Lasting Reduction of Cocaine Action in Neostriatum— A Hydrolase Gene Therapy Approach^S

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ABSTRACT

We previously found that a quadruple mutant cocaine hydrolase derived from human butyrylcholinesterase [termed cocaine esterase (CocE)] can suppress or reverse cocaine toxicity and abolish drug-primed reinstatement in rats. Here, we examined whether gene transfer of CocE reduces cocaine actions in brain reward centers. Early experiments used a standard, early region 1-deleted adenoviral vector, which, after intravenous delivery of 10^{10} plaque-forming units, caused plasma cocaine hydrolase activity to rise 25,000-fold between day 4 and day 7. During this period, under a protocol that typically induces FosB expression in the caudate nucleus, these rats and unprotected controls given only empty vector or saline were subjected to repeated twice-daily injections of cocaine (30 mg/kg i.p.). Immunohistochemistry of the neostriatum on day 7 showed many

FosB-reactive nuclei in unprotected rats but few if any in rats pretreated with active vector, which resembled rats never exposed to cocaine. Western blots confirmed this result. In contrast there was a more localized protection against cocaine-elicited FosB induction when hydrolase vector was injected directly into the ventral striatum, which generated high transgene expression in many neurons of the target area. Similar results were obtained with systemic and local injection of a more efficient helper-dependent adenoviral vector, which transduced high levels of hydrolase for at least 2 months, with lesser expression continued up to 1 year. Behavioral tests are now warranted to determine whether such effects can reduce drug-seeking behavior and lower the probability of relapse.

Efforts guided by computer-based protein engineering have yielded modified versions of human butyrylcholinesterase (hBChE) that hydrolyze cocaine rapidly enough to deserve consideration for therapeutic use (Sun et al., 2001, 2002a,b; Pancook et al., 2003; Pan et al., 2005; Zheng et al., 2008). We found accelerated cocaine metabolism and blunted cardiovascular effects of cocaine in rats given such enzymes directly (Gao and Brimijoin, 2004) or by adenoviral gene transfer (Gao and Brimijoin, 2005). Others observed that bacterial cocaine hydrolase protected rats against the lethal

effects of cocaine (Cooper et al., 2006; Ko et al., 2007). Last, we showed that a catalytically efficient hBChE-albumin fusion protein will abort cocaine-induced seizures and selectively suppress cocaine-induced reinstatement of drug-seeking behavior in rats (Brimijoin et al., 2008).

Taken together, the above-mentioned results support the idea of a gene therapy for cocaine addiction based on generating sustained levels of a hydrolase that prevents cocaine access to reward centers in the forebrain. As a step toward that goal, we have now evaluated two viral vectors encoding a quadruple mutant hBChE, with a k_{cat} value 200-fold greater than that of wild-type BChE. Each vector was given systemically or by intracerebral injection to test its effects on cocaine accumulation in brain tissue. We also investigated the ability of such treatments to suppress cocaine-induced up-regulation of Fos-related antigens in the neostriatum, a phenomenon believed to reflect genomic events in the initiation of drug addiction (McClung and Nestler, 2003). The results presented here suggest that hydrolase gene transfer can markedly restrict cocaine access to brain and seems capable of damping responses to

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ABBREVIATIONS: hBChE, human butyrylcholinesterase; BChE, butyrylcholinesterase; ANNA-1, type 1 antineuronal nuclear antibody; IHC, immunofluorescence histochemistry; iso-OMPA, tetraisopropylphosphoramidate; DFP, di-isopropylfluorophosphate; BW284c51, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; CocE, cocaine esterase; CocH, cocaine hydrolase; CMV, cytomegalovirus; AV, adenovirus; hdAV, helper-dependent adenovirus; pfu, plaque-forming unit.

this drug in central reward areas for protracted periods of time.

Materials and Methods

Drugs, Reagents, and Antibodies. Drugs were prepared in 0.9% NaCl (saline). Nonradioactive cocaine HCl was from Mallinckrodt (St. Louis, MO), whereas [^3H]cocaine (23 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). Sodium pentobarbital was from Ovation Pharmaceuticals (Deerfield, IL). Pansorbin (heat-killed, protein A-bearing *Staphylococcus aureus* cells) was purchased from Calbiochem-EMD Biosciences (San Diego, CA). Primary antibodies for immunohistochemistry (IHC) and Western blotting included mouse monoclonal antibodies B12 and B18 selective for human BChE (Brimijoin et al., 1983), rabbit polyclonal anti-FosB (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), human ANNA-1 (from Dr. V. A. Lennon, Mayo Clinic, Rochester, MN), and rabbit anti-rat IgG and anti-mouse IgG (Sigma-Aldrich, St. Louis, MO). Secondary antibodies were Cy3-labeled goat anti-human IgG, goat anti-rabbit IgG, fluorescein isothiocyanate-labeled rat anti-mouse IgG (Millipore Bioscience Research Reagents, Temecula, CA), and biotin-labeled goat anti-rabbit IgG as well as peroxidase-based ATP-binding cassette reagent (Vector Laboratories, Burlingame, CA). Other chemicals including tetraisopropylphosphoramidate (iso-OMPA), di-isopropylfluorophosphate (DFP), butyrylthiocholine iodide, and BW284c51 were purchased from Sigma-Aldrich.

