (Fig. 3A) where there was an initial increase in NT-3 expression that peaked at about 90 days with a decline towards normal values by about 200 days. The 4 animals treated with AAV-control exhibited a small increase in muscle NT-3 compared to untreated muscles (horizontal line).

We subjected these data to a 2-way ANOVA to determine whether there was significant variation in muscle NT-3 expression with duration of AAV exposure (short (21-57 days), intermediate (77-98 days) or long (154- 540 days)) or serotype (AAV1(NT-3), AAV2(NT-3), AAV5(NT-3) and AAV-control). We found significant variation according to duration ($F(2, 17) = 13.4$; $p < 0.001$); variation with serotype was not significant ($F(3,17) = 0.17$; $p = 0.18$). *Post hoc* multiple comparison tests (Tukey) for duration revealed significant increase in NT-3 expression from short to intermediate time ($p = 0.001$) and decrease from

intermediate to long time ($p < 0.001$), i.e., NT-3 reached its highest level at intermediate times after administration and then declined.

Similar trends were observed with spinal cord NT-3 expression (Fig. 3C) where serotype was not a significant factor ($F(3, 15) = 1.8$; $p = 0.19$) whereas duration was ($F(2, 15) = 3.7$; $p < 0.05$). *Post hoc* pair wise comparisons (Tukey) revealed that NT-3 expression at intermediate duration was significantly higher than at long duration ($p < 0.05$), but the difference with short duration was not significant ($p = 0.23$). The similarity of NT-3 expression in muscle and spinal cord was further confirmed by demonstrating a highly significant correlation between these values ($r = 0.78$; $p < 0.0001$, $n = 21$) (Fig. 4).

The pattern of NT-3 expression in the DRG (Fig. 3B) displayed clear differences from that observed in muscle and spinal cord. The 2-way ANOVA revealed a significant correlation with serotype ($F(3, 15) = 3.36$; $p < 0.05$) but not with duration, the opposite of what was observed for muscle and cord NT-3 expression. *Post hoc* pair wise comparisons (Tukey) revealed no significant differences although NT-3 expression from AAV2 compared to AAV1 barely missed significance ($p < 0.08$). We observed no significant correlation in NT-3 expression between muscle and DRG ($r = 0.39$; $p = 0.08$; $n = 21$) unlike what had been found for muscle and cord NT-3.

In order to determine whether spread of the injected virus occurred, we also determined NT-3 concentration in the ipsilateral synergist LGS as well as the contralateral MG muscle. We observed no significant change in NT-3 concentration in left LGS over the entire range of times (small symbols in Fig. 3A; serotype: $F(3, 17) = 1.7$; $p = 0.2$; duration: $F(2, 17) = 1.8$; $p = 0.2$). Similar findings were made for the right MG (serotype: $F(3, 17) = 2.6$; $p = 0.09$; duration: $F(2, 17) = 0.23$; $p = 0.80$), for right cord (serotype: $F(3, 10) = 1.3$; $p > 0.30$; duration: $F(2, 10) = 4.1$; $p = 0.051$), and for right DRG (serotype: $F(3, 7) = 0.13$; $p > 0.90$; duration: $F(2, 7) = 0.07$; $p > 0.90$).

It is noteworthy that the effect of duration on NT-3 levels in the right cord barely lacked statistical significance ($p = 0.051$) and thus was similar to the effect observed for NT-3 levels in the left cord. This suggests that the increase in NT-3 in the cord spread beyond the infected neurons which we assume were restricted to the ipsilateral (left) cord (as reported in Fortun et al., 2009) since the right MG displayed no significant increase in NT-3 levels.

Motoneuron parameters in AAV(NT-3) treated rats

Motoneuron properties (R_n , rheobase) were averaged over all motoneurons in each rat (range 2-17 cells/ preparation; mean = 7) and used to calculate a mean value of each parameter for each animal.

