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Abbreviations

AAV	adenoassociated virus
AChE	acetylcholinesterase enzyme
ACHE	acetylcholinesterase gene
Arc	activity-regulated cytoskeleton-associated protein
BChE	butyrylcholinesterase enzyme
BCHE	butyrylcholinesterase gene
BW284C51	1,5 bis(4-allyldimethylammoniumphenyl)-pentan-3-one; specific inhibitor of AChE
DFP	diisopropylfluorophosphate
DMEM	Dulbecco's Modified Eagle Medium
DTNB	dithiobisnitrobenzoic acid
Elisa	enzyme linked immunosorbent assay
ES	embryonic stem cells
FBS	fetal bovine serum
FLAG	8 amino acid peptide DYKDDDDK
G117H	human butyrylcholinesterase containing Histidine 117 in place of Glycine 117
HA	Hemagglutinin epitope
HEK293	Human embryonic kidney cells
IMDM	Iscoe's Modified Dulbecco's Medium
ip	intraperitoneal
Iso-OMPA	tetraisopropylpyrophosphoramidate; specific inhibitor of BChE
OP	organophosphorus toxicant
PBS	phosphate buffered saline
PCR	polymerase chain reaction
s.c.	subcutaneous
SDS	sodium dodecyl sulfate
TAT	Protein transduction domain from HIV, containing 11 amino acids
VX	O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate; nerve agent

Introduction

The goal of this work is to protect the soldier from the toxicity of nerve agents. The research plan involves finding new ways to deliver protective cholinesterase enzymes into tissues. Gene therapy and protein therapy protocols are being developed.

Test animals are being made to test the idea that an organophosphate hydrolase enzyme in the right location will provide protection against organophosphorus toxins. The G117H knockin mouse and the G117H transgenic mouse will give information on the level of protection that can be achieved by introducing the gene for an organophosphorus hydrolase.

List of tasks

Task 1. Various types of AChE enzyme will be injected into mice for the purpose of determining whether AChE enters the brain and other tissues.

1.1 Tetramers of fetal bovine AChE will be injected intraperitoneally into AChE-/- mice. *Completed in year 1.*

1.2 Monomers of human AChE will be produced in Chinese Hamster Ovary cells, purified, and injected into AChE -/- mice. *Completed in year 3*

1.3 A TAT fusion protein with human AChE will be produced in bacteria. The purified AChE fusion protein will be tested in cultured cells for ability to enter cells and fold into active enzyme.

Completed in year 2

1.4 The AChE fusion protein will be injected into AChE-/- mice to determine whether it improves their phenotype, and into wild-type mice to test the protective effect against OP.

Task 2. A gene targeted mouse substituting the G177H mutant of human BChE for the ACHE gene will be made.

2.1 A gene targeting construct will be made. *Completed in year 1.*

2.2 Homologous recombination in embryonic stem (ES) cells will substitute the human G117H BCHE gene for the mouse ACHE gene.

Completed in year 2. Repeated in year 3.

2.3 Microinjection of targeted ES cells into blastocysts and transfer into pseudopregnant mice will lead to the birth of chimeric mice.

Completed in year 2. Needs to be repeated.

2.4 Mating of chimeric mice with C57Bl/6 mice will determine which of the chimeras transmit the targeted allele in their germline. *Completed in year 3. Needs to be repeated.*

2.5 Chimeric mice that are known to transmit the targeted allele in their germline will be mated with 129sv mice to produce mice with a 129sv genetic background.

2.6 Homozygous G117H mice will be produced by mating. Their phenotype and their resistance to OP will be determined.

Task 3. A transgenic mouse that expresses human G117H BChE will be made.

3.1 A plasmid will be made that contains the mouse ACHE promoter, mouse ACHE exon 1, and mouse intron 1 attached to the cDNA of human G117H BCHE. *Completed in year 1.*

3.2 The linearized, digested, and purified DNA will be microinjected into mouse fertilized eggs of strain FVB/N. The injected embryos will be transferred into pseudopregnant mice. The live pups will be tested for the presence of the transgene. *Completed in year 2.*

3.3 Mice that carry the transgene will be tested for expression of human G117H BCHE. *Completed in year 2.*

3.4 Founder mice expressing the highest levels of G117H BCHE will be mated to produce colonies of transgenic mice. *Completed in year 2.*

3.5 Transgenic mice will be characterized with respect to tissue location of the expressed transgene and the levels of expression. *Completed in year 3.*

3.6 Transgenic mice will be tested for resistance to OP. *Completed in year 3.*

Task 4. Gene therapy with AChE.

4.1 Human AChE cDNA will be cloned into a shuttle vector. The linearized shuttle vector and pAdEasy-1 will be cotransfected into bacteria to allow homologous recombination. Colonies resistant to kanamycin will be screened by restriction endonuclease digestion. *Completed in year 1.*

4.2 The adenoviral vector containing human AChE, pAd-ACHE, will be linearized and transfected into 293 cells. Virus production will be visualized by fluorescence of green fluorescence protein and by measuring AChE activity. *Completed in year 3.*

4.3 Viral stocks will be amplified in 293 cells to obtain 10^{11} to 10^{12} plaque forming units. The virus will be purified in preparation for injection into mice. *Completed in year 3.*

4.4 Mice will be injected intravenously with various doses of adenoviral vector. The site of localization of the adenovirus will be determined. Expression levels of AChE will be determined. The duration of expression of AChE will be measured. *Completed in year 3.*

Relation to statement of work. In this third year of the project, we have made progress on tasks 1, 2, 3, and 4.

Task 1. Various types of AChE enzyme will be injected into mice for the purpose of determining whether AChE enters the brain and other tissues.

1.2 Monomers of human AChE will be produced in Chinese Hamster Ovary cells, purified, and injected into AChE $-/-$ mice.

Task 1.2

Introduction

The US Army is preparing to field a new therapeutic for protection against nerve agents, namely butyrylcholinesterase. This enzyme, purified from human plasma, is a tetramer with a molecular weight of 340,000. It is known that tetramers of human butyrylcholinesterase do not cross the blood-brain barrier. What is not known is whether monomers cross the blood-brain barrier. Chemical nerve agents enter the brain where they are unavailable for scavenging by the new enzyme therapeutic. If monomers were to enter the brain, there would be an advantage to administering monomers for protection against nerve agent toxicity.

Our goal was to test whether monomers enter the brain. A model system was chosen for this study because the model system had the best chance of giving a conclusive answer. The model system was the AChE knockout mouse treated with monomeric human AChE. Human AChE is similar in size and properties to human BChE. The advantage of using the AChE knockout mouse is that this mouse, having zero AChE activity, allows detection of tiny amounts of active AChE enzyme. There is no background AChE activity to contend with.

Methods

Recombinant human AChE. Human AChE was expressed in Chinese Hamster Ovary cells where it was secreted into serum-free culture medium. The AChE in the culture medium was purified on a procainamide-affinity column. The purified AChE was concentrated and dialyzed in an Amicon stirred cell. The activity of the dialyzed AChE was 130 units/ml. The purified AChE consisted of 50% monomers, 45% dimers, and 5% tetramers.