Viral Vectors. Viral vectors of two types were designed to transduce a version of hBChE with four mutations that enhance cocaine hydrolysis 200-fold (Pancook et al., 2003; Gao et al., 2005). This cocaine esterase or CocE (formerly designated AME) was selected over the more powerful mutant, CocH (Pan et al., 2005), after pilot experiments showed that vectors carrying CocE transduced several-fold more gene product in cell culture, generating higher total cocaine hydrolase activity. In some experiments, we used CMV-AV-CocE, a previously described type-5, early region 1-deleted adenoviral vector with cytomegalovirus promoter (Gao et al., 2005). Empty vector controls and active vector with the transgene sequence were assembled in the University of Iowa Gene Transfer Core Facility (Iowa City, IA). Other experiments used a new-generation helper-dependent adenoviral vector produced by Dr. Robin Parks (Ottawa Health Research Institute, Ottawa, ON, Canada). The CocE cDNA was put under regulation by a human ApoE hepatic control region (provided by Dr. P. Ng, Baylor College of Medicine, Houston, TX), as described previously (Kim et al., 2001). A bovine growth hormone polyadenylation sequence was appended, and the construct was cloned into a derivative of the pΔ28lacZ hdAd-backbone plasmid. The final ApoE-hdAd-CocE vector was expected to be optimal for CocE transduction in liver but also capable of transduction in brain (Simonet et al., 1993). Vector was propagated using the AdNG163 helper virus, as described previously (Parks et al., 1996), and particle titers were determined by optical density at 260 nm. Helper virus contamination, determined by plaque assay on human embryonic kidney-293 cells, was approximately 0.2% for both loaded and empty vectors.

Animal Care. Animals were handled according to the Principles of Laboratory Animal Care (National Research Council, 2003) in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care, under Institutional Animal Care and Use Committee protocol A20605. Male Wistar rats (200–250 g) were obtained from Harlan (Indianapolis, IN). Cocaine was administered intraperitoneally or through the tail vein with a rinse of isotonic NaCl (total injection volume, ~1.5 ml). Viral vectors were injected similarly through the tail vein or directly into the brain (see below). Blood samples (~100 μl) were taken from the femoral vein, never exceeding 0.7 ml/100 g body weight per 2-week interval. Tissues were obtained after sodium pentobarbital euthanasia (250 mg/kg i.p.) followed by intra-aortic perfusion with ~250 ml of isotonic NaCl.

Brain Injections. Rats were anesthetized with sodium pentobarbital (45 mg/kg i.p.) plus inhaled isoflurane in amounts sufficient to prevent leg withdrawal after foot pinch. Eyes were protected with petroleum jelly, and the surgical field was swabbed with iodine. Animals were mounted in the stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), and a midline skin incision was made over the skull under strict aseptic conditions. Subcutaneous muscle and fascia were separated to expose the bone, which was dried with sterile swabs and pressurized air, whereas minor bleeding was stopped with a low-temperature microcautery pen. After the head was leveled, the bregma was marked with ink and a small hole was drilled over the target striatal area (atlas coordinates for central caudate: 0.5 mm, forward from bregma; ± 3 mm, lateral; 6.4 mm, below dura). A 31-gauge needle was lowered slowly and, after a 3-min rest, 4 μl of vector (4×10^8 pfu, in saline with or without Evans Blue as marker dye) was introduced over 10 min. The needle was left in the brain for a further 3 min and then retracted over 5 min. In specific cases, equivalent volumes of control vector, saline, or marker dye were delivered similarly to contralateral sites. After injections, the skin incision was closed with sterile Vicryl suture (Ethicon, Somerville, NJ) and Neosporin antibiotic was applied to the surface. Recovering rats were given analgesic (buprenorphine; 0.3 mg/ml i.m.) immediately after surgery, twice the next day, and once again on day 3.

Enzyme Assays. Blood collected into heparin-treated tubes was centrifuged (10 min at 8000g) to obtain plasma. Brains were homogenized in 10 volumes of 10 mM sodium phosphate, pH 7.4, with 0.1% Tween 20, and centrifuged as described above. Cocaine hydrolase activity in duplicate 50- μl aliquots of plasma or supernatant was assayed by incubating 30 min with [^3H]cocaine (50 nCi; 18 μM) and measuring liberated [^3H]benzoic acid after partition into toluene-based fluor for scintillation counting (Brimijoin et al., 2002). A related procedure was used to determine levels of [^3H]cocaine and benzoic acid as described previously (Gao and Brimijoin, 2004), in samples prepared in 10^{-5} M DFP to prevent continued enzymatic breakdown. Native rat BChE activity was assayed by a radiometric method (Johnson and Russell, 1975) using 1 mM [^3H]acetylcholine as substrate and 10^{-5} M BW284c51 as an acetylcholinesterase inhibitor.