In preparations treated with AAV(NT-3) we observed a small decline in motoneuron R_n from 1.7 ± 0.4 Mohms in untreated preparations ($n = 5$) measured using identical procedures (Petruska et al., 2007) to 1.3 ± 0.2 Mohms ($n = 15$) ($p < 0.04$; 2-tailed t-test). This was a consistent decrease since it was observed in all individual experiments with AAV(NT-3) (untreated control values shown by arrow: Fig. 5A). Nonetheless, this result must be interpreted cautiously since treatment with AAV-control vector also resulted in a decline in R_n in 3 of 4 preparations. However, the mean value of R_n in these 4 preparations was 1.5 ± 0.2 Mohms ($n = 4$) which did not differ significantly from either the untreated or AAV(NT-3) treated preparations.

We considered the possibility that a decline in R_n occurred only in MG motoneurons because only the MG muscle was injected with no evidence of spread to the LGS muscle (Fig. 3). However, the decline in R_n over all preparations was not greater in MG

motoneurons (14% decrease) than in LGS motoneurons (15% decrease), suggesting that any NT-3 effect was not restricted to the motoneurons innervating the muscle in which the virus was injected, i.e., it was a paracrine effect. This is consistent with the spread of increased NT-3 expression to the right cord (see above).

The AAV(NT-3)-treated preparations also exhibited an increase in rheobase from 8.3 ± 0.9 nA (untreated; $n=7$) to 10.9 ± 2.5 nA (AAV(NT-3)-treated; $n=15$) (2-tailed t-test; $p < 0.02$). This was a consistent increase since all individual preparations displayed a mean rheobase greater than the mean value in untreated controls (Fig. 5B). As with Rn, AAV-control elicited changes similar to AAV(NT-3) (mean value 11.3 ± 1.6 nA, $n=4$). This suggests that at least part of the increase in rheobase may have been due to a direct effect of the AAV virus or the small increase in NT-3 associated with AAV-control (see Discussion). These results with Rn and rheobase suggest that administering the AAV virus may not be a totally neutral procedure, and this must be considered in evaluating the effects of the NT-3-expressing virus (see Discussion).

sEPSPs in AAV(NT-3)-treated rats

In untreated cords EPSP amplitude displays an inverse relationship to motoneuron Rn (Burke, 1968). A plot of mean sEPSP in each preparation as a function of mean Rn of motoneurons revealed no tendency for small mean Rn to be associated with large mean EPSPs (Fig. 6), unlike naïve animals. Rather there appeared to be 2 distinct groups of preparations, one whose mean value of sEPSP was considerably smaller than the mean sEPSP amplitude in untreated preparations (1.6 mV; arrow) studied in precisely the same way (Petruska *et al.*, 2007a), and another whose mean sEPSPs were similar to or larger than those in untreated preparations. We demonstrate below that the latter preparations displayed the most elevated levels of NT-3 in the spinal cord.

The 3-D plot of Figure 7A illustrates the relationship between sEPSP amplitude for each preparation and NT-3 concentration in the spinal cord and in the DRG. Here, we considered only rats that were studied within the initial 180 days after delivery of the virus to avoid possible ambiguities in the relationship due to the decline in NT-3 levels observed after that time (Fig. 3), as well as the changes in trkC receptor expression (Johnson *et al.*, 1999) and in synaptic processes (Boxer *et al.*, 1988) that have been reported in aged rats. Five of these preparations exhibited a relatively large mean EPSP, and from the position of their drop lines on the DRG-spinal cord NT-3 expression plane, they had relatively large values of NT-3 expression. The mean sEPSP in these 5 rats was 1.7 ± 0.1 mV which was indistinguishable from values observed in untreated preparations carried out under identical conditions (1.6 ± 0.8 mV; Petruska *et al.*, 2007). In the remaining experiments with lower values of NT-3 expression the mean EPSP was considerably smaller (1.0 ± 0.2 mV). Thus it appears that low values of NT-3 (though not necessarily lower than normal) were associated with a decline in sEPSP amplitude.

