Neuroscience 145 (2007) 314–322

α7 NICOTINIC RECEPTOR GENE DELIVERY INTO MOUSE HIPPOCAMPAL NEURONS LEADS TO FUNCTIONAL RECEPTOR EXPRESSION, IMPROVED SPATIAL MEMORY-RELATED PERFORMANCE, AND TAU HYPERPHOSPHORYLATION

K. REN,[a] J. THINSCHMIDT,[b] J. LIU,[a] L. AI,[b] R. L. PAPKE,[b] M. A. KING,[c] J. A. HUGHES[a] AND E. M. MEYER[b]*

[a]*Department of Pharmaceutics, Box 100494, University of Florida, Gainesville, FL 32610, USA*

[b]*Department of Pharmacology, Box 100267, University of Florida, Gainesville, FL 32610, USA*

[c]*Department of Neuroscience, University of Florida, Gainesville, FL 32610, USA*

Abstract—Brain α7 nicotinic receptors have become therapeutic targets for Alzheimer's disease (AD) based on their memory-enhancing and neuroprotective actions. This study investigated the feasibility of increasing neuronal α7 receptor functions using a gene delivery approach based on neuron-selective recombinant adeno-associated virus (rAAV)–derived vectors. In order to determine whether α7 receptor-mediated cytotoxicity was dependent on receptor density, rat α7 nicotinic receptors were expressed at high concentrations in GH4C1 cells as measured with nicotine-displaceable [^{3}H]methyllycaconitine (MLA) binding. The potency of GTS-21 (an α7 receptor agonist) to induce cell loss was similar in these cells to that seen in pheochromocytoma (PC12) cells expressing nine-times-lower receptor levels, suggesting that cytotoxicity was more dependent on agonist concentration than receptor density. Hippocampal transduction with rat α7 nicotinic receptors increased [^{3}H]MLA binding in this region in wild type and α7 receptor-knockout (KO) mice without apparent cytotoxicity. No difference was observed in Kd values for MLA binding between endogenous and transgenic receptors. Single cell recordings demonstrated that dentate granule cells that normally have no α7 receptor response did so following α7 receptor gene delivery in wild type mice. Recovery of α7 function was also observed in stratum oriens and stratum radiatum neurons of KO mice following gene delivery. Wild type mice exhibited improved acquisition performance in the Morris water task 1 month after bilateral hippocampal transductions with the rat α7 receptor gene compared with green fluorescent protein–transduced controls. However, both groups reached similar training levels and there was no difference in subsequent probe performance. Finally, this gene delivery approach was used to test whether α7 receptors affect tau-phosphorylation. Chronic (i.e. 2 month but not 2 week) expression of high levels of α7 receptors in hippocampus increased AT8 staining characteristic of hyperphosphorylated tau in that region, indicating that endogenous agonist-mediated receptor activation may be able to modulate this process.

Key words: α7 nicotinic receptors, recombinant adeno-associated virus.

α7 Nicotinic receptors are homomeric pentamers concentrated in hippocampus and neocortex (Seguela et al., 1993) that are involved in cell viability (Ren et al., 2005) and a variety of memory-related and attentional behaviors (Meyer et al., 1998b; Ren et al., 2005). Activation of these receptors improves spatial and avoidance memory-behaviors in rodents, avoidance behavior in rabbits, delayed matching performance in primates, and word recall in humans (Arendash et al., 1995; Briggs et al., 1997; Meyer et al., 1997; Woodruff-Pak, 2003; Marubio and Paylor, 2004). In contrast, hippocampal infusion of methyllycaconitine (MLA), an α7 nicotinic receptor antagonist, interferes with memory-related behavior (Woodruff-Pak, 2003). α7 Nicotinic receptors have accordingly become targets for treating conditions associated with memory or attentional deficits, including Alzheimer's disease (AD), psychosis, and age-related cognitive decline.

One approach to increase or restore α7 nicotinic receptor-function has been to administer selective agonists such as GTS-21, which has been effective in non-AD animal models and in healthy humans (Kitagawa et al., 2003). However, several factors may complicate this approach in AD. Total hippocampal nicotinic α7 receptor binding density is reduced in AD, and the neuronal component of this deficit may be even more severe due to the recently discovered concomitant increase in astrocytic receptor-expression in this condition (Teaktong et al., 2003). This observation also suggests that α7 receptor agonists may have fewer functional receptors to activate in AD, and moreover, that these agonists may affect non-neuronal populations as well. Other factors reducing neuronal α7 receptor function in AD include deficits in cholinergic innervation of the hippocampus (Mufson et al., 2003), as well as the potent inhibition of these receptors by amyloidogenic peptides that accumulate in AD (Liu et al., 2001; Thinschmidt et al., 2005). The combined loss of cholinergic plus GABAergic septal innervation of the hippocampus was recently found to reduce α7 receptor function in that region (Thinschmidt et al., 2005).

*Correspondence to: E. M. Meyer, University of Florida, ARB R5-110, 1600 Southwest Archer Road, Gainesville, FL 32610, USA. Tel: +1-352-392-6364; fax: +1-352-392-9696.
E-mail address: meyere@ufl.edu (E. M. Meyer).

Abbreviations: ACSF, artificial cerebrospinal fluid; AD, Alzheimer's disease; FBS, fetal bovine serum; GFP, green fluorescent protein; gp, genomic particle; KO, knockout; KRH, Krebs Ringer buffer; MLA, methyllycaconitine; PBS, phosphate-buffered saline; PC12, pheochromocytoma; rAAV, recombinant adeno-associated virus.

0306-4522/07$30.00+0.00 © 2006 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2006.11.023

We therefore investigated an alternative technique to elevate α7 nicotinic receptors for extended intervals in a neuron-selective manner, using recombinant adeno-associated virus (rAAV)–mediated delivery of α7 nicotinic receptor transgene directly into the hippocampus. rAAV vectors have been used extensively for gene delivery into brain and been found to be non-toxic, to transduce postmitotic neurons selectively in this organ, and to permit long-term transgene expression gene when combined with appropriate promoters (Peel and Klein, 2000). For this study, rAAV8/2 hybrid vectors were used for transduction of the hippocampus due to their ability to achieve gene transfer within large tissue volumes (Klein et al., 2006). High affinity ligand binding and electrophysiological assays were used to demonstrate increased expression of functional receptors in wild type and α7 knockout (KO) mice. Morris water task performance was evaluated to determine whether increased gene expression, in the absence of any agonist treatment, was sufficient to improve spatial memory–related behavior. Since very rapid exposure to high concentrations of α7 nicotinic receptor agonists may be toxic under some conditions *in vitro* (Li et al., 1999b), we also evaluated the potential effects of receptor gene delivery on cell viability with increasing agonist concentrations.

Following the characterization of this gene delivery model relative to ligand binding, electrophysiological responses, and behavior, it was used to test the hypothesis that long term hippocampal α7 nicotinic receptor expression affects tau phosphorylation as seen following chronic nicotine administration (Oddo et al., 2005). Tau hyperphosphorylation is widely believed to be an early event in the formation of neurofibrillary tangles in AD (King, 2005). Since α7 nicotinic receptors contribute to amyloid-induced tau hyperphosphorylation, we hypothesized that they may underlie the effects of nicotine on this process as well (Wang et al., 2006).