Treatment of mice. AChE $-/-$ mice received 0.3 ml AChE, 130 u/ml, intraperitoneally. Blood samples were taken at 0, 10, 30, 60, 90, 120, 150, and 165 min. Serum was separated from red blood cells by centrifugation and assayed for AChE activity. Mice were euthanized at 165 min, perfused intracardially with 100 ml PBS to wash blood out of tissues, and the brain collected.

AChE enzyme activity. AChE activity was assayed with 1 mM acetylthiocholine in 0.1 M potassium phosphate buffer pH 7.0 (Ellman et al., 1961), after inhibiting BChE with 0.1 mM iso-OMPA.

The brain was homogenized with 10 volumes of cold 50 mM phosphate pH 7.0, 0.5% Tween. The homogenate was clarified by centrifugation and the supernatant assayed for AChE activity.

Results and Discussion

Half-life in the circulation. The intraperitoneally injected AChE rapidly entered the mouse blood. Maximum activity was reached after about 1 hour and was maintained for another hour (Figure 1.1). This pattern was similar to the one obtained for tetrameric recombinant BChE (Duysen et al., 2002a) where maximum activity in blood was found about 1 hour after ip injection. The AChE in Figure 1.1 disappeared from the mouse circulation with a half-life of about 1 hour. In contrast, BChE tetramers disappeared with a half-life of about 16 hours (Duysen et al., 2002a).

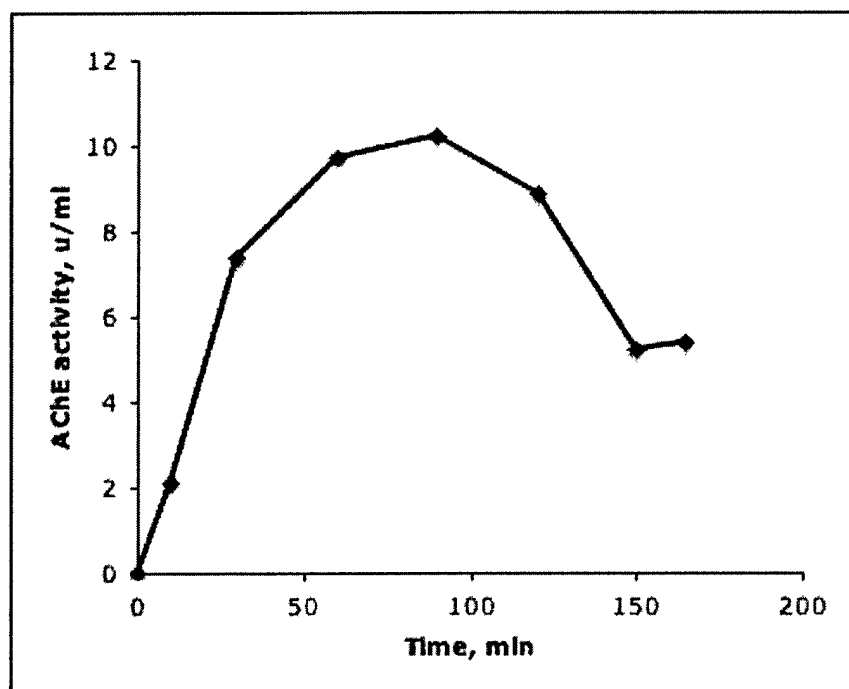


Figure 1.2.1. Appearance of AChE activity in blood after ip injection of human AChE monomers and dimers into AChE^{-/-} mouse.

Monomeric AChE did not cross the blood-brain barrier. The mouse brain contained no detectable AChE activity after the AChE knockout mouse had been treated with human AChE monomers and dimers.

No effects on behavior. Injection of human AChE into AChE^{-/-} mice caused no detectable improvement in their body tremor or muscle weakness. This is consistent with previous experiments where AChE^{-/-} mice had received repeated injections of AChE tetramers over a period of days. These repeated injections also had no effect on the phenotype of AChE^{-/-} mice. It is concluded that muscle weakness cannot be treated with soluble AChE. The AChE must be anchored in the neuromuscular junction to have an effect on muscle strength.

Task 2. A gene targeted mouse substituting the G177H mutant of human BChE for the ACHE gene will be made.

2.1 A gene targeting construct will be made.

2.2 Homologous recombination in embryonic stem (ES) cells will substitute the human G177H BCHE gene for the mouse ACHE gene.

2.3 Microinjection of targeted ES cells into blastocysts and transfer into pseudopregnant mice will lead to the birth of chimeric mice.

2.4 Mating of chimeric mice with C57Bl/6 mice will determine which of the chimeras transmit the targeted allele in their germline.

Tasks 2.3 and 2.4

Abstract

The G117H mutant of human BChE has the unique capability of being able to hydrolyze the neurotransmitter acetylcholine and to hydrolyze organophosphorus (OP) toxicants. We want to know whether substitution of G117H BChE for AChE will make the mouse resistant to OP toxicity. Last year we completed tasks 2.1 and 2.2 and identified one positive ES clone. This year the ES clone was microinjected into mouse blastocysts, leading to the birth of 11 chimeras. The chimeras were bred to C57Bl/6 mice. A total of 297 pups were born over a period of 9 months. None had an agouti coat color. This meant that none of the chimeras was a germline transmitter of the gene-targeted allele. Therefore, we had to start over by isolating additional ES clones. In the next round of screening, we identified 7 new positive ES clones. Three of these will be injected into blastocysts.

Introduction

Better protection from nerve agents would be achieved if an OP hydrolase were present in the nerve muscle junction and in neurons of the brain. Millard et al. (Millard et al., 1995; Millard et al., 1998) and Lockridge et al. (Lockridge et al., 1997) have shown that the G117H mutant of human BChE hydrolyzed the OP agents sarin, VX, echothiophate, and paraoxon, as well as butyrylthiocholine and acetylthiocholine. Potentially, G117H BChE would perform the job that AChE normally performs, that of terminating nerve impulse transmission, with the advantage that G117H BChE is resistant to inactivation by OP. To test the idea that a mouse could be OP resistant, we are making a mouse that has the human G117H BCHE gene in the mouse ACHE gene locus.

Methods

Design of gene targeting vector. We have designed and constructed the gene targeting vector to make the G117H knockin mouse. We designed the gene targeting vector so that expression of human G117H BChE would occur during development at the same time and in the same tissues as AChE in wild-type mice. The design of the

gene targeting vector aims to retain as much of the control regions of the ACHE gene as possible so as not to alter the expression pattern. Therefore, mouse ACHE intron 1, the mouse ACHE signal peptide, mouse ACHE intron 3, exon 4, intron 4, exon 5, intron 5, exon 6, and the 3' untranslated region are retained. The human BCHE in this vector is represented by amino acids Glu 1 to Gly 478 encoded by human BCHE exon 2. This plan is missing one potential control region, namely intron 2 of the ACHE gene. It was not possible to include intron 2 because it would have given unwanted recombination. Luo et al., (Luo et al., 1998) have shown that retention of either intron 2 or 3 of mouse ACHE is sufficient to control exon 4 to exon 6 splicing. Since our construct contains intron 3, it is expected that normal control of splicing will be possible.