In order to determine whether significant changes in the segmental EPSP could be attributed to DRG NT-3 or cord NT-3, we carried out multiple linear regression of sEPSP on DRG and cord NT-3 concentrations. The slope for the relationship of sEPSP amplitude vs. DRG concentration of NT-3 was not significant (slope = -0.01 ; $p=0.57$) whereas the slope for the relationship of sEPSP vs. cord concentration was significant (slope = 0.13 ; $p=0.04$). We conclude that under these conditions it was the level of cord NT-3 rather than that of DRG NT-3 that accounted for the significant NT-3 effect on EPSP amplitude (see Discussion).

We observed that the preparations yielding large values of sEPSP occurred after either short or intermediate duration of AAV(NT-3) exposure (numbers in Fig. 7A). This is consistent with our finding that short and medium duration of exposure to AAV(NT-3) resulted in no

significant difference in NT-3 expression in the cord (see Fig. 3 and associated description in the text). A possible effect of serotype cannot be ruled out, for example the absence of large sEPSPs after treatment with AAV1(NT-3) (Figs. 6, 7A), but this will require more investigation.

Stimuli in these experiments were delivered at 1.67 Hz, and it is known that EPSP amplitude is somewhat attenuated compared to maximum values observed at lower stimulus rates (Curtis & Eccles, 1960; Honig *et al.*, 1983). Therefore, we investigated whether there is any interaction between frequency of stimulation and the effects of NT-3 treatment. We recorded EPSPs elicited in the same cells by stimuli at 1.67Hz and 0.2 Hz in both untreated (26 cells) and AAV1(NT-3)-treated (23 cells) preparations. We observed a similar effect of NT-3 at both frequencies of stimulation, i.e., a mean decline of 17% in EPSP amplitude at 1.67 Hz stimulation and 11% at 0.2 Hz stimulation. We compared these differences with a repeated measures ANOVA and found that they were not significant ($p > 0.5$). This suggests that the effects of NT-3 on EPSP amplitude were not dependent on the stimulation frequency used.

cEPSPs in AAV(NT-3)-treated rats

Analysis of the mean values of cEPSP elicited in each rat by VLF stimulation using multiple linear regression revealed no significant difference correlated with cord ($p = 0.76$) and DRG ($p = 0.81$) NT-3 concentrations (Fig. 7B). The mean value of cEPSP amplitude over all experiments was 2.0 ± 0.9 mV which was smaller than the values in intact preparations (2.2 ± 0.8 mV), qualitatively similar to our finding for sEPSP.

DISCUSSION

The goal of these experiments was to determine whether the enhanced level of NT-3 in the spinal cord and/or DRG produced by a single intramuscular injection of AAV viruses engineered with the NT-3 gene would alter the electrophysiology of a simple circuit in the spinal cord. We evaluated the effect of NT-3 on the monosynaptic stretch reflex pathway because of the well established effect of this neurotrophin on the function of this synapse. We used 3 different serotypes, AAV1, AAV2 and AAV5 (see also Burger *et al.*, 2004), and demonstrated using ELISA that such treatments can elevate NT-3 levels in muscle, DRG and spinal cord. We considered the possible contribution of 2 factors to elevation of NT-3 level in the tissues examined, exposure time and AAV serotype. Using 2-way ANOVA we showed that the level of NT-3 in muscle in these experiments was determined by exposure time rather than serotype with the maximum levels occurring around 2.5 to 3 months after AAV administration followed by a decline to control values at about 6 months. A similar finding was made when cord levels of NT-3 were examined, and NT-3 levels in cord were highly correlated with those in the muscle over all experiments. However, in the same rats, the level of NT-3 in the DRG was found to be determined by serotype rather than duration of exposure with AAV2 being the serotype causing the greatest elevation in NT-3. The specificity of the different AAV serotypes is related to the different capsid proteins expressed by individual AAV serotypes; these interact with unique receptors and co-receptors in target cells as a prelude to internalization (Wu *et al.*, 2006a; Wu *et al.*, 2006b; Van Vliet *et al.*, 2008). From these experiments it appears that AAV2 may have a special affinity for some population(s) of muscle afferent fibers (but see below).