EXPERIMENTAL PROCEDURES

rAAV2 and rAAV8/2 preparation

rAAV2 and rAAV8/2 vectors were prepared and quantified using the methods of Zolotukhin et al. (1999) and Klein et al. (2006), respectively. rAAV2 and rAAV8/2 contained identical expression cassettes flanked by AAV2 terminal repeats, but differed with respect to capsid serotypes (AAV2 vs. AAV8). Expression of green fluorescent protein (GFP) or rat α7 nicotinic receptor was driven by a chicken β-actin promoter containing the human cytomegalovirus enhancer.

Plasmids were propagated in SURE cells (Stratagene, La Jolla, CA, USA) and CsCl-purified. Briefly, 70% confluent human embryonic kidney 293 cells were transfected by the calcium-phosphate method with AAV terminal repeat-containing GFP or rat α7 nicotinic receptor plasmid in equal molar ratios with the rAAV2 or rAAV8/2 helper plasmid. After 3 days, cells and media were harvested and centrifuged at 3000×*g*. The pellets were resuspended in a solution of 50 mM Tris, pH 8.3, and 150 mM NaCl, then freeze-thawed three times. The resulting suspension was put through a discontinuous iodixanol gradient followed by a heparin sulfate column purify the rAAV2. A Q-sepharose column (Sigma Chemicals, St. Louis, MO, USA) was used in place of the heparin sulfate column to purify rAAV8/2. Vector doses were expressed as genomic particles (gp).

Cell culture studies

The GH4C1 cell line is pituitary-derived and is one of the few lines that normally express the RIC-3 chaperone protein for α7 nicotinic receptors but not the receptors themselves; thus, these cells are commonly used for functional receptor transfection/transduction studies (Williams et al., 2005). GH4C1 and pheochromocytoma (PC12) cells, which normally functional express α7 nicotinic receptors, were obtained from American Type Culture Collection. (Manassas, VA, USA) GH4C1 cells were grown in F-10 nutrient mixture containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and PC12 cells were grown in an RPMI medium 1640 mixture containing 10% horse serum, 5% FBS and 1% penicillin/streptomycin. Both cell types were incubate at 37 °C in 5% CO_2 and 90–92% humidity, and were split at a 1:3 ratio every 5 days, up to 10 passages. Cell confluence at the initiation of each study was approximately 60%. PC12 cells were treated for 7 days with 100 ng/ml nerve growth factor (NGF, BD Biosciences, San Diego, CA, USA), which was added at days 1 and 3. At that time, they were treated with specified GTS-21 concentrations for another 2 days, when cell density and high affinity [^{3}H]MLA binding were determined. To initiate gene delivery in GH4C1 cells, the cell culture medium was removed and cells were exposed to a 0.05% trypsin/0.53 mM EDTA solution for 5 min. Trypsin/EDTA was removed by transferring the cellular suspension to sterile conical tubes, which were centrifuged at 3000×*g* for 5 min. Pellets were resuspended in 150 μl fresh F-10 medium. Vectors were added and incubated for 30 min at 37 °C. After incubation, cells were plated in 60 mm dishes to which 2 ml of fresh medium were added. Three days later, cells were either assayed for high affinity [^{3}H]MLA binding or treated for another 2 days with specified GTS-21 concentrations to determine its effect on cell viability. The number of cells remaining at that time was used to estimate viability, based on counting the total number of cells at three random sites/plate (Li et al., 1999a).

Stereotaxic surgeries

C57BL/6 mice and α7 KO mice from the same strain were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). All animals were housed in the animal facility of the Health Science Center at the University of Florida. Mice were anesthetized with 4% isoflurane/O_2. After mounting the head on a stereotaxic frame, measurements were taken from bregma midline and the top of the skull. The injection coordinates were −2.06 mm bregma, ±1.8 mm medial–lateral, and −2.0 mm dorsal–ventral. rAAV2-rat α7, rAAV2-GFP, rAAV8/2-rat α7 and rAAV8/2-GFP vectors were injected into hippocampus through a 27 ga cannula connected via 26 ga I.D. polyethylene tubing to a 10 μl syringe mounted to a CMA/100 microinjection pump. The pump delivered 2 μl virus (10^{10} gp) at a rate of 0.2 μl/min. The needle remained in place at the injection site for 2 min before removal. The cannula was removed slowly after the injection and the skin was sutured. All animal care and procedures were in accordance with institutional IACUC and NIH guidelines. Every effort was made to minimize the number of animals used and their suffering.

High affinity [^{3}H]MLA binding assay

Brain tissues or cell culture samples were prepared for nicotine-displaceable, high-affinity [^{3}H]MLA binding assay as described previously (Thinschmidt et al., 2005). Tissues were rapidly dissected from killed animals following 4% isoflurane/O_2 anesthesia and suspended in ice-cold Krebs Ringer buffer (KRH; in mM: 118 NaCl, 5 KCl, 10 glucose, 1 $MgCl_2$, 2.5 $CaCl_2$, 20 Hepes; pH 7.5). Ice-cold KRH was also used to wash and harvest culture cells. Brain tissues or cells were homogenized in ice-cold KRH buffer with a Polytron (setting 4 for 10 s). After two 1 ml washes with KRH at 20,000×*g*, the membranes were incubated in 0.5 ml KRH with

0.5 nM to 50 nM [^{3}H]MLA (63 Ci/mmol; Tocris, Ellisville, MO, USA), for 60 min at 4 °C, plus or minus 5 mM nicotine. Kd and Bmax values were determined by Scatchard analyses using the Statview program. Tissues were washed three times with 5 ml ice-cold KRH buffer by filtration through Whatman GF/C filters that were preincubated for 30 min with 0.5% polyethylenimine (Sigma). Liquid scintillation (EcoLite, Fisher Pharmaceuticals, Hampton, NH, USA) counting of radioactivity was conducted in a Beckman LS1800. Nicotine-displaceable binding was calculated for each sample in triplicate in each experiment, and normalized for total protein (Bio-rad assay) (Thinschmidt et al., 2005).

Immunohistochemistry

Animals anesthetized with 4% isoflurane/O_2 were perfused with 100 ml of cold phosphate-buffered saline (PBS), followed by 400 ml of cold 4% paraformaldehyde (Sigma) in PBS. The brain was removed and equilibrated in a cryoprotectant solution of 30% sucrose/PBS and stored at 4 °C. Coronal sections (50 μm) were cut on a sliding microtome with freezing stage. Antigen detection was conducted on floating sections by incubation in a blocking solution (2% goat serum/0.3% Triton X-100/PBS) for 30 min at room temperature, followed by primary antibody incubation overnight at 4 °C. The primary antibody used was anti-human AT8 (Ser-202/Thr-205) monoclonal antibody (1:200, Pierce Pharmaceuticals, Rockford, IL, USA). Sections were washed in PBS three times for 5 min each and then incubated with biotinylated anti-mouse IgG (1:1000, Dako, Carpinteria, CA, USA) for 1 h at room temperature. The sections were then washed with PBS three times for 5 min each and labeled with fluorescent streptavidin conjugate (1:200, Invitrogen) for 3 h at room temperature. The sections were then washed with PBS three times for 5 min each. Sections were mounted on Fisher Superfrost Plus slides with glycerol gelatin (Sigma).