Assays for Circulating hBChE Antibody. Pansorbin cells were saturated with rabbit anti-rat IgG for 1 h at 37°C in buffer (50 mM sodium phosphate, pH 7.4, plus 0.1% bovine serum albumin). Aliquots of loaded cells (100 μl) were centrifuged (10 min at 1500g), rinsed (two times in 1 ml of buffer), and then exposed 1 h at 37°C to rat plasma (100 μl) to adsorb IgG antibodies. The cells were then rinsed again and, after a final exposure to 5 ng of purified cocaine hydrolase in buffer and one last centrifugation, aliquots of the supernatant and the resuspended pellet fractions were assayed for cocaine hydrolase activity. This assay scheme was designed for efficient detection of circulating immunoglobulins with affinity for hBChE, either in free form and able to combine with target antigen, or already saturated with enzymatically active cocaine hydrolase.

Histology and Immunohistochemistry. After aortic perfusion with 250 ml of isotonic NaCl, brains and livers were removed and frozen. Cryostat sections (14 μm) were later cut, thawed onto slides, and postfixed 30 min in 4% paraformaldehyde, pH 7.4. Some sections for general histological study were stained with cresyl violet. Others were stained for BChE activity as described previously (Gao et al., 2005), using 3 mM butyrylthiocholine as substrate and BW284C51 (2×10^{-4} M) as a selective acetylcholinesterase inhibitor. IHC was performed on slide-mounted sections blocked 90 min at room temperature with 5% goat serum in 0.1% bovine serum albumin and 0.1% Triton X-100. Primary antibodies were applied (anti-BChE, 1:200; anti-FosB, 1:500; and ANNA-1, 1:16,000) and, after 16 h at 4°C, slides were rinsed in phosphate-buffered saline and reblocked for 90 min. For fluorescence detection with a confocal imaging system, the slides were incubated 1.5 h with a fluorescein-conjugated, goat anti-mouse IgG (1:100) or goat anti-rabbit IgG (1:400). Final

specimens were mounted in glycerol and 50 mM NaHCO₃ (1:1). For light microscopy and immunoperoxidase detection, slides were incubated 1.5 h with biotinylated goat anti-rabbit IgG (1:500) followed by avidin-biotin complex reagent, and they were mounted in DPX mounting medium (Electron Microscopy Sciences, Hatfield, PA).

Western Blots. Brain samples comprising microdissected regions of the mid-caudate nucleus were prepared for Western blotting by homogenization in 10 volumes of the following buffer: 20 mM HEPES, 0.4 M NaCl, 20% glycerol, 5 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P40, 5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. Samples were boiled and then subjected to SDS electrophoresis on 10% polyacrylamide gels, electrotransferred to nitrocellulose membranes, blocked with reconstituted dry milk in phosphate-buffered saline Tween, and incubated sequentially with primary antibody (rabbit anti-FosB, 1:2500) and secondary antibody (peroxidase-conjugated goat anti-rabbit IgG, 1:4000).

Quantitative Morphometry. For unbiased determination of the relative intensity of FosB expression in the brain, two frozen sections from each experimental animal were assigned primary code numbers. These sections were then recorded by an individual unaware of treatment status, who prepared digital photomicrographs from two fields of the central portion of the caudate nucleus under a 10× objective. Each micrograph was again coded for analysis by another observer. The analysis, using Image version 1.57 (National Institutes of Health, Bethesda, MD), measured the apparent mean density of every clearly identifiable nucleus after subtracting general background (sampled in extranuclear areas). Total summed nuclear stain density was recorded for each slide and, after codes were broken, the two values from each rat were averaged to yield one data point per animal for statistical analysis.

Statistical Analysis and Pharmacokinetics. Treatment effects were subjected to analysis of variance using StatView 4.5 (Abacus Concepts, Berkeley, CA). Post hoc testing used both the Scheffé and the Bonferroni/Dunn methods, with concordant results; $p < 0.05$ was considered statistically significant. Concentration-time profiles of plasma enzyme activity were analyzed with SigmaPlot 4.1 (Systat Software, Inc., San Jose, CA). Data on recovery of hydrolase activity after irreversible inhibition by iso-OMPA were fitted to the standard asymptotic exponential equation $E = 100 \times (1 - e^{-at})$, where E is activity as a percentage of the mean in samples taken immediately before iso-OMPA and t is time in hours. Finally, to evaluate the likelihood of simultaneous expression of hBChE and FosB in brain neurons, established principles of combinatorial mathematics (Hoel, 1962) were used to obtain a "coincidence probability equation" applicable to specific outcomes (see Supplemental Data for derivation information).

$$P = (1 - p)^n + \sum_{j=1}^k \binom{n}{j} \cdot p^j \cdot (1 - p)^{n-j} \quad (1)$$

Here, P is the probability of observing no more than k instances of double labeling in a sample population of n cells, and p is the product of the (presumed independent) probabilities of the two single labels (as estimated from the observed frequency of each marker).

