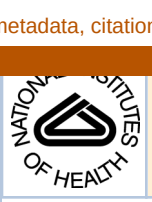

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Therapeutic Liabilities of *in Vivo* Viral Vector Tropism: Adeno-Associated Virus Vectors, NMDAR1 Antisense, and Focal Seizure Sensitivity

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Abstract

The *N*-methyl-D-aspartic acid (NMDA) receptor provides a potential target for gene therapy of focal seizure disorders. To test this approach, we cloned a 729-bp NMDA receptor (NMDAR1) cDNA fragment in the antisense orientation into adeno-associated virus (AAV) vectors, where expression was driven by either a tetracycline-off regulatable promoter (AAV-tTAK-NR1A) or a cytomegalovirus (CMV) promoter (AAV-CMV-NR1A). After infection of primary cultured cortical neurons with recombinant AAV-tTAK-NR1A, patch clamp studies found a significant decrease in maximal NMDA-evoked currents, indicative of a decrease in the number of NMDA receptors. Similarly, infusion of AAV-tTAK-NR1A (1 μl) into the rat temporal cortex significantly decreased NMDAR1-like immunoreactivity in layer V pyramidal cells. When AAV-tTAK-NR1A vectors were infused into the seizure-sensitive site of the rat inferior collicular cortex, the seizure sensitivity increased significantly over a period of 4 weeks. However, collicular infusion of AAV-CMV-NR1A vectors caused the opposite effect, a significant decrease in seizure sensitivity. Subsequent collicular coinfusion of vector encoding green fluorescent protein (GFP) driven by the tetracycline-off promoter (AAV-tTAK-GFP) and vector encoding β-galactosidase driven by the CMV promoter (AAV-CMV-LacZ) transduced distinct neuronal populations with only partial overlap. Thus, differing transduction ratios of inhibitory interneurons to primary output neurons likely account for the divergent seizure influences. Although AAV vector-derived NMDAR1 antisense can influence NMDA receptor function both *in vitro* and *in vivo*, promoter-related tropic differences dramatically alter the physiological outcome of this receptor-based gene therapy.

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Keywords

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Introduction

In the central nervous system (CNS), adeno-associated virus (AAV) vectors can transduce neurons for long periods of time with little concomitant toxicity [1-3]. AAV preferentially binds neurons *in vivo* [4], and this physical property, combined with promoter tropisms, contributes to the differential patterns of transduction observed across various brain structures [2]. Although not all neurons are transduced, the level of gene expression has proven sufficient, in several instances, to change physiological function. For example, Kaplitt *et al*. [1] showed that AAV vectors could deliver tyrosine hydroxylase to a dopamine depleted caudate and produce enough of the gene product to reverse the behavioral deficit associated with the dopamine depletion. Similarly, Klein *et al*. [5] recently reported that transduction of basal forebrain neurons with AAV-nerve growth factor vectors retarded age-related decreases in the Morris water task 9 months later. Certainly, physiologically relevant, functional gene product can be derived from AAV vectors.

A promising therapeutic target for gene therapy is focal epilepsy, particularly where the site of seizure genesis is well defined. With a circumscribed site of seizure initiation, it is quite feasible that viral vectors could transduce enough neurons to significantly influence the seizure focus. Also, because neurons are the source of seizure activity, AAV vectors' *in vivo* preference for neurons [4] should provide direct access to mechanisms that modulate focal seizure activity. Finally, there are numerous potential molecular targets that could directly influence focal seizure activity, ranging from neurotransmitter receptors to ion channels. For example, it is well known that increasing local γ-amino-butyric acid (GABA) function or decreasing local *N*-methyl-$_D$-aspartic acid (NMDA) receptor function can attenuate seizure sensitivity [6]. Thus, if a gene therapeutic strategy increased local GABA activity, or decreased local NMDA receptor function, attenuation of local seizure sensitivity could be attained.

The NMDA receptor presents an obvious therapeutic target for seizure attenuation, in which a reduction in the number of functional NMDA receptors should diminish NMDA receptor-mediated excitation in the CNS. Because the NMDA receptor (NMDAR1) subunit protein is essential to the formation of a fully functional NMDA receptor, removal of this subunit *in vivo* should significantly decrease NMDA receptor-mediated excitation [7,8]. In fact, both Chapman *et al*. [9] and Zapata *et al*. [10] have shown that pretreatment with NMDAR1 antisense oligonucleotides reduces seizure sensitivity. However, effective treatment of focal epilepsies will require a chronic reduction in NMDA receptor function, a goal not compatible with current oligonucleotide treatments.