Structure of the gene targeting vector. See Figures 2.1. The gene targeting vector contains two selectable markers: the TK gene (2 kb) for negative selection, and the PGK-NEO gene (1.6 kb) for positive selection. The 34 nucleotide loxP sequence was placed on both sides of the NEO gene to allow deletion of the NEO gene by Cre recombinase.

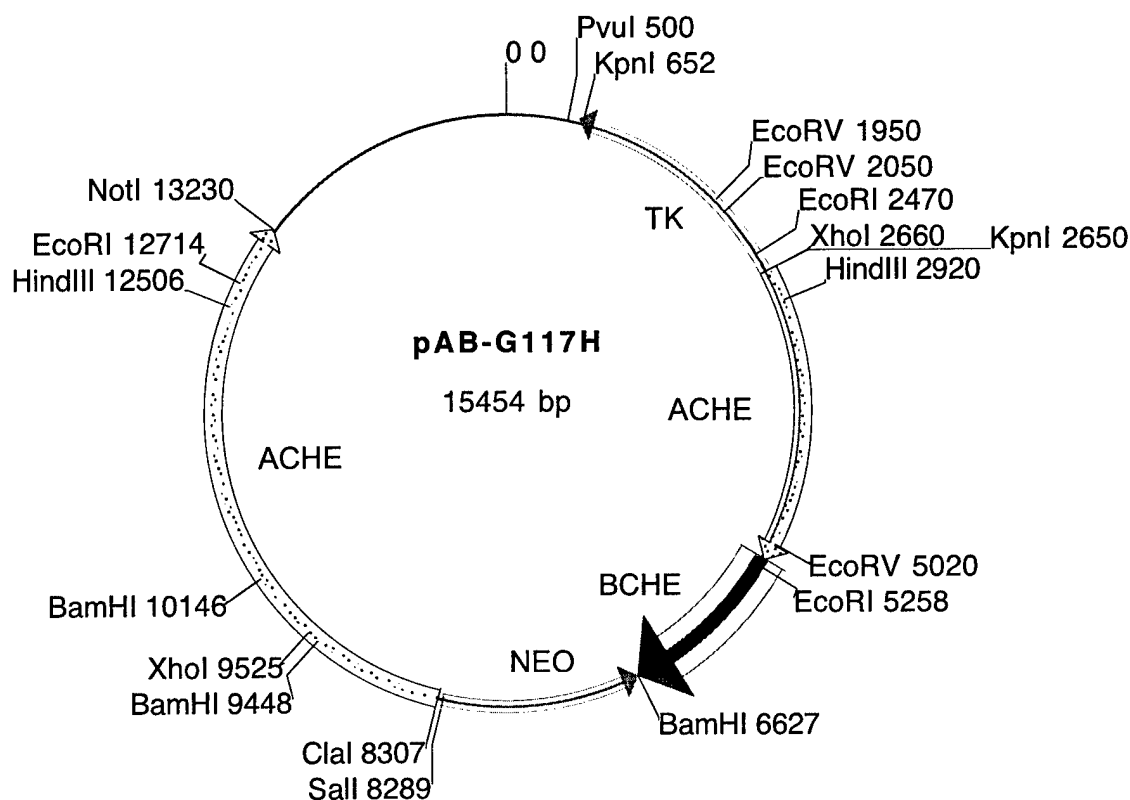


Figure 2.1. Map of the gene-targeting vector. The selectable markers are the TK gene (2 kb) and loxP NEO (1.66 kb). The 2502 bp of mouse ACHE are followed by 1433 bp of human BCHE, 1662 bp of loxP NEO, and 4923 bp of mouse ACHE. Human BCHE contains the G117H mutation.

Screening ES cells for homologous recombination. The gene-targeting vector, linearized with Not I, was transfected into R1 mouse embryonic stem cells by the University of Michigan Transgenic Animal Model Core Facility directed by Dr. Thom Saunders. 480 colonies resistant to G418 and gancyclovir were picked into triplicate 96-well plates. DNA was purified from two sets of plates and sent to us for screening. The DNA was digested with EcoRI and electrophoresed on 0.8 % agarose gels. The DNA was transferred to Zeta-probe membrane (Bio-Rad catalog 162-0159) and hybridized with a P32-labeled probe. The 255 bp probe was located outside the targeted region, on the 5' side of the ACHE gene. See Figure 2.2. The expected size of the EcoRI fragment was 10.3 kb for the gene that had undergone homologous recombination, and 16.5 kb for the wild-type gene.

One positive clone was found (see Figure 2.3). The positive clone had the expected two bands of 16.5 and 10.3 kb after hybridization of EcoRI digested genomic DNA with the 5' probe. The positive clone was expanded to yield more DNA for additional testing. The right panel in Figure 2.3 used a 3' probe and different restriction enzymes. The results confirmed that this positive clone was indeed the result of homologous recombination and that the clone did not contain random insertions.

Chromosome analysis revealed that this positive clone had the correct chromosome count (85.7% of spreads had 40 chromosomes) and was therefore suitable for blastocyst injection.

Blastocyst injection and birth of chimeras. ES clone 1C3 was microinjected into mouse blastocysts by the University of Michigan Transgenic Animal Model Core Facility. The blastocysts were implanted into the uterus of pseudopregnant mice and live pups were born. Mice that had some agouti color in their coats were chimeras. 5 male and 6 female chimeras arrived on October 28, 2003 from the University of Michigan.