Results

Overview. Having previously demonstrated strong if brief transduction of cocaine hydrolase by a CMV-AV vector (Gao and Brimijoin (2005, 2006), our goal was to assess the effect of vector treatment on cocaine actions in the brain. Initially, we administered this vector into the caudate nucleus (intracerebral), or systemically (intravenous), and used FosB induction after 4 days of twice-daily cocaine injections as a major endpoint. Positive outcomes led us to repeat key experiments with a better tolerated ApoE-hdAV vector for sustained transduction. Results below are grouped by delivery route, then by vector type, and last by observation category (see outline in Table 1).

Experiment 1: Intracerebral AV, hBChE Transduction, and Cocaine Metabolism. Intracerebral delivery was used in a pilot study of two rats with unilateral injection of CMV-AV-CocE vector (4×10^8 pfu) in the central caudate nucleus. Four days later, a time associated with optimal transduction, brains were harvested to determine the cellular locus of the transgene. Needle tracks were noticeable, as were infiltrations of small cells at the center of these mechanical lesions (data not shown). Farther away but still within 0.5 mm of the injection site, the brain parenchyma seemed undisturbed, and immunofluorescence staining showed high reactivity for hBChE in moderate-to-large cells (Fig. 1). Approximately 60% of these cells also stained with the neuron-specific antibody ANNA-1, identifying them as striatal neurons.

To determine whether brain-transduced enzyme could alter the striatal accumulation of systemically administered cocaine, nine additional rats were given stereotaxic injections of the same vector along with an inert marker dye and, on the contralateral side, dye alone. Four days were allowed for transduction, and [³H]cocaine was then injected intravenously. Five minutes later, both dye-marked injection zones were dissected for assay of cocaine and its breakdown product benzoic acid (Fig. 2). The assays showed 25% less radiolabeled cocaine on the vector side compared with the dye-only side ($p < 0.01$) and a 4000% rise in the benzoic acid/cocaine ratio ($p < 0.001$).

Experiment 2: Intracerebral AV and FosB Induction. To test our hypothesis that the increased local cocaine metabolism indicated by these results would hinder cocaine in activating striatal neurons and inducing immediate early genes, we used a version of the Δ -FosB induction paradigm (Hope et al., 1994). Four rats were desensitized for 4 days with twice-daily i.p. saline injections intended to accustom the animals to handling. Immediately afterward (day 1), CMV-AV vector encoding active hydrolase was injected into the left or right caudate (selected at random), whereas empty

TABLE 1
Experimental outline

Expt	Route	Vector	Observations	Rats
1	i.c.	CMV-AV	BChE expression, neuronal localization, local metabolism	11
2	i.c.	CMV-AV	Pilot study of FosB induction 6 days after vector (IHC)	6
3	i.c.	ApoE-hdAV	BChE expression in striatum, FosB induction 18 to 19 days after vector (IHC)	4 4
4	i.v.	CMV-AV	FosB induction, 7 days after vector (Western blot and IHC)	22
5	i.v.	ApoE-hdAV	FosB induction, 18 to 19 days after vector (IHC only)	22
6	i.v.	ApoE-hdAV	Year-long study of plasma hydrolase levels and turnover, immune response, endpoint liver histology	3

Fig. 1. Transduction of cocaine hydrolase by striatal neurons and non-neural cells after local delivery of viral vector. Representative confocal fluorescence images of central portion of rat caudate nucleus injected 4 days earlier with CMV-AV-CocE. Top, cocaine hydrolase transgene, anti-hBChE fluorescein isothiocyanate stain. Middle, neuron-specific Hu antigen, ANNA-1 Cy-3 stain. Bottom, overlay.

vector was injected contralaterally. Twice-daily cocaine injections (30 mg/kg i.p.) began on day 4, continued on days 5 and 6, and ended with a final injection on the afternoon of day 7. The next morning (~18 h after the last dose), brains were

Fig. 2. Altered local cocaine metabolism after intracerebral injection of hydrolase vector. Levels of [^3H]cocaine and [^3H]benzoic acid were determined in dye-marked regions of caudate nuclei microdissected from nine rats, 5 min after injection of [^3H]cocaine via the tail vein. Dissected tissue weights were 40 ± 6 and 44 ± 6 mg in the dye-vector and dye only samples, respectively. Represented are values from nucleus receiving CMV-AV-CocE, 4 days earlier, as a percentage of values from contralateral nucleus receiving dye only. Asterisks denote statistical significance between vector and dye-only measures: *, $p < 0.05$; **, $p < 0.01$.

harvested to detect long-lived FosB-reactive proteins by immunohistochemistry. For baseline comparisons, brains were also taken from two sham-operated rats that received only saline injections throughout.