A less used, but nonetheless effective, gene therapy approach is the delivery and expression of antisense constructs. Like traditional antisense oligonucleotides, it is possible to target specific genes, but unlike traditional oligonucleotides, AAV vector-derived antisense RNA can be produced nontoxically *in vivo* for long periods of time [11]. Using this approach, Xiao *et al*. [12] found that acute expression of AAV-derived antisense to the $GABA_A$ receptor α1 subunit caused a selective increase in inferior collicular seizure duration. Moreover, Shafron *et al*. [13] have shown that an AAV plasmid containing a NMDAR1 antisense fragment reduced MK-801 binding in primary cortical cultures. Because focal seizure genesis in the inferior colliculus is sensitive to local manipulation of NMDA receptor function [14] and because this brain area is permissive to AAV vector transduction

[2], the following studies sought to determine if AAV vector-derived expression of an NMDAR1 antisense could influence receptor protein, receptor function, and seizure sensitivity.

Results

AAV Vectors

The following studies used five different AAV vectors (Fig. 1). The first AAV vector used a previously described, self-contained tetracycline-off construct [15] in which a 729-bp fragment of the rat NMDAR1 receptor cDNA was inserted in the antisense orientation (AAV-tTAK-NR1A) and gene expression was driven by the tetracycline regulated promoter (pTet). The second AAV vector was identical to the first except that the cDNA for protein inhibitor of nitric oxide synthase (PIN) [16], instead of the NMDAR1 fragment, was inserted in an antisense orientation (AAV-tTAK-PINA). The third AAV vector contained the NMDAR1 cDNA in an antisense orientation, but had gene expression driven by a CMV promoter (AAV-CMV-NR1A). The final two AAV vectors contained the reporter genes enhanced green fluorescent protein (*gfp*) or *lacZ*, with gene expression driven by either the pTet or CMV promoters, respectively (AAV-tTAK-GFP, AAV-CMV-LacZ).

Reduced NMDA Responsiveness of Primary Cultured Cortical Neurons after AAV-tTAK-NR1A Transduction

Primary cultured neurons were infected with AAV-tTAK-NR1A (multiplicity of infection (MOI) of 100), which has proven sufficient to transduce approximately 75% of the neurons (unpublished data). After 14 to 15 days, the responsiveness to NMDA was assessed using whole-cell patch clamp techniques, and the maximal responsiveness to NMDA was significantly diminished in the infected cultures ($n = 9$) compared with that in uninfected controls ($n = 9$; Fig. 2). The most likely explanation for this attenuated response to NMDA is a reduction in the number of functional NMDA receptors.

In Vivo Reduction of NMDAR1 Protein by AAV-tTAK-NR1A Treatment

Given the vector-induced change in NMDA responsiveness *in vitro*, the next step was to determine if AAV-tTAK-NR1A transduction produced a decrease in NMDAR1 subunit protein *in vivo*. Rat temporal cortex is a brain area where substantial AAV-induced transduction can be achieved, and NMDAR1-like immunoreactivity can be clearly localized. NMDAR1-like immunoreactivity is highly concentrated in the pyramidal cell bodies of layer V in the temporal cortex (Fig. 3A), but 3 weeks after infusion of the AAV-tTAK-NR1A vector, significant reductions were found in the optical density of this NMDAR1-like immunoreactivity versus that in the uninjected contralateral side (17 ± 6% of control side, mean ± SEM; $P < 0.01$; Fig. 3B).

In Vivo Alteration of Focal Seizure Sensitivity after AAV-NR1A Infusion

The inferior colliculus is quite permissive to AAV transduction [2] and contains a well-characterized site of focal seizure genesis [17,18]. A key property of this seizure model is that without external perturbation, the threshold for seizure genesis remains stable for a long period of time. Thus, 1 μl of the AAV-tTAK-NR1A was infused just before the electrode implantation. Subsequently, the threshold current for wild running seizures was determined at 1-week intervals. Surprisingly, the threshold for seizure initiation decreased significantly over a 4-week post-treatment time frame (Fig. 4). No significant change in seizure threshold current was found in rats that received similar amounts of AAV-tTAK-GFP virus (Fig. 4) or in rats that received an AAV-tTAK virus that contained an antisense fragment to PIN (data not shown). Thus, contrary to the predicted outcome, the rats actually became more seizure

sensitive. Because a previous publication showed that an AAV-CMV-GABAα1 antisense vector produced the expected direction of change in seizure sensitivity [12], we infused an AAV-CMV-NR1A vector into the inferior colliculus before electrode implantation. In direct contrast to AAV-tTAK-NR1A results, the seizure threshold significantly increased over a 4-week post-treatment time frame (Fig. 4). Thus, a modest change in the promoter resulted in opposite effects on focal seizure sensitivity.