One uncertainty in interpreting these findings is the inability to distinguish between NT-3 protein manufactured by viral vector-infected neurons in the DRG and spinal cord, and NT-3 synthesized and released by infected myocytes and then transported to these target tissues. Since AAV2(NT-3) treatment led to the greatest relative NT-3 levels whereas RT-PCR data from animals indicated that infection of the cord and DRG was in the order

AAV1>AAV5>>AAV2 at 8-9 weeks, it seems likely that at least some of the NT-3 found in the DRG and spinal cord was made in the muscle.

We observed several electrophysiological changes in these preparations. Most individual preparations displayed a decrease in mean motoneuron Rn and an increase in mean motoneuron rheobase compared to untreated preparations (Fig. 5). NT-3 administration has been shown to increase cell surface area in cells in layer 6 visual cortex whose dendritic branching increases after exposure to NT-3 (McAllister *et al.*, 1997). Such a change in motoneuron surface area would be expected to decrease Rn and increase rheobase of the motoneurons. A problem in interpreting these changes strictly in terms of NT-3 is that mean Rn was not proportional to the measured levels of cord NT-3. Since most preparations treated with AAV(control) exhibited similar changes in Rn and rheobase (Fig. 5), it is conceivable that the AAV virus itself was responsible for the changes. However, AAV(control) also resulted in small increases in NT-3 levels in the spinal cord, which may be the result of an inflammatory processes initiated by multiple injections into the muscle rather than the AAV virus itself (Oddiah *et al.*, 1998)(but see (Towne *et al.*, 2004). This increase in NT-3 expression may have been sufficient to elicit the changes in motoneuron properties. The AAV virus itself is unlikely to cause an immune reaction by interacting with the host tissue (Peden *et al.*, 2004; Tenenbaum *et al.*, 2004). Even if this were to occur, regulation of NT-3 appears insensitive to numerous models of inflammation, allergy, asthma, etc. in contrast to NGF and BDNF (Kemi *et al.*, 2006; Rochlitz *et al.*, 2006). Further work is needed to determine how AAV(control) elicited its effects.

The mean amplitude of the monosynaptic sEPSP was generally smaller in treated preparations than in untreated controls except for several preparations with greatly enhanced levels of cord NT-3 in which mean sEPSP amplitude reached a value similar to untreated controls. Our interpretation of this is that sEPSP amplitude was determined by two (groups of) factors, one factor which decreased sEPSP amplitude, and the other factor that increased it. A factor contributing to the decline in amplitude at low concentration of NT-3 is the decrease in motoneuron Rn (Burke, 1968; Peshori *et al.*, 1998) although this was probably not the only factor since mean EPSP amplitude was not proportional to mean Rn (Fig. 6). Motoneuron rheobase was consistently increased (Fig. 5), indicating an increased threshold for discharge. Interestingly, if similar threshold changes were occurring in presynaptic membrane channels, transmitter release would decline due to conduction block in the terminals resulting in reduced EPSP amplitude.

As for the second factor (increasing sEPSP), statistical analysis of the graph in Fig. 7A suggests that the significant increase in amplitude of the sEPSPs from depressed (at low [NT-3]) to normal (at high [NT-3]) was associated with increases in spinal cord NT-3, and that DRG NT-3 was not a significant factor. The elevated cord levels of NT-3 might have elicited effects on the terminals of trkC-expressing spindle afferent fibers. The ability of chronically delivered NT-3 to enhance EPSPs produced by group Ia fibers, known to express the trkC receptor (McMahon *et al.*, 1994), has been reported previously under different conditions (Mendell *et al.*, 1999; Arvanian *et al.*, 2003). It has also been established that spinal NT-3 administered either locally into the spinal cord (Schnell *et al.*, 1994) or retrogradely from nerve (Zhou *et al.*, 2003) can cause terminal sprouting of trkC-expressing corticospinal axons. It will be necessary to study group Ia fiber afferent terminals directly under these conditions to see if they also undergo sprouting. It will also be valuable to determine whether direct application of NT-3 to the cord can mimic the effects of muscle application of NT-3, and whether application to the DRG is ineffective as suggested by our current results.