Sections of caudate nucleus from sham-operated saline controls contained no hBChE-immunoreactive neurons and few neurons with appreciable FosB immunoreactivity (data not shown). Samples from rats given cocaine, however, showed both hBChE and FosB immunoreactivity, but with a differential spatial distribution (Fig. 3). On the side treated with empty vector, where hBChE was absent, many nuclei exhibited intense FosB reactivity. By contrast FosB-reactive neuronal nuclei were much fewer on the side treated with active vector, especially in regions with many contiguous cells expressing hydrolase transgene.

Experiment 3: Intracerebral hdAV Vector and FosB Induction. To confirm and extend the above-mentioned results under conditions with less tissue damage, we incorporated the hydrolase transgene into a new vector designed to arouse weaker immune responses and sustain longer transduction. Initial trials of this ApoE-hdAV-CocE showed robust hBChE expression with minimal signs of inflammation for at least 2 weeks after intracerebral injection (Fig. 4). Consequently, four additional rats were given unilateral injections of ApoE-hdAV-CocE and subjected to the FosB induction protocol. Taking advantage of the longer window for transgene expression, drug treatments were delayed for 14 days. Brain samples harvested 18 or 19 days after vector treatment again showed little FosB expression near the injection site, where hBChE-expressing cells were clustered. Farther away, FosB-reactive nuclei were intermixed with the sparser transgene-expressing neurons (Fig. 5). Fields within 0.5 mm of the center had 27 CocE-positive cells out of 265 neurons, none with FosB immunoreactivity. Outside that zone, 91 of 1320

Fig. 3. Local suppression of FosB induction in rats exposed to cocaine after pretreatment with hydrolase vector. CMV-AV-CocE was delivered into the caudate nucleus on one side, whereas “empty vector” CMV-AV was delivered into the contralateral nucleus. Immunohistochemistry for FosB and transgene (hBChE) was then performed after a 4-day course of twice-daily cocaine injections. Representative fields: active vector, BChE stain (A); active vector, FosB stain (B); empty vector, BChE stain (C); and empty vector, FosB stain (D). Scale bar, 50 μm (in B, applies to all images).

Fig. 4. Abundant expression of BChE activity in caudate nucleus and adjacent structures, 2 weeks after intracerebral injection of ApoE-hdAV-CocE. Frozen section of rat brain stained for BChE activity. Right, injected hemisphere. Left, uninjected hemisphere. Darkly stained areas have high BChE activity, normally very weak in the rat neostriatum.

observed neurons displayed transgene, 299 displayed FosB, and 13 displayed both. These numbers indicate a 0.23 probability of FosB expression after cocaine (299/1320), a 0.10 probability of neuronal CocE near the center (27/265), and a 0.069 probability at the periphery (91/1320). With those estimates, the coincidence equation (eq. 1 in *Materials and Methods*, under Statistical Analysis) yielded probabilities of 0.002 for zero double positives in the central sample (highly significant) and 0.049 for 13 or fewer double positives in the peripheral sample (marginally significant). In essence, local vector treatment suppressed FosB induction where hBChE expression was widespread but seemed less effective in isolated cells.

Fig. 5. Differential location of transgene and FosB reactivity in caudate nucleus of rats receiving APO-hdAV-CocE, 19 days earlier. A and D, hBChE stains of active- and empty-vector sides, respectively. B and E, FosB stains of active- and empty-vector sides. C and F, overlays. FosB-reactive nuclei occur in a higher proportion of hBChE-negative cells than hBChE-positive cells.

Experiment 4: FosB Induction after Systemic CMV-AV. Seeking a less invasive therapeutic approach, we examined systemic intravenous vector delivery, found previously to reduce drug uptake into brain (Gao et al., 2005) but not tested on the FosB induction paradigm. As in the intracerebral injection study, four rats habituated to handling by twice-daily saline treatment received 10^{10} pfu of active CMV-AV vector, four more rats received empty vector, and 14 rats received only saline. Plasma cocaine hydrolase activity was barely detectable after empty vector or saline but in the active-vector rats it rose nearly 25,000-fold (to 2.2 ± 0.66 U/ml, or 1.1 mg/ml hBChE protein). All vector-treated rats and half of the saline-controls underwent the standard FosB-induction paradigm with intraperitoneal cocaine injections starting on day 4. The remaining seven controls received intraperitoneal saline instead. Brains were harvested on day 7 and the medial portion of the caudate nucleus from one hemisphere was isolated under a dissection microscope for homogenization in SDS buffer, gel electrophoresis, and blotting with FosB antibody. In samples from unprotected rats (saline or empty vector pretreatment) the major Fos-reactive band at ~ 40 kDa increased consistently after cocaine exposure ($p < 0.01$), but this effect was completely blocked in rats pretreated with active vector (Fig. 6).