Divergent Patterns of AAV-Mediated Gene Expression *in Vivo*

An explanation for these seemingly disparate results would be that the tropism of the two AAV vectors was such that in one case the transduction pattern favored primary inhibitory interneurons, whereas in the other case the transduction pattern favored primary output neurons. However, this interpretation would require that the two AAV vectors have different patterns of neuronal transduction. It has been shown that striatal infusion of two AAV vectors containing different transgenes, but the same promoter (1:1 mixture), resulted in over 90% of the cells expressing both transgenes [19]. When an AAV-tTAK-GFP vector was mixed 1:1 with an AAV-CMV-LacZ vector, subsequent infusion into the inferior colliculus produced different patterns of transduction for the two vectors (Fig. 5). When the transduced neurons were counted across the area of transduction, 41% were positive for GFP alone, 24% were positive for β-galactosidase alone, and 35% were positive for both reporter genes. These percentages diverge dramatically from those observed in the study that used two vectors with different transgenes but the same promoter [19]. Another indication of this divergence was seen in the axons that project to the contralateral colliculus through the commissure of the inferior colliculus. In the commissure and the contralateral colliculus, axons were clearly positive for GFP, but in no case were any axons positive for β-galactosidase (data not shown).

Discussion

The present studies have established that virus vector delivery of antisense can alter receptor function, diminish the amount of endogenous receptor protein, and influence physiological function in the brain. More importantly, these studies vividly illustrate how a modest change in the promoter element can change the *in vivo* tropism to such a degree as to produce opposite physiological effects.

Both *in vitro* and *in vivo* studies established the efficacy of the vector-derived NMDAR1 antisense fragment. Following transduction with AAV-tTAK-NR1A vectors, primary cultured cortical neurons were not as responsive to NMDA application as the untreated control neurons. That this decrease did not approach zero can be explained by several factors. First, approximately 75% of the primary cultured neurons are transduced, so it is likely that a mixed population of neurons was tested. Second, the cultures were tested 2 weeks after infection, so, given the lag for vector-derived gene expression and the normal half-life of the receptor, one would not expect a total loss of receptors. Thus, in agreement with a previous report [13], the findings clearly show that the AAV-mediated expression of this NMDAR1 antisense fragment can reduce significantly NMDA receptor function in primary cultured cortical neurons. Similar conclusions can be drawn from the subsequent *in vivo* study. Infusion of AAV-tTAK-NR1A vectors into the rat temporal cortex significantly reduced NMDAR1-like immunoreactivity, a finding that highly suggests that vector-mediated expression of the antisense reduced NMDAR1 protein levels. Moreover, the extent of reduced NMDAR1-like immunoreactivity closely matched the expected area of transduction from AAV vectors, providing additional support that AAV vector-derived antisense effectively reduced NMDAR1 subunit protein levels *in vivo*.

Although the *in vivo* studies demonstrated clearly the ability to modulate seizure sensitivity by vector-derived NMDAR1 antisense, the studies also showed that modest changes in the promoter element can alter the tropism in a manner that produced dramatically divergent effects. Infusion of the AAV-CMV-NR1A did cause a significant reduction in focal seizure sensitivity over a post-treatment period of 4 weeks, the predicted direction of change based upon the direct influence of NMDA upon inferior collicular seizure sensitivity [14,20]. However, the pTet promoter construct caused the opposite effect, a significant increase in seizure sensitivity. It has been shown that in the brain, NMDA receptor excitation can drive GABA inhibition [21], so removal of NMDA receptor excitation from these inhibitory interneurons would create a state of hyperexcitability. Therefore, if the preponderance of transduced neurons favored these inhibitory interneurons, then seizure sensitivity would increase. In both instances, though, the changes likely accrued from the NMDAR1 antisense, because neither GFP nor PIN antisense expression caused any change in seizure sensitivity.