Taken together these considerations suggest a complex effect of AAV(NT-3) on the sEPSP elicited by group Ia fibers in motoneurons. The decrease in motoneuron R_n elicited by NT-3 and possible changes in voltage threshold of the afferent terminals would act to cause the observed decrease in sEPSP at low values of cord NT-3. Elevated levels of NT-3 would tend to increase sEPSP back towards normal values perhaps as a result of sprouting of the afferent terminals.

The findings in the present studies were surprising because they differ substantially from earlier work where chronically delivered NT-3 yielded uniformly larger EPSPs (Mendell *et al.*, 1999; Arvanian *et al.*, 2003). However, the conditions were very different in those experiments, i.e., the peripheral nerve was chronically axotomized or the rats were treated during neonatal development. In addition, NT-3 was delivered either intrathecally or over a long period via osmotic minipump attached directly to a cut peripheral nerve. It would be interesting to know if the NT-3 was distributed differently under those conditions, for example to stimulate more extensive sprouting of the sensory afferents than might have occurred in the present experiments.

The cEPSP elicited by VLF stimulation exhibited no systematic variation with spinal NT-3 concentration in contrast to the increase toward normal in sEPSP observed at high values of cord NT-3 (Fig. 7). In the presence of AAV(NT-3) the small decline in motoneuron R_n might have been expected to reduce mean amplitude of the cEPSP as in the case of the sEPSPs. However, in the case of the cEPSP this decline would not be counterbalanced by any putative presynaptic effect since VLF fibers express trkB receptors (Merlio *et al.*, 1992; Menei *et al.*, 1998; Jin *et al.*, 2002) rather than trkC. Another possible contributing factor might be that any sprouting of inputs responsible for the enlarged sEPSP might have displaced the synaptic input evoking the cEPSP. These issues require further study.

Recent observations in the intact cervical cord (Fortun *et al.*, 2009) demonstrated much smaller levels of NT-3 than those we measured in the untreated lumbar cord under similar conditions. Lumbar cord supplies a greater mass of muscle than cervical cord, possibly resulting in larger spinal NT-3 levels derived from peripheral or spinal sources. Fortun *et al.* also reported a much greater percentage increase in NT-3 in cervical cord after AAV5(NT-3) administration than we have observed in lumbar cord after similar AAV5(NT-3) administration although the arithmetic increase was very small. In our experiments we did observe a substantial increase in cord NT-3 in 1 of 4 experiments with AAV5(NT-3), and NT-3 levels exhibited a similar increase in expression in several experiments using the AAV1 or AAV2 serotypes (Fig. 3C). Incubation time is an important variable that may not have been optimized in our experiments with AAV5(NT-3) (Fig. 3).

We have shown that administration of AAV(NT-3) elevates NT-3 and alters the electrophysiological properties of spinal synapses in a way that is correlated with NT-3 level. A major problem still to be overcome is that both the electrophysiology and the ELISAs provide average results over the entire lumbar enlargement of the spinal cord. Such studies would be improved by the development of techniques to enable determination of local NT-3 levels and to measure the effects of such changes on neighboring cells and synapses. Furthermore, the viral vectors do not infect all neurons (Fortun *et al.*, 2009); thus additional analytical power would be gained by the development of electrophysiological real-time indicators of the infection status of the recorded neurons. Such an advance would enable the separation of electrophysiological results from infected and non-infected neurons in the same preparation.

Despite these difficulties in interpretation, it is encouraging that function of a specific neural circuit in the mammalian spinal cord is subject to long lasting change after a single

intramuscular injection of viral vector. This finding suggests great translational potential, and is attractive because there may be no need to expose the spinal cord in order to deliver neurotrophins effectively. An important question is whether behavior of the animal can be affected by these treatments. There is evidence that intrathecal administration of neurotrophins can affect locomotor performance (Blits *et al.*, 2003; Boyce *et al.*, 2007). AAV(NT-3) administered to forelimb muscles has also been reported to improve forelimb motor behavior after dorsal lesions of the cervical spinal cord (Fortun *et al.*, 2009). Our preliminary data indicate treatment with hindlimb intramuscular AAV1(NT-3) after thoracic spinal transection is associated with improved stepping and standing behavior (Petruska *et al.*, 2007b); these findings will be addressed in detail elsewhere.