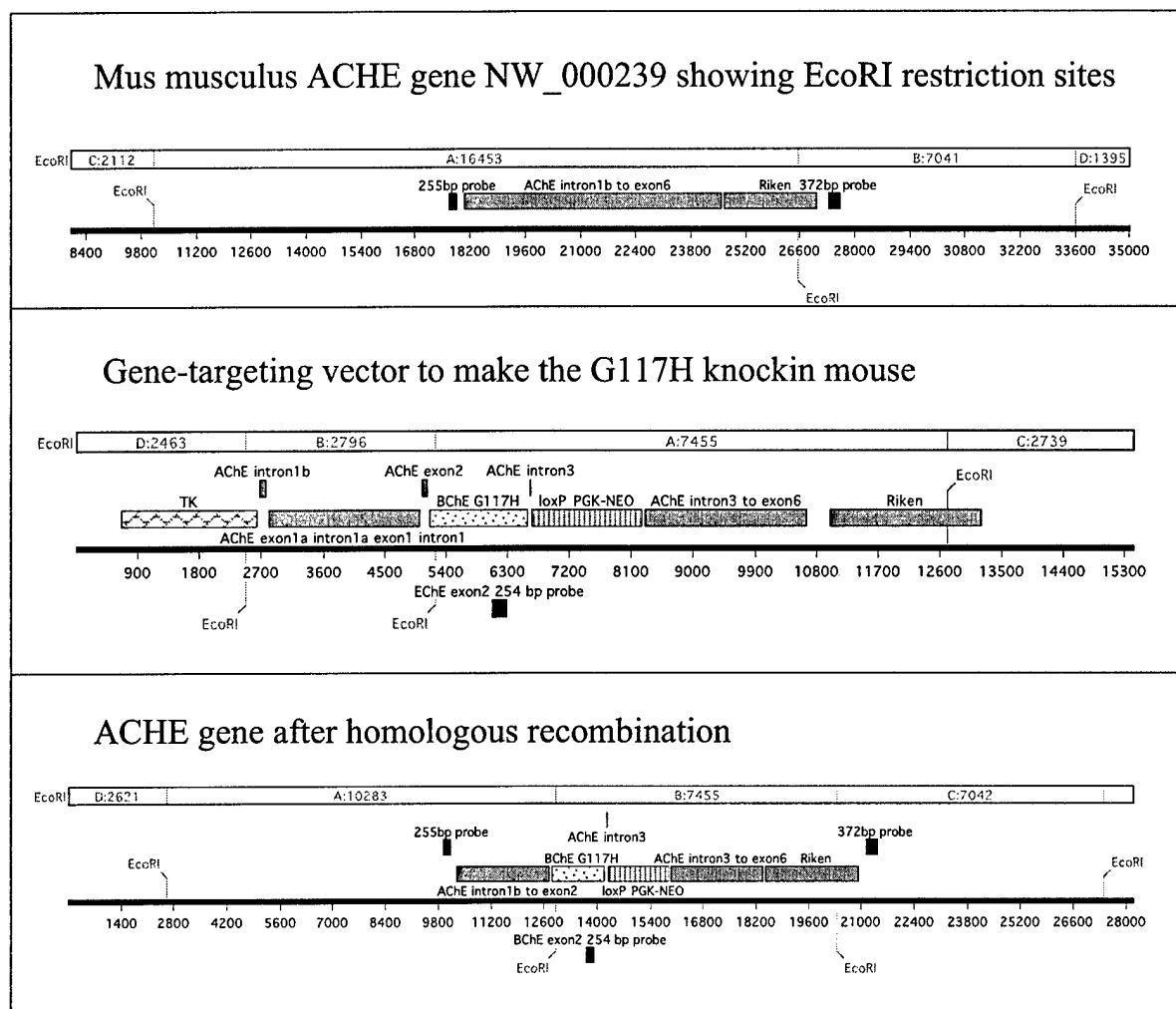


Figure 2.2. Restriction map of the mouse ACHE gene and location of probes. The top panel represents the ACHE gene in the wild-type mouse; the EcoRI restriction fragment probed with the 5' probe contains 16453 bp. The center panel represents the gene-targeting vector; the 5' probe does not recognize any sequence in the gene-targeting vector. The bottom panel represents the ACHE gene after it has undergone homologous recombination to substitute the human BCHE G117H gene for ACHE; the EcoRI fragment probed with the 5' probe contains 10283 bp. The probes for screening and confirming homologous recombination in ES cells were outside of the targeted region: the 5' probe contained 255 bp and the 3' probe contained 372 bp. A 254 bp probe located inside the human BCHE gene is intended for genotyping mice. Macvector software was used to make this figure.

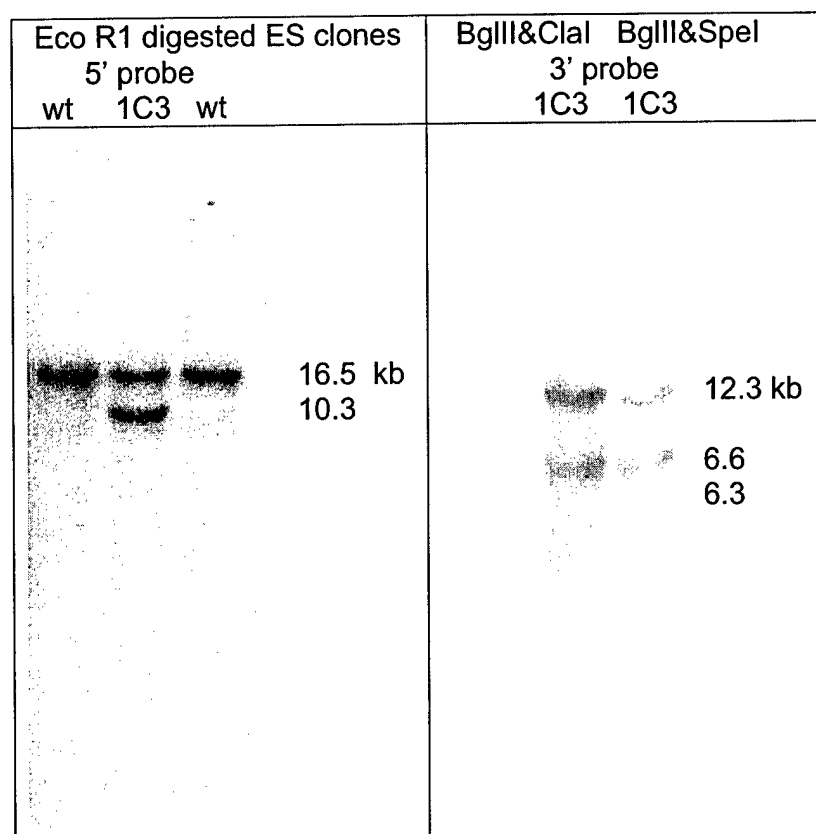


Figure 2.3. Southern blot showing positive ES clone 1C3. The bands in the left panel were obtained by digesting genomic DNA with EcoRI and hybridizing with a 5' probe. One clone, called 1C3, shows two bands; the 10.3 kb band contains the desired recombination. Two clones show a single band of 16.5 kb; these contain only wild-type DNA. To confirm that homologous recombination had taken place, the positive clone 1C3 was digested with additional restriction enzymes and hybridized with a 3' probe. The right panel shows 12.3 and 6.3 kb bands for a Bgl II and Cla I digest, and 12.3 and 6.6 kb bands for a Bgl II and Spe I digest of clone 1C3. These results confirm that clone 1C3 has one allele that has undergone homologous recombination.

Breeding to look for germ line transmission. The chimeras were bred to C57Bl/6 mice. The principle of the method is that pups with an agouti coat color have a 50% chance of carrying the gene-targeted allele, whereas pups with a black coat color have 0% chance of carrying the gene-targeted allele. The agouti coat color shows the presence of genes from ES cells. The birth record for the 11 chimeras is in Table 2.1.

Table 2.1 Birth record for chimeras carrying genes from ES clone 1C3.

ID	Sex	% agouti	# of viable litters	total # living pups	# agouti pups	# non-viable litters
YWK1 M1c	male	15	2	14	0	4
YWK1 M2c	male	5	6	38	0	3
YWK1 M3c	male	10	9	68	0	0
YWK1 M4c	male	70	10	82	0	1
YWK1 M5c	male	40	0	0	0	0
YWK1 F1c	female	40	0	0	0	5
YWK1 F2c	female	50	3	12	0	2
YWK1 F3c	female	40	2	6	0	1
YWK1 F4c	female	70	0	0	0	2
YWK1 F5c	female	20	7	68	0	0
YWK1 F6c	female	80	1	9	0	4
		total	40	297	0	22

Table 2.1 shows that 297 pups were born but none had an agouti coat color. One male chimera, YWK1 M5C, was sterile. Two female chimeras, YWK1 F1c and YWK1 F4c, produced 7 litters but all pups died shortly after birth due to maternal neglect. Four male chimeras fathered 202 pups. Four female chimeras gave birth to 95 pups. It took 9 months of breeding to produce the mice in Table 2.1. The breeding was stopped on July 22, 2004, when all mice in this colony were euthanized.