Further substantiation of this altered tropism was obtained using a mixture of two AAV vectors. It is clear that AAV-CMV-LacZ and AAV-tTAK-GFP vectors transduce different neuronal populations, albeit with some overlap between the two populations (Fig. 5). Certainly, some difference might be expected from random variation, but a previous study has shown that 1:1 mixtures of AAV vectors with the same promoter, but different transgenes, transduced over 90% of the same cells in the striatum [19]. Thus, the transduction patterns reflect a difference in tropism, not random variation. This conclusion is further reinforced by the observation that pTet expression, but not CMV expression, labeled axons coursing to the contralateral inferior colliculus, projections that have been characterized as GABAergic [22]. The most likely source of this divergent tropism is the promoters of the two vectors. Both constructs contain the minimal CMV promoter (mP, Fig. 1) but different enhancer regions. The CMV constructs contain the human CMV immediate early enhancer, whereas the pTet construct contains a seven-repeat unit of the *tet* operator sequence (tetO). The presence of these regions upstream of the minimal promoter could differentially attract factors that shut down or promote gene expression. Alternatively, the presence of the tetracycline transactivator (tTAk) or the *neo* gene could have indirect influences on gene expression in different neurons. However, the *neo* gene is unlikely to be a major contributor, because it is not present in AAV-CMV-LacZ, which still has significantly different tropism from the AAV-tTAK-GFP. Further experiments are needed to determine the elements that contribute to tropism and where they have their effects during the transduction process.

Certainly, these studies validate the use of vector-derived antisense as a means to alter function in the brain, but the differences between the two different vectors point out an important concern for gene therapy applications in the brain. Any approach that depends upon cell-specific transduction, such as modulation of neurotransmitter receptors or ion channels, must be validated with the specific vector. Even with such validation, it is still possible that differences between experimental animals and humans may produce undesired results. Also, there are instances in which two or more transgenes need to be expressed in the same cell with different promoters, as is the case with many gene expression regulation systems. Due to the nature of these regulation systems, the same promoter can not be used for the transactivator and the transgene, so differential cellular tropism could result in significantly diminished gene expression. Clearly, such liabilities must be considered before proposing the clinical application of any gene therapy directed at the CNS.

MATERIALS AND METHODS

Construction of pAAV-NR1A and rAAV virus production

We cloned a 729-bp fragment (from bp 140 to 869) from a rat NMDAR1-1a plasmid (clone pNMDAR1-1a, GenBank acc. no. U08261; provided by Steve Heineman of the Salk Institute) using a "sticky end" PCR technique [23]. Using this technique, two PCR products are created and, when denatured and reannealed, 25% contain a 5′ *Not*I overhang and a 3′ *Age*I overhang. Next, an *Age*I and *Not*I digest of the AAV-tTAK-eGFP plasmid [15] excised enhanced *gfp* gene, and the NMDAR1 fragment was ligated into the AAV-tTAK plasmid in the antisense orientation (AAV-tTAK-NR1A). In addition, a fragment of the coding sequence for PIN (from bp 41 to 490, GenBank acc. no. U66461) was cloned from rat inferior colliculus cDNA using the sticky end PCR technique and subsequently ligated into the AAV-tTAK plasmid in the antisense orientation (AAV-tTAK-PINA) as described for the NMDAR1 antisense. Finally, the *Age*I/*Not*I fragment from AAV-tTAK-NR1A plasmid was removed and cloned into the *Age*I and *Not*I sites of an AAV-CMV-eGFP plasmid. All of the AAV plasmids were made into recombinant AAV virus by the UNC Virus Vector Core as described [24]. The titers of the four viruses were approximately 10^{12} particles per ml.

Primary cortical neuronal culture

Primary neuronal cultures were prepared as described [25]. Briefly, cortices from 10 1-day-old rat pups were dissected, digested with trypsin, and gently dissociated. The cells were plated at a density of 1×10^6 cells per well and on the next day, infected with AAV-tTAK-NR1A (MOI of 100). It was crucial to infect the cultures before the introduction of cytosine arabinoside (ARA-C), because AAV is a single strand DNA virus that requires second strand synthesis. On day 3, the cells were treated with ARA-C, which was removed on day 5. The cells remained in culture until day 14 or 15, when patch clamp studies were performed. This MOI has been shown to transduce approximately 75% of the neurons in culture but have no deleterious effects upon culture viability (unpublished data).