FluoroJade staining

FluoroJade staining of degenerating neurons (Zuch et al., 2000) was conducted in floating brain sections with 100% ethanol for 3 min, followed by 70% ethanol and dH_2O for 1 min each, 0.06% potassium permanganate for 15 min, and a wash with dH_2O for 1 min. The sections were treated in the dark with 0.001% FluoroJade for 30 min and then mounted on slides, air-dried and coverslipped with glycerol gelatin.

Morris water task

Morris water maze was performed to test spatial learning and memory of mice injected rAAV8/2-rat α7 vectors using a water tank (1 m diameter) designed for mice with a fixed platform 1 cm below the water surface (Monteiro et al., 2005). Mice received three blocks of training of eight trials daily for 3 days followed 1 day later by a probe trial. During training, mice were hand-guided to the platform if they did not reach it during a 60 s interval. The mouse was allowed 30 s on the platform. For each trial, latency to find the platform, path length to the platform, and swim speed were recorded by a video-tracking/computer-digitizing system (HVS Image, Hampton, UK). The probe consisted of a 60 s interval in which the mice searched for the removed platform. The swim distance and percentage of time spent in each quadrant were recorded. The total daily times required for each animal to reach the platform and the total times for the 3 day training intervals were determined and compared between the two GFP- and receptor-transduced groups by two-way Student's *t*-test, which was also used to compare the main effects of probe performance between the treatment groups.

Electrophysiological recordings

Fresh transverse brain slices (300 μm) were prepared using a vibratome (Pelco, Redding, CA, USA). Slices were incubated at 30 °C for 30 min and then maintained submerged at room temperature. The artificial cerebrospinal fluid (ACSF) used for cutting and incubating slices contained in mM: 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 2.5 $MgSO_4$, 10 D-glucose, 1 $CaCl_2$, and 25.9 $NaHCO_3$, saturated with 95% O_2/5% CO_2. Following incubation, slices were transferred to a recording chamber where they were superfused at a rate of 2 ml/min with ACSF at 30 °C containing in mM: 126 NaCl, 3 KCl, 1.2 NaH_2PO_4, 1.5 $MgSO_4$, 10 D-glucose, 2.4 $CaCl_2$, and 25.9 $NaHCO_3$, saturated with 95% O_2/5% CO_2. Individual neurons were identified with infrared differential interference contrast microscopy (IR DIC) using a Nikon E600FN microscope (Nikon Instruments, Melville, NY, USA). Whole-cell patch-clamp recordings were made with pipettes pulled on a Flaming/Brown electrode puller (Sutter Instruments, Novato, CA, USA). Pipettes were typically 3–5 MΩ when filled with an internal solution that contained in mM: 125 K-gluconate, 1 KCl, 0.1 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 2 MgATP, 0.3 NaGTP, and 10 Hepes. All internal solutions were approximately 285 mOsm and were pH adjusted to 7.3 with KOH.

For experiments involving fluorescence microscopy, GFP-expressing neurons were visualized using light from a mercury lamp filtered at 510–560 nM. For all local application experiments, a picospritzer (General Valve, Fairfield, NJ, USA) was used to apply a solution containing 1 mM ACh from pipettes identical to those used for whole-cell recording or from double-barreled pipettes made using theta tubing (Sutter Instruments). It should be noted that the actual concentration of ACh that reached these cells prior to their receptor-desensitization was much less than the 1 mM contained in the pipette (Papke, 2006). An Axon Multiclamp 700A amplifier (Axon Instruments, Union City, CA, USA) was used to amplify voltage and current records. The data were sampled at 20 kHz, filtered at 2 kHz, and recorded on a computer via an Axon Digidata 1322 analog-to-digital converter using Clampex version 9.0 (Axon Instruments). Data were analyzed using Clampfit version 9.0 (Axon Instruments), Microsoft Excel and GraphPad Prism v. 3.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

GH4C1 cells expressed high affinity [^{3}H]MLA binding (750±62 fmol/mg protein, mean±S.E.M. of three separate experiments, each conducted in triplicate) 3 days after transduction with 2.5×10^9 gp of the rAAV2 α7 nicotinic receptor expression vector. In contrast, no binding was seen in these cells after transduction with the same dose of the control GFP-vector. GTS-21 had no effect on cell density in GFP-transduced GH4C1 cells when added 3 days post-transduction but did reduce cell density in α7- receptor transduced GH4C1 cells in a concentration-dependent manner (Fig. 1). GTS-21 also reduced the density of NGF-differentiated, non-dividing PC12 cells over this 2 day interval at similar or slightly higher concentrations (Fig. 1). These differentiated PC12 cells expressed a much lower nicotine-displaceable [^{3}H]MLA binding level (78±6 fmol/mg protein, mean±S.E.M.; N=3 experiments) at the time of GTS-21 administration than receptor-transduced GH4C1 cells did, indicating that the agonist-induced toxicity may be more dependent on agonist-concentration than receptor-density.

High affinity [^{3}H]MLA binding was observed on the side of the hippocampus of KO mice injected with 1.5×10^{10} gp of the rAAV2 α7 receptor vector after 2 weeks, but not in

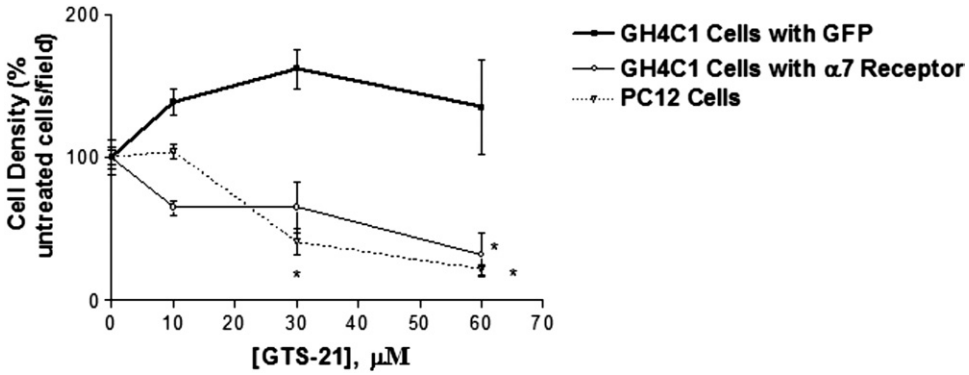

Fig. 1. Effects of GTS-21 on the density of α7 receptor- and GFP-transduced GH4C1 cells, as well as differentiated PC12 cells. GH4C1 cells were transduced with GFP or α7 nicotinic receptors (2.5×10^9 gp/200 μl) and 3 days later exposed to specified concentrations of GTS-21 for 2 days. PC12 cells were differentiated with 100 ng/ml NGF for 1 week, and then also exposed to GTS-21 for 2 days. Cell density is expressed as the percentage of mean values for untreated cells (no GTS-21) for the same experiment; each value is the mean±S.E.M. of three separate experiments, each conducted in triplicate. * $P<0.05$ compared with same cell type with no GTS-21 (one-way ANOVA).

the contralateral hippocampus (Fig. 2). Wild type mice also had much higher Bmax levels of hippocampal [^{3}H]MLA binding on the α7 receptor vector injected side of the hippocampus than on the contralateral uninjected side, indicating higher receptor concentrations. Hippocampal [^{3}H]MLA binding on the transduced side of the wild type mice was also higher than that of the transduced KO mice to an extent that appeared more than additive with endogenous ligand binding.