Screening for new ES clones. After a few months of breeding, when no agouti pups had been born, we undertook the task of isolating new ES clones. R1 cells were again transfected with the same gene-targeting vector. 480 colonies on five 96-well plates were screened with P32-labelled probes by Southern blotting as illustrated in Figure 2.3. This time 7 positive ES clones were identified. The colonies were expanded, the DNA was digested with various restriction enzymes, and probed on Southern blots with 3' and 5' probes. All 7 colonies were confirmed to be positive for homologous recombination. The seven expanded colonies were analyzed for chromosome counts. Clones BL8H9, BL6E10, BL10G11, BL8G11, and BL10B11 were found to be euploid and suitable for blastocyst injection. Clones BL6F2 and BL10C12 were aneuploid.

Conclusion. This task is still in progress. Three of the euploid ES clones will be microinjected into blastocysts to produce chimeras.

Task 3. *A transgenic mouse that expresses human G117H BChE will be made.*

3.1 A plasmid will be made that contains the mouse AChE promoter, mouse AChE exon 1, and mouse intron 1 attached to the cDNA of human G117H BCHE.

3.2 The linearized, digested, and purified DNA will be microinjected into mouse fertilized eggs of strain FVB/N. The injected embryos will be transferred into pseudopregnant mice. The live pups will be tested for the presence of the transgene.

3.3 Mice that carry the transgene will be tested for expression of human G117H BCHE.

3.4 Founder mice expressing the highest levels of G117H BCHE will be mated to produce colonies of transgenic mice.

3.5 Transgenic mice will be characterized with respect to tissue location of the expressed transgene and the levels of expression.

3.6 Transgenic mice will be tested for resistance to OP.

Tasks 3.1, 3.2, 3.3, 3.4, 3.5, 3.6

Resistance to organophosphorus agent toxicity in transgenic mice expressing the G117H mutant of human butyrylcholinesterase

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Toxicol Appl Pharmacology (2004) 196: 356-366

Abstract

Organophosphorus toxicants (OP) include chemical nerve agents and pesticides. The goal of this work was to find out whether an animal could be made resistant to OP toxicity by genetic engineering. The human butyrylcholinesterase (BChE) mutant G117H was chosen for study because it has the unusual ability to hydrolyze OP as well as acetylcholine, and it is resistant to inhibition by OP. Human G117H BChE, under the control of the ROSA26 promoter, was expressed in all tissues of transgenic mice. A stable transgenic mouse line expressed 0.5 µg/ml of human G117H BChE in plasma as well as 2 µg/ml of wild-type mouse BChE. Intestine, kidneys, stomach, lungs, heart, spleen, liver, brain and muscle expressed 0.6 to 0.15 µg/g of G117H BChE. Transgenic mice were normal in behavior and fertility. The LD₅₀ dose of echothiophate for wild-type mice was 0.1 mg/kg sc. This dose caused severe cholinergic signs of toxicity and lethality in wild-type mice, but caused no deaths and only mild toxicity in transgenic animals. The mechanism of protection was investigated by measuring acetylcholinesterase (AChE) and BChE activity. It was found that AChE and endogenous BChE were inhibited to the same extent in echothiophate treated wild-type

and transgenic mice. This led to the hypothesis that protection against echothiophate toxicity was not explained by hydrolysis of echothiophate. In conclusion, the transgenic G117H BChE mouse demonstrates the factors required to achieve protection from OP toxicity in a vertebrate animal.

Introduction

Organophosphorus nerve agents and pesticides (OP) are highly toxic chemicals. Pretreatment with native human BChE protects monkeys, guinea pigs, mice, and rats from toxicity (Wolfe et al., 1987; Raveh et al., 1989; Ashani et al., 1991; Broomfield et al., 1991; Maxwell et al., 1992; Wolfe et al., 1992; Brandeis et al., 1993; Maxwell et al., 1993; Raveh et al., 1993; Castro et al., 1994; Genovese and Doctor, 1995; Raveh et al., 1997; Allon et al., 1998; Matzke et al., 1999), but has the limitation that native BChE is a stoichiometric scavenger so that a molar equivalent of BChE is required for each mole of OP. An OP hydrolase, like G117H BChE (Millard et al., 1995; Lockridge et al., 1997; Millard et al., 1998), is expected to be more efficient since it would destroy many molecules of OP per molecule of BChE. A transgenic mouse expressing human G117H BChE was made to test the protective effect of this OP hydrolase in mice.

The ROSA26 promoter was selected because this promoter directs expression of the transgene in most tissue types throughout embryonic development and in adult tissues (Zambrowicz et al., 1997; Kisseberth et al., 1999). It was assumed that widespread distribution of G117H BChE activity had the best chance of providing a protective shield against OP toxicity.

OP resistance has not been reported in other transgenic mice that might have been expected to have this property, for example in mice expressing human paraoxonase (Oda et al., 2002; Tward et al., 2002) or human AChE (Andres et al., 1997). Active human BChE enzyme has not been previously expressed in a transgenic mouse (Beerli et al., 1994). This is the first OP resistant vertebrate animal created by genetic engineering.

Methods

Construction of the transgene

A plasmid pBROAD (3.2 kb) containing the ROSA26 promoter (Zambrowicz et al., 1997; Kisseberth et al., 1999; Soriano, 1999) was purchased from InvivoGen (San Diego, CA). A chimeric intron from pCI-neo (Promega) composed of the 5'-donor site from the first intron of the human beta-globin gene and the branch and 3'-acceptor site from the intron of an immunoglobulin gene heavy chain variable region (161 bp) was inserted into pBROAD between NcoI and Bgl II. The intron was added to pBROAD because the presence of an intron enhances expression of the transgene (Choi et al., 1991; Palmiter et al., 1991). The human BCHE cDNA encoded the 28 amino acid signal peptide and 574 amino acids of the full-length BChE protein. A single amino acid mutation at codon 117 substituted His for Gly to make the G117H mutation. Human BCHE was inserted between the Bgl II and Nhe I sites of pBROAD. A FLAG tag encoding amino acids DYKDDDDK was placed at the 3' end of BCHE. The 3'untranslated region and polyadenylation sequence of the human elongation factor 1-

alpha gene were in the pBROAD plasmid. The transgene was excised from the plasmid by digestion with Pac I to make a transgene of 3063 bp. See Figure 3.1.