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ABBREVIATIONS

AAV	adeno-associated virus
ANOVA	Analysis of variance
βGal	βGalactosidase
cEPSP	EPSP produced by stimulation of the spinal ventrolateral white matter
DRG	dorsal root ganglion
GFP	green fluorescent protein
LGS	lateral gastrocnemius-soleus
MG	medial gastrocnemius
NT-3	neurotrophin-3
Rn	input resistance
sEPSP	EPSP produced by stimulation of the heteronymous peripheral nerve
VLF	ventrolateral funiculus of the spinal cord

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Figure 1.

Schematic of experimental preparation (A) and examples of electrophysiological records used in the analyses. B) Segmental EPSP (sEPSP) elicited by stimulation of the nerve to the heteronymous muscle. Amplitude measured from baseline to peak. C) Record of cEPSP produced in the same motoneuron by stimulation of the ventrolateral funiculus at 2x threshold. D) Determination of input resistance. Record is the depolarization produced in the motoneuron by a 1 nA current square wave. Depolarization measured from baseline to peak value. Resistance was determined by dividing the measured voltage by the known current. E) Rheobase determination in a different motoneuron to show the records evoked by increasing levels of depolarization. This motoneuron had a rheobase of about 6 nA. Calibrations 1mV, 2 ms for B and C; 2 mV, 3 ms for D. Calibration pulse at the onset of E is 2 mV, 2 ms.

Figure 2.

The different AAV serotypes display different profiles of gene transfer. All bar graphs indicate the level of gene expression ($\pm$ SD) relative to AAV2, the most commonly used serotype. The top graphs show the expression of the transgene (β -Galactosidase) 2 weeks after injection. The bottom graphs show the expression of the transgene (GFP) in the indicated structure 8 weeks after injection.

Figure 3.

Graphs of NT-3 concentration measured by ELISA in several sites as a function of time after a single AAV(NT-3) administration into the left MG muscle. Each point represents values from a single treated preparation. A) NT-3 concentration in injected MG muscle (large symbols) and ipsilateral LGS muscles (small closed symbols) and contralateral MG (small open symbols). Horizontal line indicates NT-3 level in untreated MG muscle (n=3). Different colors indicate the different serotypes and the control AAV. The increase in NT-3 in the injected MG reaches a maximum at about 3 months and falls back towards control values about 6 months. B) Similar presentation for left (large symbols) and right (small symbols) L4/L5 DRGs from the same injections. Legend for B and C is in panel C. Not all DRGs were available. The NT-3 level in DRGs from the untreated preparations was not available. C) Similar presentation of NT-3 levels in the left and right spinal cord for these same preparations. Horizontal line indicates NT-3 level in spinal cord from untreated animals (n=3). Further description and statistical treatment of these data is in the text.

Figure 4.

Correlation between NT-3 levels in left cord and the injected left MG muscle. Further details and statistics are in the text.

Figure 5. Distribution of mean input resistance and rheobase for individual AAV(NT3)- and AAV(control)- treated preparations. Arrows indicate mean values in untreated preparations (from Petruska et al., 2007a).

Figure 6.

Plot of mean sEPSP amplitude as a function of mean input resistance shows that there are 2 groups of preparations: one with large mean sEPSP, and another with smaller mean EPSPs. Input resistance is not a determinant of sEPSP amplitude. Arrow indicates mean value from naïve preparations.

Figure 7.

Plot of mean sEPSP (A) and cEPSP (B) as a function of spinal cord and DRG NT-3 levels. Each point represents mean data from an individual experiment. Further detail and statistics are in text. The numbers above some of the points denote the number of days after AAV(NT-3) administration that the determinations were made.