In an attempt to increase α7 receptor gene delivery and expression further, a higher dose of the rAAV8/2-α7 receptor vector, 3.6×10^{10} gp, was injected into wild type mouse hippocampus. However, the level of hippocampal high affinity [^{3}H]MLA binding in these mice was similar (926±57 fmol/mg protein, mean±S.E.M. of six animals) 2 weeks post-injection to that seen with the lower dose of rAAV2-α7 vector above. Wild type mice injected with the

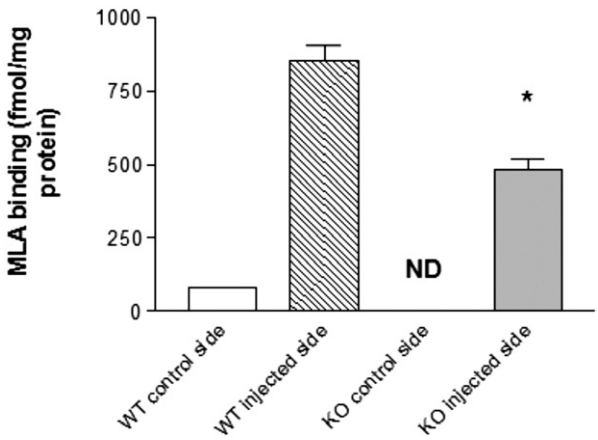

Fig. 2. [^{3}H]MLA binding in wild type (WT) and α7 receptor KO mouse hippocampus. 1.8×10^{10} gp of rAAV2-rat α7 receptor were unilaterally injected into WT or KO mouse hippocampus. Two weeks later, each hemisphere of the hippocampus was assayed separately for nicotine displaceable [^{3}H]MLA binding as described in the text, and expressed as the mean±S.E.M. of at least four animals per group. * $P<0.05$ compared with same genotype, untreated side (*t*-test); ND: not detectable.

rAAV8/2-GFP vector expressed much lower levels of binding (23±2.5 fmol/mg protein, $N=7$ animals) than receptor-transduced animals did, reflecting endogenous receptor expression. Scatchard analyses revealed monophasic binding slopes for [^{3}H]MLA binding in the rAAV8/2-α7 receptor and rAAV8/2-GFP transduced hippocampus, and essentially identical Kd values of 2.2 nM and 2.0 nM, respectively, after these treatments. Thus, it appeared that the transduced rat receptor had similar binding characteristics to the endogenous mouse α7 receptor. No FluroJade labeling of dying neurons was observed in the injection region for at least 2 months after vector injection ($N=4$ animals/group; not shown).

To evaluate the function of transgenic α7 receptors electrophysiologically, 1.8×10^{10} gp of rAAV8/2-rat α7 vector was injected unilaterally into wild type or KO mouse hippocampus, along with 0.6×10^{10} gp of rAAV8/2-GFP to label likely transduced neurons. The contralateral side received 0.6×10^{10} gp of rAAV8/2-GFP only. When ACh was applied to GFP-labeled dentate granule cells from a pressure application pipette containing 1 mM ACh in the α7 receptor-transduced hemisphere, there was a significant MLA-sensitive response (Fig. 3B). Control dentate granule cells transduced with GFP-only on the contralateral side had no MLA-sensitive responses to 1 mM ACh, as expected (Fig. 3A). A typical response from striatum radiatum interneurons, which normally do express α7 receptors, is shown in Fig. 3C. GFP-labeled stratum radiatum neurons from the α7 receptor transduced side responded to ACh with a current amplitude that was about 50 times that of the conventional, α7 receptor type current in the contralateral control side (Fig. 3D).

α7 Receptor gene delivery also restored receptor function in 2 month old α7 KO mice (Fig. 3E). GFP-labeled stratum radiatum and stratum oriens neurons had α7 nicotinic receptor responses evoked by pressure application from pipettes containing 1 mM ACh that were blocked with 50 nM MLA (Fig. 3C). These results demonstrate that α7 receptors could be expressed functionally in KO mice in