Patch clamp procedures

Electrophysiological studies were performed under voltage-clamp in the whole-cell configuration using an Axopatch-1D amplifier. Recording pipettes were fabricated from N51A capillary glass (Drummond Scientific). The internal solution used for measuring ion currents induced by NMDA included 150 mM KCL, 3.1 mM $MgCl_2$, 15 mM HEPES, 2 mM K-ATP, 5 mM EGTA, 15 mM phosphocreatine, and 50 U/ml creatine phosphokinase. Inclusion of the last two items regenerates ATP and GTP, decreasing cell rundown [26]. This solution was adjusted to pH 7.4 and had an osmolality of 310 (adjusted with sucrose). Seals were formed on the neurons with electrodes having a tip resistance of 2–4 MΩ. Data were displayed on an oscilloscope, digitized at 50 milliseconds/sample, and stored on a personal computer. Recordings were performed at room temperature in a bath where the neurons were superfused at 0.5–1.0 ml/minute with standard external solution (145 mM NaCl, 5 mM KCl, 10 mM Hepes, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose, pH = 7.4, 340 mosmoles). NMDA (1,10,100, and 1000 μM) was applied by a U-tube placed 25–50 μm from the neuron for 4-second intervals with a minimum of 1 minute between applications. This technique allowed a brief cellular application and rapid removal of the drugs.

Recombinant AAV infusion and inferior collicular electrode implantation

All of the animals were pathogen-free male Sprague-Dawley rats obtained from Harlan (Indianapolis, IN). The animals were maintained in a 12-hour light-dark cycle and had free access to water and food. All care and procedures were in accordance with the Guide for the

Care and Use of Laboratory Animals (DHHS Publication No. [NIH]85-23), and all procedures received prior approval by the University of North Carolina Institutional Animal Care and Use Committee.

For infusions and implants, all animals were first anesthetized with 50 mg/kg of pentobarbital and then placed into a stereotaxic frame. Using a 32-gauge stainless steel injector and a Sage infusion pump, animals received 1 ml of AAV-tTAK-NR1A (n = 8) over 9 minutes into the temporal cortex (IAL 3.2 mm, lateral 6.5, vertical 6.3, according to the atlas of Paxinos and Watson [27]). The injector was left in place for 1 minute post-infusion to allow diffusion from the injector. The animals were perfused 3 weeks later, and their brain sections were processed for NMDAR1 immunohistochemistry. A second group of infusions were performed in concert with inferior collicular electrode implants. These animals received a 1-μl infusion over 9 minutes of either AAV-tTAK-NR1A (n = 6), AAV-CMV-NR1A (n = 5), AAV-tTAK-GFP (n = 5), or AAV-tTAK-PINA (n = 4) into the seizure-sensitive area of the inferior collicular cortex (IAL 0.2 mm, lateral 1.6 mm, vertical 3.5 mm, according to the atlas of Paxinos and Watson [27]). The injector was left in place for 1 minute post-infusion to allow diffusion from the injector. After removal of the injector, a tripolar, stimulating electrode (0.015 inches) stainless steel, insulated except for the tip cross section, 400 μM vertical tip separation) was implanted into the site of AAV infusion, and the electrode was secured to three screws in the skull with cranioplastic cement. The animals were allowed at least 5 days to recover from the surgery before any seizure threshold tests were conducted.

Seizure threshold testing procedure

The baseline seizure stimulation threshold current and wild running duration were determined 5 and 7 days after surgery as described [17]. Briefly, animals were connected to a Grass Model SD9 stimulator and stimulation (30 Hz, 1.5 millisecond duration, monophasic square wave) was initiated at 80 μA (all stimulation currents were continuously monitored on an oscilloscope by measuring the voltage drop across a 10Ω resistor). The stimulation current was increased 20 μA every 5 seconds until the first appearance of wild running behavior. At this instance, the stimulation was terminated, and the post-stimulus wild running duration was timed. Following these first two seizure threshold determinations, the animals were tested once every 7 days to evaluate the effects of the gene transfer and expression on seizure threshold. Under control, unperturbed conditions, the seizure threshold remains stable over an extended period of repeated testing [17]. The seizure thresholds were determined once a week for 4 weeks after AAV vector infusion.

Finally, three rats received 1 μl infusions of a 1:1 mixture of AAV-tTAK-GFP and AAV-CMV-LacZ vectors into the inferior colliculus. The animals were perfused, and their brains were processed for β-galactosidase immunohistochemistry 1 week post-infusion.