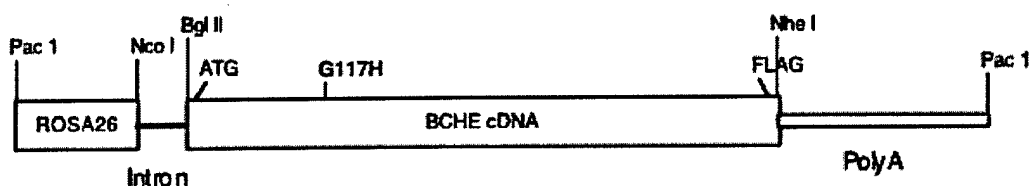


Figure 3.1. Schematic representation of the BCHE transgene. The ROSA26 promoter (382 bp) is followed by a chimeric intron (161 bp), the human BCHE cDNA (1859 bp) including a FLAG tag, and a poly A addition site (661 bp). The Pac I restriction site was used to produce a 3063 bp transgene for injection into fertilized mouse eggs. GenBank accession numbers are U83173 for ROSA26, and M16541 for human BCHE cDNA.

Injection into fertilized mouse eggs and generation of transgenic mice

The DNA was microinjected into fertilized eggs C57BL/6 x (C57BL/6xSJL)F1 by the University of Michigan Transgenic Animal Model Core facility (Ann Arbor, MI) [Margaret Van Keuren mvkeuren@umich.edu and Dr. Thomas Saunders tsaunders@umich.edu; <http://www.med.umich.edu/tamc>]. The eggs were transferred into pseudopregnant recipient females. Out of 51 pups born, 13 carried the transgene. All procedures involving mice were approved by the appropriate Institutional Animal Care and Use Committees of the University of Nebraska and the University of Michigan. Animal care was provided in accordance with the principles and procedures outlined in the National Research Council *Guide for the Care and Use of Laboratory Animals*.

Mouse strain

Founder mice were bred to the F1 cross of C57BL/6 X SJL called strain B6SJL/F1Tac (purchased from Taconic). Brother-sister matings of pups with the highest G117H BChE activity produced the F2 and subsequent generations.

Identification of mice that carry the transgene

For routine genotyping, DNA was extracted from hair roots by incubating hair in 50 mM NaOH at 95°C for 15 min (Schmitteckert et al., 1999). Animals were about two weeks old when hair was pulled for genotyping. Genomic DNA was tested for the presence of the BCHE transgene by PCR. The sense primer matched the chimeric intron, the antisense primer matched human BCHE. The PCR product had 392 bp. The 34mer sense primer was 5'GGTTTAAGGAGACCAATAGAACTGGGCTTGTCG, the 28mer antisense primer was 5'CCAAATATCAGACCACTTGGTCAGAGAC.

Southern blot

Genomic DNA was purified from tail snips with QIAamp DNA Mini Kit from Qiagen (Chatsworth, CA) and digested with EcoRI. Blots were probed with a single stranded P32-labeled probe containing 254 bases of the transgene. The 254 base probe encoded human BChE from Asp304 to Ala388. Blots were hybridized at 60°C in ExpressHyb solution from Clontech.

BChE activity assay

Blood was collected from the leg vein (Saphenous vein) of transgenic mice into heparinized tubes. Plasma was tested for BChE activity by the Ellman method (Ellman et al., 1961) using 1 mM butyrylthiocholine, 0.5 mM dithiobisnitrobenzoic acid, 0.1 M potassium phosphate pH 7.0, at 25°C. Increase in absorbance at 412 nm was recorded in a Gilford spectrophotometer. Slopes were converted to μ moles substrate hydrolyzed per min per ml by using the extinction coefficient $13,600 \text{ M}^{-1} \text{ cm}^{-1}$.

Tissue extracts were preincubated with 0.5 mM dithiobisnitrobenzoic acid in 0.1 M potassium phosphate buffer to react free sulfhydryl groups before addition of butyrylthiocholine.

Assay for G117H BChE activity

To assay G117H BChE activity, advantage was taken of a special property of the G117H BChE enzyme, that is, its resistance to inhibition by OP. By contrast, wild-type BChE was completely inhibited by OP. G117H activity in plasma was measured in the Ellman assay (Ellman et al., 1961) by adding echothiophate to a final concentration of 0.1 mM. G117H activity in tissues was measured after inhibiting wild-type BChE with 0.001 mM diisopropylfluorophosphate. The residual rate of butyrylthiocholine hydrolysis after addition of OP was due to G117H BChE activity. Echothiophate iodide was from Wyeth-Ayerst, Rouses Point, NY.

Echothiophate and butyrylthiocholine yield the same product, thiocholine, after being hydrolyzed by G117H BChE. However, the thiocholine from hydrolysis of echothiophate does not interfere with quantitation of the thiocholine from butyrylthiocholine hydrolysis, because echothiophate contributes only one molecule of thiocholine out of every 10,000 molecules of thiocholine that come from butyrylthiocholine. There is a 10,000 fold difference in the rates of hydrolysis of these two substrates by G117H BChE (Lockridge et al., 1997).

AChE activity assay

AChE activity was measured in the Ellman assay (Ellman et al., 1961) using 1 mM acetylthiocholine after preincubating the sample for 30 min in 0.1 mM iso-OMPA (Sigma catalog #T1505, tetraisopropylpyrophosphoramidate) to inhibit wild-type BChE. Since G117H BChE was resistant to inhibition by iso-OMPA and since G117H BChE hydrolyzes acetylthiocholine, the contribution of G117H BChE was evaluated by inhibiting AChE activity with 0.1 mM BW284C51 (Sigma catalog #A9013; 1,5 bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide), a specific AChE inhibitor. The reported AChE activity has been corrected for acetylthiocholine hydrolysis by G117H BChE.

Units of activity for AChE, BChE, and G117H are defined as micromoles of substrate hydrolyzed per minute.

ELISA to measure FLAG epitope

The human G117H BChE has the 8 amino acid FLAG epitope at the carboxy-terminus. The presence of the FLAG epitope was measured in transgenic mouse blood with ELISA. In the first trial a 96-well plate coated with anti-FLAG antibody was purchased from Sigma (catalog # P2983). 3 to 30 μ l of mouse serum from the test mice

and from wild-type mice were placed into each well and allowed to bind to the anti-FLAG antibody. The positive controls were known amounts of wild-type human BChE-FLAG or G117H BChE-FLAG in culture medium harvested from Chinese Hamster Ovary cells. Wells were washed to remove unbound material, and then received butyrylthiocholine and dithiobisnitrobenzoic acid to reveal BChE activity. No signal from transgenic mouse serum was found when Sigma precoated plates were used.

The assay was repeated, but this time we coated our own 96-well plate with M2 anti-flag antibody (Sigma), 6 µg/well. The antibody in each well was diluted to 200 µl with Tris buffered saline pH 7.4 and incubated overnight at 4°C. Unbound antibody was washed out by rinsing 3 times with 0.05% Tween-20 in Tris buffered saline. The wells were blocked with 250 µl of 0.05% Tween-20 in Tris buffered saline, 1% non-fat dried milk at room temperature for one hour, and then rinsed 3 times with 0.05% Tween-20 in Tris buffered saline. 13 µl of mouse plasma from founder mouse M816T was diluted to 200 µl and allowed to bind at room temperature for one hour. Plasma volumes from other mice (M837T, M838T, M844T, F860 wild-type control) ranged from 40 to 60 µl. The plate was rinsed three times with 0.05% Tween-20 in Tris buffered saline and then assayed for bound BChE activity by adding butyrylthiocholine and dithiobisnitrobenzoic acid in buffer. The yellow color was allowed to develop for 1 hour and then it was read in a microtiter plate reader at 405 nm.