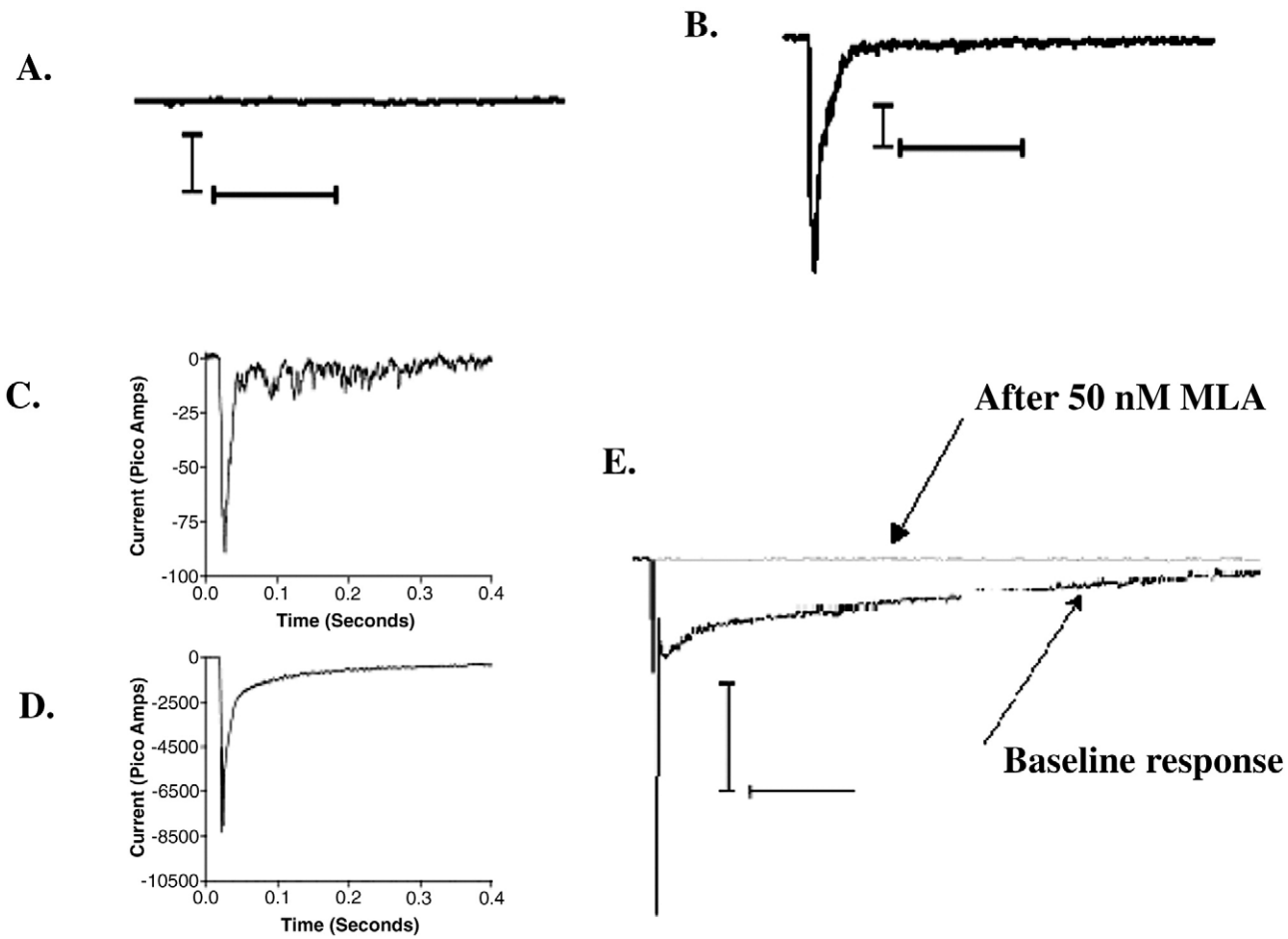

Fig. 3. Hippocampal electrophysiological responses in mice transduced with rat α7 nicotinic receptors. (A, B) Responses of wild type mouse dentate granule cells to 1 mM ACh in GFP- and α7 receptor-transduced sides of hippocampus, respectively, with N=6 positive-response cells on receptor-transduced side out of six GFP-labeled neurons; N=4 negative-response cells out of four GFP-labeled cells on the contralateral, GFP-only transduced side (scale bars=100 pA vertical/horizontal 100 ms). (C) One millimolar ACh produces a typical α7 receptor current in a hippocampal stratum radiatum interneuron in GFP-only transduced wild type mouse. (D) Neuron located in the hippocampus injected with the α7 receptor gene labeled with GFP. This response is nearly 50 times greater in peak amplitude than that shown in C. (E) Two-month-old α7 KO mouse injected with the α7 receptor gene plus GFP vector. GFP-identified neuron had a response to 1 mM ACh that was completely blocked with 50 nM MLA (scale bars=1 nA and 100 ms). For stratum radiatum and stratum oriens combined, N=4 positive responses from five GFP-labeled neurons; in contrast, no responses were seen in GFP-only transduced hippocampus (N=6).

the same types of neurons of that normally express them in wild type animals.

In the Morris water task, wild type mice receiving bilateral injections of the 1.8×10^{10} gp of rAAV8/2 α7 vector had improved acquisition performance compared with GFP-transduced animals over the 3 day training interval (Fig. 4). No difference in swim speed or distance traveled was observed between these groups. Both groups eventually reached similar performance levels by the end of the training. There was no difference in the probe performance between these groups measured 24 h after the end of the training, with the times spent in the correct quadrant for the receptor-transduced (N=8 animals) and GFP-transduced (N=3 animals) groups being 19.3±3 s and 14.5±4 s, respectively.

Transduction with the rAAV8/2-α7 receptor vector (3.6×10^{10} gp) in the hippocampus of wild type 2 month old mice (N=4) led to significant immunohistochemical AT8-staining (591±102 neurons/hippocampus, mean±S.E.M., N=4) in this region 2 months later (Fig. 5). AT8 staining was not apparent in adjacent neocortex and was limited to the injection area. No AT8 staining was observed 2 weeks post-transduction with this receptor (N=3; data not shown), or at 2 months after GFP-transduction (N=4; Fig. 5).

DISCUSSION

The results of this study indicate that α7 nicotinic receptor expression and function can be modulated in the hippocampus for extended intervals by gene delivery. Gene delivery can increase α7 receptor densities in wild type animals and restore α7 receptor functions in receptor KO animals. Since this approach depends on endogenous neurotransmission to activate transgenic receptors, it likely permits phasic, neuronal activity-dependent receptor stimulation that is more physiological than the

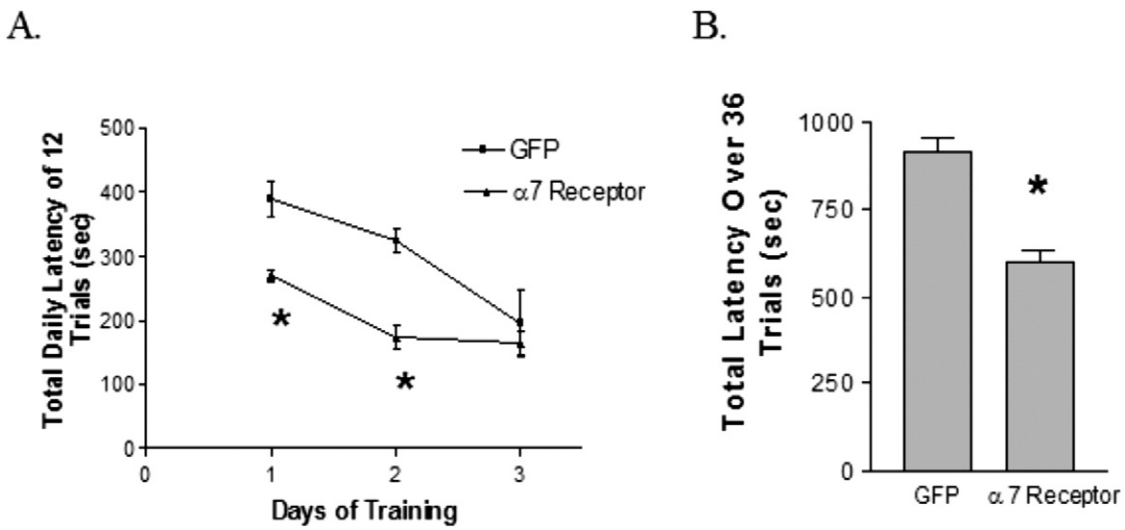

Fig. 4. Morris water task test-performance in wild type mice receiving bilateral injections of rAAV8/2-GFP or rAAV8/2-α7 (1.8×10^{10} gp of each). (A) Daily means±S.E.M. of total times-to-trial for eight trials in mice 1 month after gene delivery. (B) Total times-to-trial for acquisition performance over 3 days (24 trials). * $P<0.05$ compared with corresponding GFP-transduced groups, one-way ANOVA. $N=3$ GFP-transduced and $N=8$ α7 receptor-transduced animals.

tonic receptor activation induced by agonist administration. This provides an alternative approach for studying the role of these receptors in normal and dysfunctional brain function. The incorporation of neuron-specific rAAV vectors in this procedure also permits selective enhancement of neuronal α7 receptor function without activating astrocytic receptors.