Immunohistochemistry and density imaging procedures

Animals that received cortical infusions of AAV-tTAK-NR1A were anesthetized with 100 mg/kg pentobarbital intraperitoneally and perfused transcardially with icecold 100 mM sodium phosphate-buffered saline (PBS), pH 7.4, followed by 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.4. After overnight fixation in the paraformaldehyde-phosphate buffer, vibratome sections (40 μm thick) were taken and rinsed in PBS. Tissue sections were incubated in 10% normal horse serum and 0.2% Triton X-100 in PBS for 30 minutes. Next, sections were incubated with a monoclonal antibody to the NMDAR1 receptor protein (1:1000 dilution; Chemicon, Temecula, CA) in 3% normal horse serum, 0.2% Triton X-100, and PBS for 48 hours at 4°C. Tissue sections were then rinsed in PBS and processed through secondary biotinylated horse anti-mouse antibody and avidin-biotin complex using a

Vectastain Mouse Elite ABC Kit (Vector Laboratories, Burlingame, CA). Visualization of NMDAR1-like immunoreactivity was achieved by nickel/cobalt enhancement of 3,3′-diaminobenzidine tetrahydrochloride. The sections were imaged on an Olympus IX 70 microscope and digitized using a Hammamatsu Orca digital camera and QED image acquisition software. The cell density of immunoreactive product for at least eight cells per section was assessed in the area of the infusion and the contralateral side using National Institutes of Health image software. This provided a relative density of the NMDAR1-like immunoreactivity, with the contralateral side serving as the within-subject control.

For the group that received the mixture of AAV-tTAK-GFP and AAV-CMV-LacZ vectors, the same perfusion and sectioning procedure described above was performed 1 week post-infusion. Then, tissue sections containing the inferior colliculus were incubated in 10% normal goat serum and 0.2% Triton X-100 in PBS for 30 minutes. Next, sections were incubated with a monoclonal antibody to β-galactosidase (1:1000 dilution; Chemicon, Temecula, CA) in 3% normal goat serum, 0.2% Triton X-100, and PBS for 48 hours at 4°C. Tissue sections were rinsed in PBS, incubated in 10% normal goat serum for 1 hour, and then incubated for 1 hour at 4°C with a fluorescently labeled goat-anti-mouse secondary antibody (Alexa 594 goat-anti-mouse IgG; Molecular Probes, Eugene, OR). Following three rinses in PBS, the sections were mounted on slides and coverslipped with fluorescent mounting media. The enhanced GFP and Alexa 594 fluorescence were visualized on an Olympus IX 70 fluorescence microscope.

Statistics

For the patch clamp studies, GraphPad Prism software (version 2.0, GraphPad Software) was used to fit the data to sigmoidal concentration-response curves. A two-way analysis of variance (ANOVA) was used to test for a significant effect between the control and virus treated cultures, followed by a post-hoc Duncan Range test for the specific NMDA concentrations. For the seizure threshold comparisons, each animal served as its own control, so changes were evaluated using a paired *t*-test. The densities of the NMDAR1 protein immunoreactivity were compared using a Student's *t*-test. $P < 0.05$ was considered significant.

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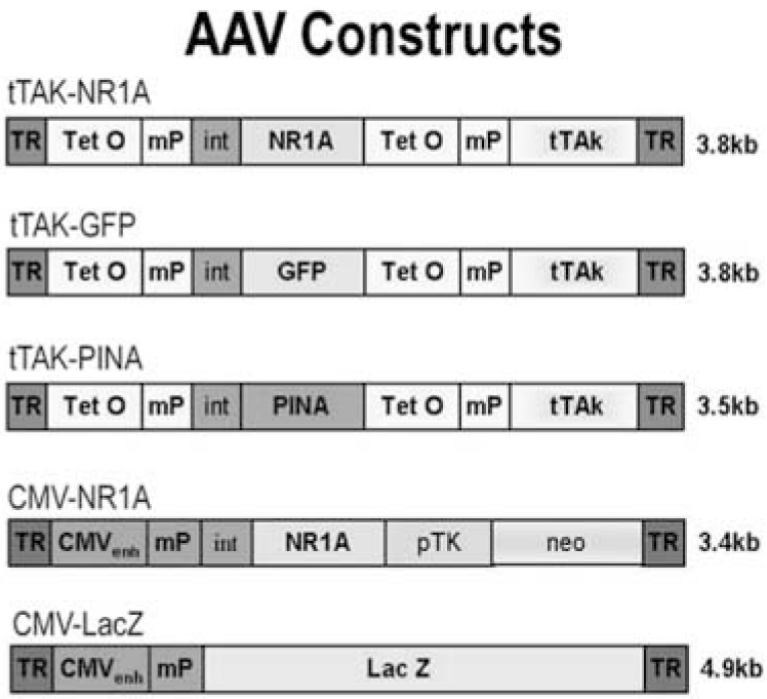

FIG. 1.