Immunohistochemistry in the contralateral hemispheres localized the changes in FosB immunoreactivity to neuronal nuclei of the neostriatum, as expected. In particular, in the central caudates of the unprotected rats, cocaine caused FosB-reactive nuclei to increase markedly over the levels in saline-treated controls (Fig. 7A). By contrast cocaine did not increase the abundance of FosB-reactive nuclei in rats protected with active hydrolase vector, which remained similar to the saline-only controls. Double-blind image analysis con-

Fig. 6. Western blot analysis of weakened cocaine induction of Δ -FosB after systemic treatment with CMV-AV-CocE. Samples were dissected caudate nuclei from rats exposed to cocaine or saline (see text). A, representative blot, arrow at putative Δ -FosB band. B, relative Δ -FosB band densities from four to seven rats per group. **, $p < 0.01$, significantly different from saline-only and from CocE + cocaine groups.

Fig. 7. Immunohistochemical analysis of weakened cocaine induction of Δ -FosB after systemic CMV-AV-CocE. A, representative FosB immunostains of regions contralateral to those used for Western blot. B and C, quantitated area and density products of FosB-immunoreactive nuclei. A, pretreatment with active or empty AV vector, 4 days before cocaine exposure. B, pretreatment with activity or empty hdAV vector, 16 days before cocaine exposure. $\ddagger$, $p < 0.01$, statistically significant versus saline only; $*$, $p < 0.01$, statistically significant versus saline-cocaine and empty vector-cocaine.

firmed these qualitative conclusions (Fig. 7B). After cocaine, the product of nuclear area and pixel density rose 80 and 90% in the saline and empty vector groups ($p < 0.01$), respectively, but only 19% in the hydrolase vector group ($p < 0.01$ versus unprotected groups; N.S. versus saline control). This outcome closely matched the changes seen on Western blots. Thus, 4 to 7 days after injection of active hydrolase vector, cocaine failed to induce increased expression of long-lived FosB-like antigens in the forebrain.

Experiment 5: FosB Induction after Systemic ApoE-hdAV. Because the hdAV-CocE vector was superior to the AV vector after intracerebral injection, we suspected it would provide more durable antagonism of cocaine actions after systemic delivery. For confirmation, 22 rats were subjected to the multiday FosB induction protocol beginning 16 days after treatment with active or empty ApoE-hdAV vector (10^{10} pfu). Plasma cocaine hydrolase activity during this time averaged 1.4 U/ml in the active vector group, rivaling the peak values recorded in the previous experiment. Western blots were not performed, but measures of FosB expressing nuclei in samples harvested on day 18 and 19 were virtually identical to those obtained with the earlier vector on day 8 (Fig. 7C). That

is, vector treatment caused selective, near-total suppression of gene induction in the tested time frame.

Experiment 6: Duration of hdAV Hydrolase Transduction, Immune Reactions, and Pathology. Given the potential value of a long-acting therapy for cocaine abuse, an extended time course for hydrolase transduction by the ApoE-hdAV vector was established with three rats, one followed for 9 months and two others for a full year after intravenous delivery, respectively, of 2×10^9 , 6×10^9 , and 10^{10} pfu. Cocaine hydrolase activity was measured at multiple time points in tail-vein plasma, with periodic screens for immune response. With only one animal per dose, no statements can be made about dose-response relationships. Actual expression varied 10-fold but all rats showed substantial enzyme activities (up to 0.4 U/ml). Most striking was the greatly prolonged period of high expression in all three subjects (Fig. 8), remaining near 80% of maximum for 60 days (versus 3 days with AV vector) and still 10% of maximum after a year (20% if adjusted for the progressive increase in body weight).

Additional tests with an immunoadsorption assay for circulating IgG antibodies against hBChE (see *Materials and Methods*) were made to determine whether the long-continued hydrolase expression reflected a failure to mount effective immune responses against the transgene. Assays at 3, 20, and 30 weeks after vector treatment showed no significant anti-hBChE activity in any of the three tested rats. At 35 and 50 weeks, serum samples bound at most 10% of the test antigen. Thus, specific antibodies, if present, were low in titer or affinity. Nonetheless a further study was performed at 39 weeks to detect "occult" reactions that might shorten

Fig. 8. Hydrolase transduction time course and turnover rates. A, cocaine hydrolase activity (milliunits per milliliter) in rat plasma drawn at indicated times after tail-vein injection of CMV-AV-CocE (dashed line; mean of six rats) or ApoE-hdAV-CocE (solid lines with data symbols; individual rats). Enzyme activity expressed in milliunits (nanomoles of product per hour) per milliliter of plasma. B, plasma cocaine hydrolase activities after irreversible inhibition are shown as percentage of pretreatment levels in same three rats given iso-OMPA, 35 weeks after tail-vein delivery of ApoE-hdAV-CocE. Means $\pm$ S.E.M.