Nicotinic α7 receptor gene delivery caused large increases in receptor binding in the hippocampus and appeared to increase both the number of neurons expressing this receptor and the size of these electrophysiological responses in neurons that normally expressed the receptor. It appeared that there was a plateau level of expression reached with the doses used in this study, since [^{3}H]MLA binding was similar in animals injected with 1.8×10^{10} rAAV2 and 3.6×10^{10} rAAV8/2 gp, despite the latter vector recently being found to lead to higher expression in hippocampus (Klein et al., 2006), suggesting that other factors such as RIC3 expression may become limiting.

Dentate granule cells normally do not respond to α7 receptor agonists, which is consistent with our present results with GFP-transduced neurons (Thinschmidt et al., 2005). However, these cells did express typical α7 receptor responses after delivery of this gene into the region. The ability to confer α7 receptor function to cells such as these projection neurons that normally do not express the receptor has potential for protecting them from apoptotic insults when combined with neuroprotective α7 receptor agonists (Shimohama et al., 1998; Ren et al., 2005). Activation of endogenous α7 receptors with GTS-21 has been found to protect against ischemic damage (Shimohama et al., 1998) and trans-neuronal degeneration (Meyer et al., 1998a) *in vivo*, and this type of protection may now be extended to other types of neurons with this gene delivery approach.

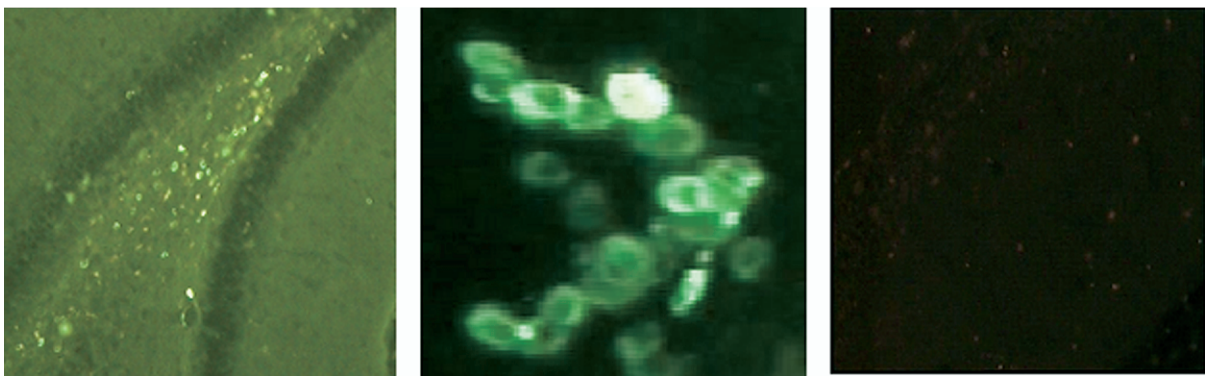

Fig. 5. Immunohistochemical AT8 staining in mouse hippocampus 2 months after rAAV8/2-mediated rat α7 (3.6×10^{10} gp) ($N=4$ animals) or GFP gene delivery (3.6×10^{10} gp) ($N=4$ animals) in that region. Left panel: dentate granule cell layer staining after α7 receptor gene delivery (10×); middle panel: CA1 staining after receptor α7 receptor gene delivery (20×); right panel: lack of hippocampal AT8 staining after rAAV8/2-GFP gene transfer (10×).

While α7 nicotinic receptors are typically neuroprotective at low agonist concentrations, higher agonist concentrations may be cytotoxic under some conditions. The lack of neurotoxicity following overexpression of these receptors in hippocampus may be explained by their biophysical properties (i.e. peak and duration of responses), which are highly dependent on agonist concentration (Papke et al., 2000). High agonist concentrations also trigger rapid desensitization and termination of receptor function (Papke et al., 2000). Neurotoxicity therefore depends on rapid administration of the agonist that is feasible *in vitro* (Li et al., 1999b) but not apparently *in vivo*, accounting for the lack of any toxicity observed after peripheral agonist injections in animals or humans.

With respect to α7 receptor gene delivery, we hypothesized based on these biophysical properties of the receptor that there was also a greater dependency of receptor function on agonist concentration than on receptor density. This was supported by the similar dose-dependency of receptor-transduced GH4C1 cells and PC12 cells to the cytotoxic effects of the α7-receptor agonist GTS-21, despite the former cell type expressing a much higher level of this receptor. This observation suggests that supra-normal levels of receptor expression may be no more toxic to cells than physiological levels are. This appears to be different from what is seen with the neuroprotective actions of α7 receptors, which have been demonstrated to be dependent on receptor density in PC12 cells (Jonnala and Buccafusco, 2001). Therefore, elevating the levels of receptor expression may have the desirable profile of increased neuroprotective activity at low agonist doses combined with no increase in toxicity at high agonist doses.

Although KO mice express ectopic α7 receptors after rAAV gene delivery, this level of transgenic expression appeared to be lower than was seen in identically treated wild type mice. This may be due to changes in intrinsic modulatory factors recently found to affect these receptors such as the level of RIC-3, a protein involved in receptor-processing (Williams et al., 2005). Alternatively, other compensatory factors involving different nicotinic receptor subunits may also occur developmentally in the α7 KO mouse. This is suggested by the observation that while neither the α7 nor the β_2 nicotinic receptor subunit KO mouse shows significant memory-related deficits, the combination KO does (Paylor et al., 1998; Marubio and Paylor, 2004).

The α7 receptor MLA binding affinity (Kd) in hippocampus was comparable between rAAV8/2 transgenic and wild type α7 receptors, despite much higher Bmax values for the vector-treated mice. This finding is significant because it indicates that the processing of the plasma membrane transgenic receptor is comparable to that of the wild type animal. From a therapeutic perspective, it also suggests that the binding affinities of drugs for wild type receptors will be predictive of their activities at brain transgenic receptors.

Pharmacological activation of α7 nicotinic receptors modulates hippocampal synaptic plasticity (Radcliffe and Dani, 1998; Chen et al., 2006) and improves memory-related behaviors, including spatial Morris water task performance (Arendash et al., 1995; Meyer et al., 1997). Memory-related behavioral improvements have been reported following nicotine-administration or treatment with selective α7 agonists in intact and memory-dysfunctional animals (Briggs et al., 1997; Meyer et al., 1997; Attaway et al., 1999). We did observe highly significant improvements in acquisition performance following gene-delivery, which may be due to the chronic nature of our model, with mice having elevated α7 nicotinic receptor levels for several weeks before initiating the Morris water task. While nothing is known about the chronic effects of selective α7 receptor activation on acquisition performance in this spatial memory–related paradigm, chronic nicotine administration does improve this performance more than is seen with acute treatment (i.e. during training exercises only) (Bernal et al., 1999).

