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

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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).

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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.

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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