the lifetime of the transgene product. For this purpose, we examined enzyme turnover in the same rats by measuring recovery from irreversible inhibition by iso-OMPA, a moderately selective organophosphate inhibitor of mammalian BChE. The injected dose (3 mg/kg i.p.) produced no overt signs of cholinergic toxicity but caused >95% inhibition of cocaine hydrolase activity in plasma samples collected 1 and 5 h after treatment. Free iso-OMPA, detected by the inhibition of an internal BChE standard, was apparent in plasma drawn at 1 h but not at 5 or 24 h. After establishing that spontaneous recovery of enzyme activity was negligible *in vitro* (<1% per day), we used the time course of recovery from 24 to 96 h *in vivo* as a measure of natural enzyme replacement (Fig. 9). The half-life calculated by fitting data to an asymptotic exponential recovery function (see *Materials and Methods*, under Statistical Analysis) was 58 ± 6 h. A similar

test on three rats never exposed to viral vector yielded an estimated half-life for native BChE activity of 34 ± 3 h. Hence, the mutated hBChE transduced in the host by viral vector was at least as stable as the endogenously produced rat enzyme.

Postmortem liver samples for general histopathology and BChE histochemistry were taken after the time course study. Cresyl violet staining showed no scarring or cellular infiltrates in one sample at 39 weeks. Two other samples harvested at 52 weeks had occasional cell infiltrates (two to three per section), but these were much smaller than seen previously in rats exposed to standard AV vector. The enzyme activity stain, in contrast, revealed active BChE throughout the liver as 1) a modest general background in nearly all hepatocytes, well above levels in control liver; and 2) intense staining in occasional hepatocytes. Thus, the morphologic picture was consistent with continued hepatic transgene expression, accompanied by modest inflammation but little structural damage.

Discussion

Several of our observations support the eventual use of hydrolase gene transfer to treat cocaine abuse. First is the generation of high levels of plasma enzyme with impressive ability to hydrolyze the drug. Second is the demonstration that enzyme transduction, even when confined to the periphery, can abrogate cocaine induction of a key Fos-related antigen in brain. Third is the indication that helper-dependent adenoviral gene transfer can sustain hydrolase expression in rats without serious adverse effects for much of an animal's lifetime.

Duration of Expression. Continued transgene expression for a full year after initial transduction with helper-dependent vector represents a dramatic improvement over the brief hBChE expression obtained with standard AV in our laboratory (Gao et al., 2005) and others (Chilukuri et al., 2008). An extended window of transduction matches previous experience with hdAV constructs, which have sustained 12 weeks of β -galactosidase expression in mouse muscle (Chen et al., 1997), 18 weeks of phosphodiesterase expression in mouse retina (Kumar-Singh and Farber, 1998), and 10 months of α -1 antitrypsin expression in baboons (Morral et al., 1999). Similar vectors have also supported life-long correction of genetic ApoE deficiency in mice (Kim et al., 2001) and uridine glucuronyl transferase deficiency in rats (Toietta et al., 2005). Regardless, the prolonged hBChE transduction in rats raises interesting questions. In rat liver hepatocytes with an average lifetime of 12 months (Zheng et al., 2005), the transgene should gradually dilute toward zero over a year unless the virally encoded DNA survives mitosis or replicates *in situ*. It has been speculated that some chromosomal integration might occur (Morral et al., 1999). Another potential explanation for long-persisting transduction is a physical association of vector DNA with the host chromosome, which draws it into one of the two daughter cell nuclei (Ehrhardt et al., 2003). This issue deserves further exploration.

As a practical matter, we suspect that effective antagonism of cocaine actions in rats may last only 2 to 3 months after vector treatment, while plasma cocaine hydrolase activity remains above 75% of its initial peak. Extrapolating across

Fig. 9. Liver histology with activity-based BChE histochemistry. A and B, representative low- and high-power views of sections from rat treated 1 yr earlier with ApoE-hdAV-CocE. C, low-power view from untreated control rat. Dark granules (largely absent from stained control sample) represent product of BChE-catalyzed hydrolysis of acetylthiocholine. Scale bars, 25 μ m.

species, from rats to humans, is always problematic. However, many metabolic parameters, including plasma protein lifetime, are found to scale roughly with the 0.75 power of body weight (Mordenti et al., 1991). Applying that rule to viral transduction, recognizing the uncertainty involved, then 2 months of CocE expression in a 0.3-kg rat could correspond to 120 months in a 75-kg person. A second dose of vector might extend transduction, given the weak reactions to the transgene in rats and its probable lack of immunogenicity in humans. Immune responses to coat proteins ordinarily block repeated vector entry into cells, but one can avoid this problem by immunosuppression or "serotype switching" (Mastrangeli et al., 1996; Smith et al., 1996).