At present, it is not clear to what extent this behavioral improvement is due to increased receptor levels and function in neurons that normally express the receptor (e.g. stratum radiatum neurons) versus novel ectopic receptor expression in neurons that do not (e.g. dentate granule cells). Since these animals were not agonist-treated, receptor activation depended on the endogenous agonists ACh and choline. The extent to which ectopic α7 receptors are activated by these ligands on normally α7 non-expressing neurons is difficult to predict and likely varies according to the proximity of cholinergic synapses that mediate non-α7 receptor transmission. Extracellular choline is found ubiquitously in the brain, though normally at low concentrations (1–10 μM) relative to those necessary to activate α7 receptors, except perhaps near cholinergic synapses where it is produced by ACh-hydrolysis (Uteshev et al., 2003). One method to determine which neuronal cell types in the hippocampus account for this improvement in acquisition performance following α7 nicotinic receptor gene delivery may be to use cell-type specific promoters as was done previously to distinguish the functions of different hippocampal neuronal populations (Buono et al., 2004).

Our results indicate that chronic expression of high levels of α7 receptors leads to the hyperphosphorylation of endogenous tau at sites associated with eventual neurofilament tangle formation, though only local to the gene delivery and only after an extended interval of sometime between 1 and 2 months. AT8 staining recognizes a form of hyperphosphorylated tau that is a component of neurofibrillary tangles in AD (Yang and Ksiezak-Reding, 1995). Little to no AT8 immunohistochemical staining appears to occur normally in adult mouse hippocampus (Distl et al., 2003). α7 Nicotinic receptor activation may account at least in part for similar tau hyperphosphorylation seen following chronic nicotine treatment. This is supported by the finding that selective α7 receptor agonists as well as nicotine stimulate p38/mitogen-activated protein kinase (MAPK) (Wang et al., 2006), which in turn phosphorylates tau (Oddo et al., 2005). Several other effects of α7 receptor activation may also trigger tau phosphorylation, including: 1) receptor-mediated activation of AKT, which has been reported to increase tau phosphorylation (Ksiezak-Reding et al., 2003); and 2) receptor-mediated increases in gluta-

mate release and subsequent NMDA receptor activation, which have also been found to increase tau phosphorylation (Guo et al., 2005). Unfortunately, currently available α7 receptor antibodies give false positive immunohistochemical staining in the mouse (Herber et al., 2004). It is therefore difficult to co-label neurons expressing high levels of this receptor and hyperphosphorylated tau, which would help distinguish between these intracellular versus trans-neuronal mechanisms of action.

It should be noted that relatively little is known about the effects of increased phosphorylation of endogenous, non-mutant tau in rodents. Studies demonstrating adverse effects of tau hyperphosphorylation have typically used transgenic mice expressing mutant human tau such as P301L, which is associated with a prefrontal cortical dementia. Even hyperphosphorylation of P301L tau in mice can improve memory-related behavior and elevate hippocampal LTP until tauopathies appear (Boekhoorn et al., 2006). The increase in wild type hippocampal tau phosphorylation seen in α7 receptor–transduced mice may therefore have beneficial effects, at least transiently. Conversely, it is conceivable that activation of α7 nicotinic receptors elicits a combination of beneficial effects (e.g. improved memory-related behaviors, reduced amyloid load, and neuroprotection), along with a detrimental action on tau-phosphorylation, that can be differentiated by appropriate dosing with agonists or genes. Another intriguing possibility is that α7 receptor–mediated increase in tau phosphorylation may be attenuated by amyloid peptides such as Abeta 1–42, which have been found to block these receptors very potently (Wang et al., 2000).

The present results indicate that receptor gene delivery alone is sufficient to increase α7 receptor function in brain via activation by endogenous agonists. However, it remains to be determined whether α7 receptor-modulated memory-related behaviors or other α7 receptor functions in brain such as auditory gating can be enhanced further by combining receptor agonist-treatments with gene delivery. This possibility is presently being evaluated due to its future therapeutic potential as well as providing a novel method for evaluating receptor function.

Acknowledgments—The authors thank Craig Meyers for technical assistance. Funding for this project was provided by NIH grant PO1 AG10485.

REFERENCES

Arendash GW, Sengstock GJ, Sanberg PR, Kem WR (1995) Improved learning and memory in aged rats with chronic administration of the nicotinic receptor agonist GTS-21. Brain Res 674:252–259.

Attaway CM, Compton DM, Turner MD (1999) The effects of nicotine on learning and memory: a neuropsychological assessment in young and senescent Fischer 344 rats. Physiol Behav 67:421–431.

Bernal MC, Vicens P, Carrasco MC, Redolat R (1999) Effects of nicotine on spatial learning in C57BL mice. Behav Pharmacol 10:333–336.

Boekhoorn K, Joels M, Lucassen PJ (2006) Increased proliferation reflects glial and vascular-associated changes, but not neurogenesis in the presenile Alzheimer hippocampus. Neurobiol Dis 24: 1–14.

Briggs CA, Anderson DJ, Brioni JD, Buccafusco JJ, Buckley MJ, et al. (1997) Functional characterization of the novel neuronal nicotinic acetylcholine receptor ligand GTS-21 in vitro and in vivo. Pharmacol Biochem Behav 57:231–241.

Buono P, Barbieri O, Alfieri A, Rosica A, Astigiano S, Cantatore D, Mancini A, Fattoruso O, Salvatore F (2004) Diverse human aldolase C gene promoter regions are required to direct specific LacZ expression in the hippocampus and Purkinje cells of transgenic mice. FEBS Lett 578:337–344.

Chen L, Yamada K, Nabeshima T, Sokabe M (2006) alpha7 Nicotinic acetylcholine receptor as a target to rescue deficit in hippocampal LTP induction in beta-amyloid infused rats. Neuropharmacology 50:254–268.

Distl R, Treiber-Held S, Albert F, Meske V, Harzer K, Ohm TG (2003) Cholesterol storage and tau pathology in Niemann-Pick type C disease in the brain. J Pathol 200:104–111.

Guo JZ, Liu Y, Sorenson EM, Chiappinelli VA (2005) Synaptically released and exogenous ACh activates different nicotinic receptors to enhance evoked glutamatergic transmission in the lateral geniculate nucleus. J Neurophysiol 94:2549–2560.

Herber DL, Severance EG, Cuevas J, Morgan D, Gordon MN (2004) Biochemical and histochemical evidence of nonspecific binding of alpha7nAChR antibodies to mouse brain tissue. J Histochem Cytochem 52:1367–1376.

Jonnala RR, Buccafusco JJ (2001) Relationship between the increased cell surface alpha7 nicotinic receptor expression and neuroprotection induced by several nicotinic receptor agonists. J Neurosci Res 66:565–572.

King ME (2005) Can tau filaments be both physiologically beneficial and toxic? Biochim Biophys Acta 1739:260–267.

Kitagawa H, Takenouchi T, Azuma R, Wesnes KA, Kramer WG, Clody DE, Burnett AL (2003) Safety, pharmacokinetics, and effects on cognitive function of multiple doses of GTS-21 in healthy, male volunteers. Neuropsychopharmacology 28:542–551.

Klein RL, Dayton RD, Leidenheimer NJ, Jansen K, Golde TE, Zweig RM (2006) Efficient neuronal gene transfer with AAV8 leads to neurotoxic levels of tau or green fluorescent proteins. Mol Ther 13:517–527.