Diagrammatic representation of the five different AAV constructs used in the present studies. CMVenh, CMV enhancer. mP, CMV minimal promoter. Tet-O, seven repeats of the *tet* repressor binding sequence. int, SV40 intron. NR1A, 729-bp NMDAR1 fragment in antisense orientation. *gfp*, Enhanced green fluorescent protein gene. PINA, a 449-bp PIN fragment in antisense orientation. *lacZ*, β-Galactosidase gene. *neo*, Neomycin resistance gene. TR, AAV terminal repeat.

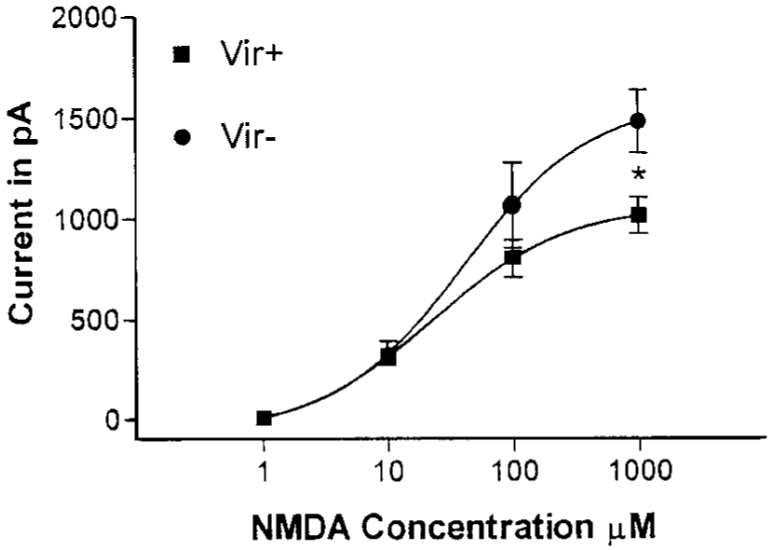

FIG. 2.

The effects of AAV-NR1A infection on current responses of primary cultured cortical neurons following NMDA application (n = 9 for each condition). GraphPad Prism software (version 2.0, GraphPad Software) was used to fit the data to sigmoidal concentration-response curves. A two-way ANOVA showed a significant effect of NMDA concentration ($F_{3,64} = 55.1$; $P < 0.05$) and a significant effect of the presence or absence of virus ($F_{1,64} = 5.96$; $P < 0.05$). A post-hoc Duncan Range test showed that the infected cultures showed a significantly lower current gated by the peak 1 mM concentration of NMDA.

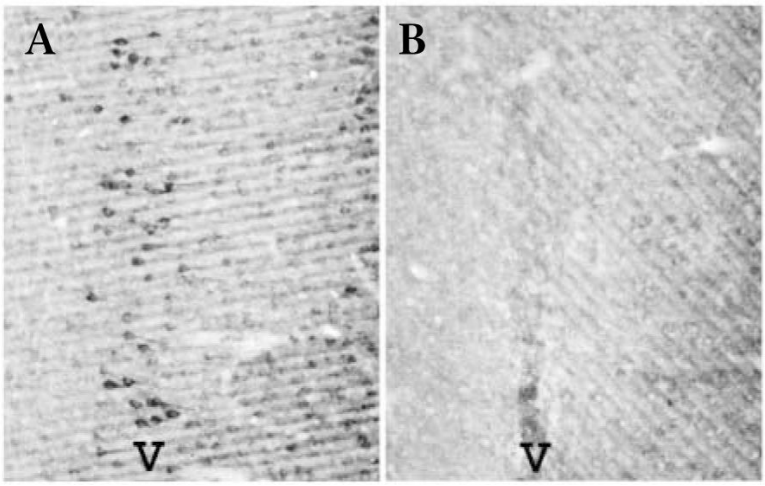

FIG. 3.