Therapeutic Potential. Preventing cocaine from up-regulating a Fos-related antigen in striatum is one sign of therapeutic potential. We used the same anti-FosB antibody characterized by Nestler and collaborators (McClung and Nestler, 2003), and we likewise delayed sample harvesting to allow decay of short-lived Fos-related antigens. Hence, the effects on our major 40-kDa band probably also reflect changes in long-lived Δ -FosB. The data therefore indicate that interference with cocaine access to target sites in neostriatum suppresses at least one of the early gene effects linked to the establishment of drug-seeking behavior (Nestler, 2005). It was interesting that hydrolase transduction in the periphery affected FosB induction as strongly or more so than transduction in the striatum itself. This outcome may simply reflect a greater power to drive enzyme expression in liver, or it could mean that metabolic interception of drug is more effective in the circulation than at the ultimate target site. Perhaps transduced neurons or glia do not secrete quite enough enzyme to block cocaine access to synaptic amine transporters. That could explain why some isolated hBChE-positive cells at the periphery of vector injection sites became FosB-positive after repeated cocaine exposure, and why the divergence between FosB and hBChE expression was only marginally significant in that zone.

A future priority is to establish whether hydrolase gene transfer reduces drug-primed reinstatement and other behaviors associated with cocaine abuse. There are two reasons for optimism. First, direct injection of a cocaine hydrolase can abolish cocaine-primed reinstatement in rats that previously self-administered that drug (Brimijoin et al., 2008). Second, gene transfer leads to enzyme blood levels comparable to those obtained by direct injection. Nonetheless, because cocaine use typically involves dose routes with rapid delivery kinetics, it is worth exploring ways to generate still higher levels of enzyme activity. Transduced activity reflects both enzyme quantity and intrinsic catalytic activity. The hBChE in the present studies was better transduced but less catalytically efficient than the CocH mutant (Pan et al., 2005; Zheng et al., 2008). If the transduction efficiency of CocH could be raised toward similar levels of protein expression, circulating hydrolase activity might rise by up to 5-fold.

In the search for new treatments of cocaine abuse, vaccines are an alternative to enzyme-based therapy. Anti-cocaine antibodies delivered directly or by active immunization in rodent models have been able to slow cocaine penetration into brain and reduce the neurobehavioral effects of drug exposure (Ettinger et al., 1997; Carrera et al., 2000). Recently, cocaine vaccines have completed phase I human trials with encouraging results (Kosten and Owens, 2005; Orson et

al., 2008). Vaccines and hydrolase gene transfer need not be mutually exclusive therapies. We predict that cocaine hydrolases and antibodies will act in synergy to reduce peak blood levels of free cocaine on a rapid time scale, while dramatically accelerating drug clearance from the body. The result could be long-lasting elimination of the potential for cocaine-induced "highs," even when drug absorption and brain transfer is rapid. This prediction is now testable.

Host Toxicity and Safety. Safety is a critical issue with any new therapy. From that standpoint, our data are also encouraging but not conclusive. It was reassuring to observe little immune response in rats with prolonged expression of a human serum protein. This surprising outcome may reflect the fact that hBChE is very highly glycosylated (Saxena et al., 1998), coated with carbohydrates that, when generated in a rat, may mask peptide epitopes from its immune system. In humans, immune responses to mutated hBChE are likely to be weaker or absent, because the modified amino acids are confined to the interior of the catalytic gorge (Pancook et al., 2003), poorly available to immune surveillance. It should also be emphasized that extensive research in animals as well as human subjects has revealed no toxicity from hBChE protein (Clark et al., 2005; Saxena et al., 2006). Therefore, the principal concern in hBChE-based gene therapy must be the direct effects of the vector per se along with immune responses to the viral protein or novel DNA. Such risks seem to be relatively modest and should shrink as gene transfer technology improves.

The minimal histopathology in our hdAV-treated rats agrees with other reports showing much less inflammation with this vector than with typical E-1 deleted AV (Morrall et al., 1998). However, despite low levels of acquired immunity, innate immune responses operate to sense the presence of viral packages and perhaps of foreign DNA (Morrall et al., 1999; Muruve et al., 2008). Hence, it remains possible that gene transfer with the best currently available vectors might cause slight liver pathology. That possibility should be weighed against the fact that cocaine itself is hepatotoxic (Shuster et al., 1988) as are norcocaine and certain other products of cytochrome P450-catalyzed cocaine metabolism (Ndikum-Moffor et al., 1998). BChE actually protects against this toxicity (Duysen et al., 2002). It is therefore conceivable that gene therapy with hBChE would reduce the chances of liver damage in cocaine abusers.

In conclusion, cocaine abuse may be uniquely susceptible to enzyme-based treatments that reduce drug delivery to critical reward-mediating centers in the brain. Enzymes offer an added advantage of eliminating hepatotoxic cocaine metabolites. Direct administration of an optimal cocaine hydrolase is likely to be effective in the short run, but systemic viral gene transfer is an attractive means to obtain sustained high-level expression of circulating enzyme. Cocaine hydrolases might also be expressed in adult stem cells designed to incorporate into vascular endothelia. In addition, cocaine vaccines, which are advancing as potential therapies for addictive disorders, may well prove to act synergistically with enzymes to trap and destroy cocaine molecules en route to brain reward centers. Although none of these approaches would affect the sense of craving immediately, they could eliminate reward effects and lead directly to cue-based extinction of drug seeking. Combined treatment modes may make this difficult goal attainable.

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