ELISA to measure BChE protein

Monoclonal antibody Mab7 in ascites fluid was a gift from Dr. Jacques Grassi (Checler et al., 1990). Wells in a 96-well plate were coated with 10 µl of the antibody in 200 µl of 0.1 M NaHCO₃ pH 9.5 buffer, blocked, and hybridized with 10 µl of human serum, 10 µl of plasma from wild-type mouse, or 10 µl of plasma from G117H BChE mouse. After washing to remove unbound antigen, the bound BChE was assayed for activity with butyrylthiocholine and dithiobisnitrobenzoic acid (Ellman et al., 1961).

Tissue extraction

Mice were narcotized with CO₂ and then perfused with phosphate buffered saline through the heart to wash the blood out of tissues. Perfused tissues were removed and frozen. Tissues were homogenized in 10 volumes of ice-cold 50 mM potassium phosphate pH 7.4 containing 0.5% Tween-20 in a Polytron (Brinkmann Instruments) for 10 seconds. The suspension was centrifuged 10 minutes in a microfuge at 4°C and the supernatant assayed for enzyme activity. 100 µl of tissue extract was assayed in a total reaction volume of 2 ml.

Toxicity studies

Eight mice in the F4 generation of M844T were 45-110 days old at the time of the study; there were 5 female and 3 male mice. Control wild-type mice 8-10 weeks old were purchased from Taconic. The wild-type mice were the F1 cross of C57BL/6 X SJL and are called strain B6SJL/F1Tac. There were 6 female and 2 male wild-type mice. Mice weighed 18-26 g.

Mice were injected sc with 0.1 mg/kg echothiophate dissolved in saline solution. The injection volume was about 20 µl. Mice were observed for signs of toxicity including salivation, lacrimation, whole body tremor, abnormal gait, impaired mobility, flattened

posture, response to being handled, and decrease in body temperature. Body temperature was measured with a surface thermometer (Thermalert model TH-5 and a surface Microprobe MT-D, type T thermocouple, Physitemp Instruments Inc., Clifton, N.J.). Mice were euthanized 2 h after receiving echthiophate. Tissues were collected after intracardial perfusion with phosphate buffered saline. Tissue extracts were prepared as described above and assayed for AChE, BChE, and G117H BChE activity.

Results

Transgenic founder mice

Out of 51 live births, 13 mice carried the human G117H BChE gene (25%). Transgenic founder M846T was lost during quarantine. The 12 other transgenic founders were bred to wild-type mice (strain B6SJL/F1Tac), and maintained by subsequent brother-sister matings. Three male founder mice, M837T, M844T, and M853T have produced stable transgenic lines (Table 3.1).

Table 3.1 shows that 2 of the founders did not transmit the G117H gene in their germline. Five founders had the human G117H gene but did not express the G117H enzyme at significant levels. Expression in the offspring of founder M838T was unstable even in the fifth generation. The founder with the highest expression level, M816T, impregnated one female when he was very young, but has been infertile since then. The male and female offspring from this one litter have been allowed to mate with each other and with wild-type mice for a year, but have produced no offspring.

Table 3.1. G117H activity in plasma of transgenic founder mice. Lack of correlation between gene copy number and transgene activity.

Founder transgenic mouse	BChE activity, u/ml	G117H activity, u/ml	G117H, μ g/ml	Transgene copy number	Germline transmitter	Generation
F813T	2.04	0.000	0	10-20	Yes	F2
F825T	1.68	Not tested		Not tested	No	Not germline
F829T	2.18	0.000	0	Not tested	Yes	F2
F835T	1.93	0.002	0.013	2	Yes	F1
F855T	2.23	Not tested		Not tested	No	Not germline
F857T	Not tested	0.000	0	Not tested	Yes	
F859T	2.45	0.008	0.053	1	Yes	F3
M816T	1.49	0.640	4.27	1	Yes	F1
M837T	1.18	0.020	0.133	5-15	Yes	F5 stable
M838T	1.25	0.005	0.033	Not tested	Yes	F5 unstable
M844T	1.10	0.030	0.200	3-5	Yes	F6 stable
M846T	1.16	Not tested		Not tested	Dead	Dead
M853T	1.33	0.023	0.153	5-15	Yes	F3 stable

BChE activity is the activity measured with 1 mM butyrylthiocholine and includes both wild-type and G117H BChE activity. For comparison, the plasma BChE activity was 0.65 and 1.31 u/ml in two male wild-type mice, and 1.87 in one female wild-type mouse of strain B6SJL/F1Tac. Units/ml of G117H activity were converted to μ g/ml using a specific activity of 0.15 units/ μ g for pure G117H BChE (Lockridge et al., 1997). The G117H u/ml and μ g/ml values have been corrected for the 30% inhibition of G117H BChE by 0.1 mM echthiophate.

Table 3.1 shows no correlation between BChE activity measured with butyrylthiocholine and G117H activity. G117H activity was detected by measuring activity after inhibiting native BChE with echothiophate. The measured activity for G117H was quite low with 1 mM butyrylthiocholine, being 0.1-2.7% that for wild-type BChE under the same conditions, even after correction for the fraction inhibited by echothiophate. This comparison does not accurately reflect the relative amounts of G117H and wild-type BChE, since it is known that G117H has reduced binding affinity for butyrylthiocholine compared to wild-type BChE and a lower turnover number, 9000 min^{-1} versus 24,000 min^{-1} (Lockridge et al., 1997). This partly explains why, under standard conditions G117H does not contribute significantly to BChE activity. A more informative way to describe G117H content is to convert G117H activity (units/ml) into G117H protein concentration ($\mu\text{g/ml}$). The specific activity of pure G117H of 0.15 units/ μg was used for this conversion (Lockridge et al., 1997). Wild-type mouse BChE has a specific activity of about 0.7 u/ μg . In terms of micrograms protein per ml, founder M835T represents the low range of measurable G117H concentration, at 0.013 $\mu\text{g/ml}$, while M844T reflects the high range, at 0.20 $\mu\text{g/ml}$ G117H. The highest amount of G117H occurred in founder mouse M816T, which had 4.27 $\mu\text{g/ml}$ G117H and 0.8 $\mu\text{g/ml}$ wild-type BChE, that is 5 times more human BChE than mouse BChE. This founder mouse was fertile only when he was very young. His offspring were sterile. The sterility could be a consequence of where the transgene inserted into the chromosome. The three stable transgenic lines express 25% as much human BChE protein as mouse BChE protein in plasma.

Transgene copy number

The number of copies of the transgene integrated into mouse chromosomal DNA was estimated by Southern blotting.