Ksiezak-Reding H, Pyo HK, Feinstein B, Pasinetti GM (2003) Akt/PKB kinase phosphorylates separately Thr212 and Ser214 of tau protein in vitro. Biochim Biophys Acta 1639:159–168.

Li Y, King MA, Grimes J, Smith N, de Fiebre CM, Meyer EM (1999a) Alpha7 nicotinic receptor mediated protection against ethanol-induced cytotoxicity in PC12 cells. Brain Res 816:225–228.

Li Y, Papke RL, He Y, Millard W, Meyer EM (1999b) Characterization of the neuroprotective and toxic effects of alpha7 nicotinic receptor activation in PC12 cells. Brain Res 816:225–230.

Liu Q, Kawai H, Berg D (2001) beta-Amyloid peptide blocks the response of alpha 7-containing nicotinic receptors on hippocampal neurons. Proc Natl Acad Sci U S A 98:4734–4739.

Marubio LM, Paylor R (2004) Impaired passive avoidance learning in mice lacking central neuronal nicotinic acetylcholine receptors. Neuroscience 129:575–582.

Meyer E, King M, Meyers C (1998a) Neuroprotective effects of 2,4-dimethoxybenzylidene anabaseine (DMXB) and tetrahydroaminoacridine (THA) in neocortices of nucleus basalis lesioned rats. Brain Res 786:252–254.

Meyer EM, Tay ET, Zoltewicz JA, Meyers C, King MA, Papke RL, Fiebre CM (1998b) Neuroprotective and memory-related actions of novel alpha7 nicotinic agents with different mixed agonist/antagonist properties. J Pharmacol Exp Ther 284:1026–1032.

Meyer EM, Tay ET, Papke RL, Meyers C, Huang G, De Fiebre C (1997) 3-[2,4-Dimethoxybenzylidene] anabaseine (DMXB) selectively activates rat alpha7 receptors and improves memory-related behaviors in a mecamylamine-sensitive manner. Brain Res 768: 49–56.

Monteiro SC, Matte C, Bavaresco CS, Netto CA, Wyse AT (2005) Vitamins E and C pretreatment prevents ovariectomy-induced memory deficits in water maze. Neurobiol Learn Mem 84:192–199.

Mufson EJ, Ginsberg SD, Ikonomovic MD, DeKosky ST (2003) Human cholinergic basal forebrain: chemoanatomy and neurologic dysfunction. J Chem Neuroanat 26:233–242.

Oddo S, Caccamo A, Green KN, Liang K, Tran L, Chen Y, Leslie FM, LaFerla FM (2005) Chronic nicotine administration exacerbates tau pathology in a transgenic model of Alzheimer's disease. Proc Natl Acad Sci U S A 102:3046–3051.

Papke R (2006) Estimation of both the potency and efficacy of alpha7 nAChR agonists from single-concentration responses. Life Sci 78:2812–2819.

Papke RL, Meyer E, Nutter T, Uteshev VV (2000) alpha7 Receptor-selective agonists and modes of alpha7 receptor activation. Eur J Pharmacol 393:179–195.

Paylor R, Nguyen M, Crawley J, Patrick J, Beaudet A, Orr-Urtreger A (1998) Alpha7 nicotinic receptor subunits are not necessary for hippocampal-dependent learning or sensorimotor gating: a behavioral characterization of Acra7-deficient mice. Learn Mem 5: 302–316.

Peel AL, Klein RL (2000) Adeno-associated virus vectors: activity and applications in the CNS. J Neurosci Methods 98:95–104.

Radcliffe KA, Dani JA (1998) Nicotinic stimulation produces multiple forms of increased glutamatergic synaptic transmission. J Neurosci 18:7075–7083.

Ren K, Puig V, Papke RL, Itoh Y, Hughes JA, Meyer EM (2005) Multiple calcium channels and kinases mediate alpha7 nicotinic receptor neuroprotection in PC12 cells. J Neurochem 94:926–933.

Seguela P, Wadiche J, Dineley-Miller K, Dani JA, Patrick JW (1993) Molecular cloning, functional properties, and distribution of rat brain alpha 7: a nicotinic cation channel highly permeable to calcium. J Neurosci 13:596–604.

Shimohama S, Greenwald DL, Shafron DH, Akaika A, Maeda T, Kaneko S, Kimura J, Simpkins CE, Day AL, Meyer EM (1998) Nicotinic alpha 7 receptors protect against glutamate neurotoxicity and neuronal ischemic damage. Brain Res 779:359–363.

Teaktong T, Graham A, Court J, Perry R, Jaros E, Johnson M, Hall R, Perry E (2003) Alzheimer's disease is associated with a selective increase in alpha7 nicotinic acetylcholine receptor immunoreactivity in astrocytes. Glia 41:207–211.

Thinschmidt J, Frazier CJ, King MA, Meyer EM, Papke RL (2005) Septal innervation regulates the function of alpha7 nicotinic receptors in CA1 hippocampal interneurons. Exp Neurol 195:342–352.

Uteshev VV, Meyer EM, Papke RL (2003) Regulation of neuronal function by choline and 4OH-GTS-21 through alpha7 nicotinic receptors. J Neurophysiol 89:1797–1806.

Wang H, Lee D, Davis C, Shank R (2000) Amyloid peptide Abeta(1–42) binds selectively and with picomolar affinity to alpha7 nicotinic acetylcholine receptors. J Neurochem 75:1155–1161.

Wang Y, Wang Z, Zhou Y, Liu L, Zhao Y, Yao C, Wang L, Qiao Z (2006) Nicotine stimulates adhesion molecular expression via calcium influx and mitogen-activated protein kinases in human endothelial cells. Int J Biochem Cell Biol 38:170–182.

Williams ME, Burton B, Urrutia A, Shcherbatko A, Chavez-Noriega LE, Cohen CJ, Aiyar J (2005) Ric-3 promotes functional expression of the nicotinic acetylcholine receptor alpha7 subunit in mammalian cells. J Biol Chem 280:1257–1263.

Woodruff-Pak DS (2003) Mecamylamine reversal by nicotine and by a partial alpha7 nicotinic acetylcholine receptor agonist (GTS-21) in rabbits tested with delay eyeblink classical conditioning. Behav Brain Res 143:159–167.

Yang LS, Ksiezak-Reding H (1995) Calpain-induced proteolysis of normal human tau and tau associated with paired helical filaments. Eur J Biochem 233:9–17.

Zolotukhin S, Byrne BJ, Mason E, Zolotukhin I, Potter M, Chesnut K, Summerford C, Samulski RJ, Muzyczka N (1999) Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. Gene Ther 6:973–985.

Zuch CL, Nordstroem VK, Briedrick LA, Hoernig GR, Granholm AC, Bickford PC (2000) Time course of degenerative alterations in nigral dopaminergic neurons following a 6-hydroxydopamine lesion. J Comp Neurol 427:440–454.

(Accepted 9 November 2006)
(Available online 9 January 2007)