The effects of AAV-tTAK-NR1A virus infusions on temporal cortex layer V pyramidal cells. (A) NMDAR1-like immunoreactivity in layer V pyramidal cells in the uninjected, contralateral temporal cortex. (B) Most, if not all, of the NMDAR1-like immunoractivity is absent in layer V pyramidal cells 3 weeks after infusion of AAV-tTAK-NR1A, and, for the entire treatment group, the injected side layer V density was 17 ± 6% of that in the control side (mean ± SEM; $P < 0.01$).

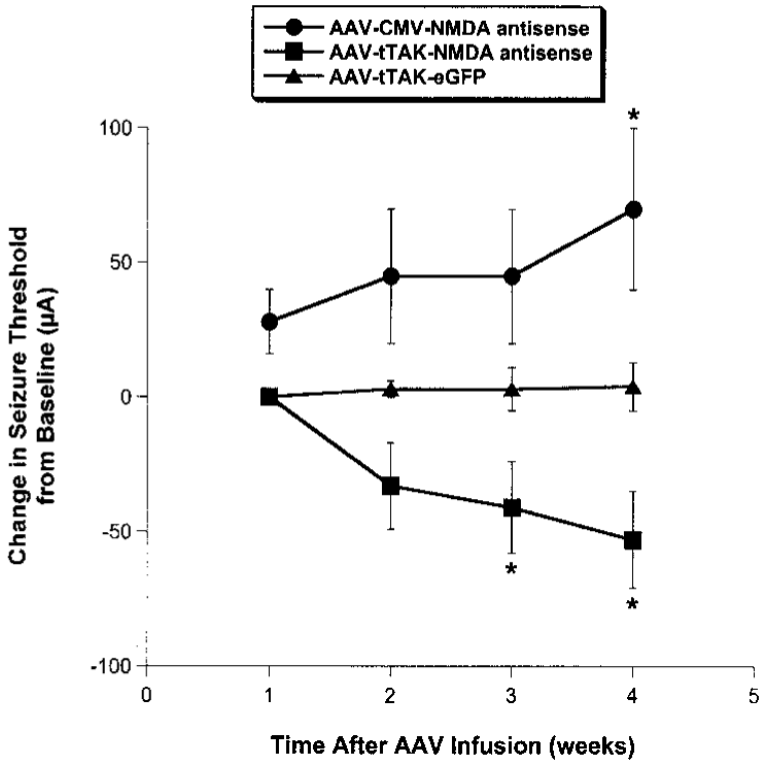

FIG. 4.

The effect of inferior collicular infusion of AAV-CMV-NR1A, AAV-tTAK-NR1A, or AAV-tTAK-GFP on seizure threshold sensitivity. Following virus infusion and electrode implant, the seizure initiation threshold was determined weekly. As seen across weeks 2–4, the AAV-tTAK-NR1A group exhibited a significant decrease in seizure threshold compared with the baseline (*$P < 0.01$), whereas no significant changes were found after AAV-tTAK-GFP infusion. In marked contrast, infusion of AAV-CMV-NR1A caused a significant increase in the seizure threshold by week 4 (*$P < 0.01$).

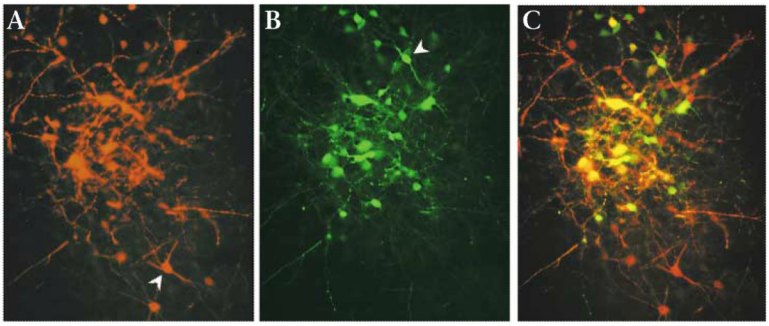

FIG. 5.

The transduction patterns 1 week after infusion of a 1:1 mixture of AAV-tTAK-GFP and AAV-CMV-LacZ vectors into the inferior colliculus. In the same section are shown *lacZ*-positive cells (A) (red), *gfp*-positive cells (B) (green), and both (C) using a dual pass filter. It can be seen that, although several cells express both gene products (yellow), several cells express only one of the two gene products. The arrows in (A) and (B) illustrate the difference. When the transduced neurons were counted across the area of transduction, 41% were positive for *gfp* alone, 24% were positive for *lacZ* alone, and 35% were positive for both reporter genes. This transduction pattern illustrates the likely difference in *in vivo* tropism between the two similar AAV vectors.