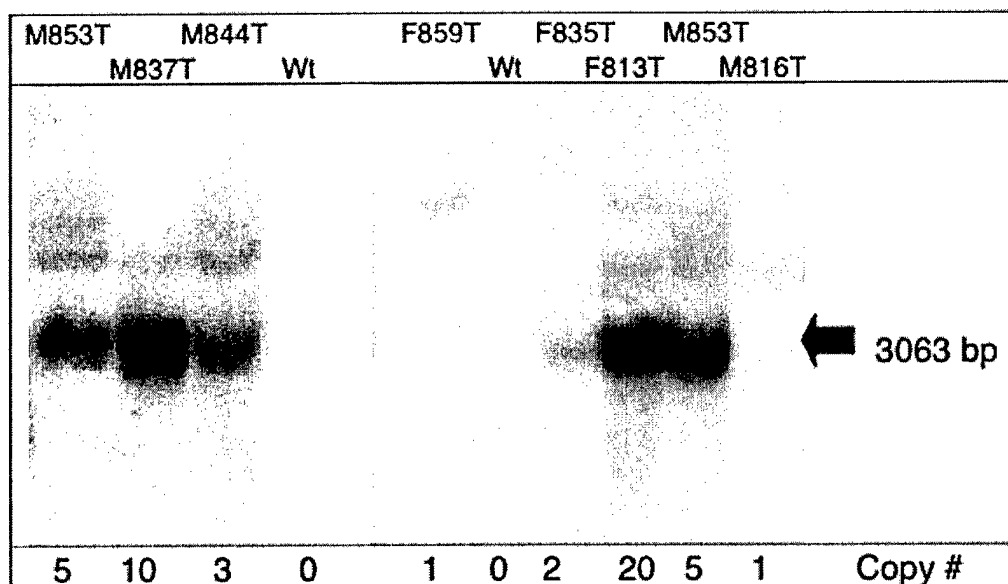


Figure 3.2. Southern blot of genomic DNA from transgenic mice. The DNA was digested with EcoRI. The P32 labeled probe is specific for the transgene. The transgene specific band

has a size of 3063 bp. The number of copies of the transgene is 0 in wild-type mice, and up to 20 in the F813T mouse.

Table 3.1 and Figure 3.2 show that founder M816T had the highest level of expression of G117H BChE but only one copy of the transgene. Three founders (F813T, M837T, and M853T) had multiple copies of the transgene. The F813T founder had the highest number of copies, but no detectable G117H activity. There was no correlation between gene copy number and level of expression. However, the mouse lines that stably expressed the transgene had multiple copies of the transgene.

Establishing stable transgenic lines

All pups were genotyped by PCR at 2 weeks and tested for G117H BChE activity in plasma when they were 4-6 weeks old. Figure 3.3 shows that the F2 generation had a range of activities. This was expected since mice in the F2 generation could be homozygous or hemizygous for the transgene or not inherit the transgene. A male and female with the highest G117H activity were mated to produce the F3 generation. 4 sets of males and females from the F3 generation were mated to produce the F4 generation. Animals with the highest activity were mated to produce the F5 generation. Similar levels of G117H activity were found in all animals in the fifth generation, suggesting that both chromosomes carried the transgene. All mice in the F5 generation had higher G117H activity than the founder mouse, supporting the conclusion that the F5 generation was homozygous for the transgene. Homozygosity was confirmed by breeding mice in the F5 generation to wild-type mice. All 20 pups from this breeding carried the transgene.

Each of the three stable transgenic lines originated from one founder: M837T, M844T, or M853T. Progeny from different founders have not been bred to each other.

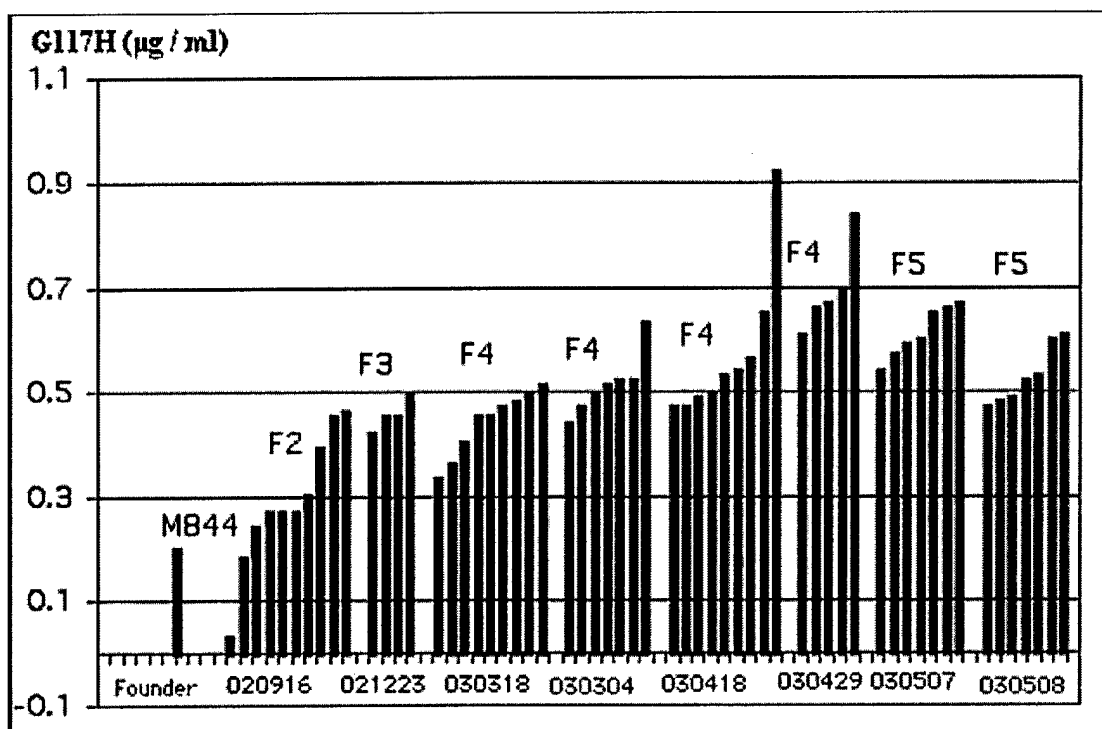


Figure 3.3. Establishment of a stable transgenic line from founder M844T. Stable G117H BChE activity in plasma was achieved by the fifth generation. The date of birth for each litter is on the x-axis. G117H activity has been corrected for 30% inhibition by 0.1 mM echthiophate.

Tissues that express G117H BChE

Tissues from adult mice in transgenic line M844T were tested for G117H activity. All tissues expressed G117H activity (Figure 3.4). The highest G117H activity was in intestine and plasma, the lowest in brain and muscle. There was a 5-fold difference in expression between the highest and lowest tissues. By contrast, levels of wild-type BChE in the same transgenic line had a different pattern of expression (Figure 3.5). The highest wild-type BChE activity was in the intestine and liver, and the lowest in brain and muscle. There was a 60 fold difference in expression between the highest and lowest. These results support the finding of others (Zambrowicz et al., 1997; Soriano, 1999) that the ROSA26 promoter directs expression in most tissue types. The level of expression seems to be regulated not only by the ROSA26 promoter but also by the site of insertion of the transgene, since one transgenic founder, M816T, had higher G117H BChE activity than wild-type BChE activity.

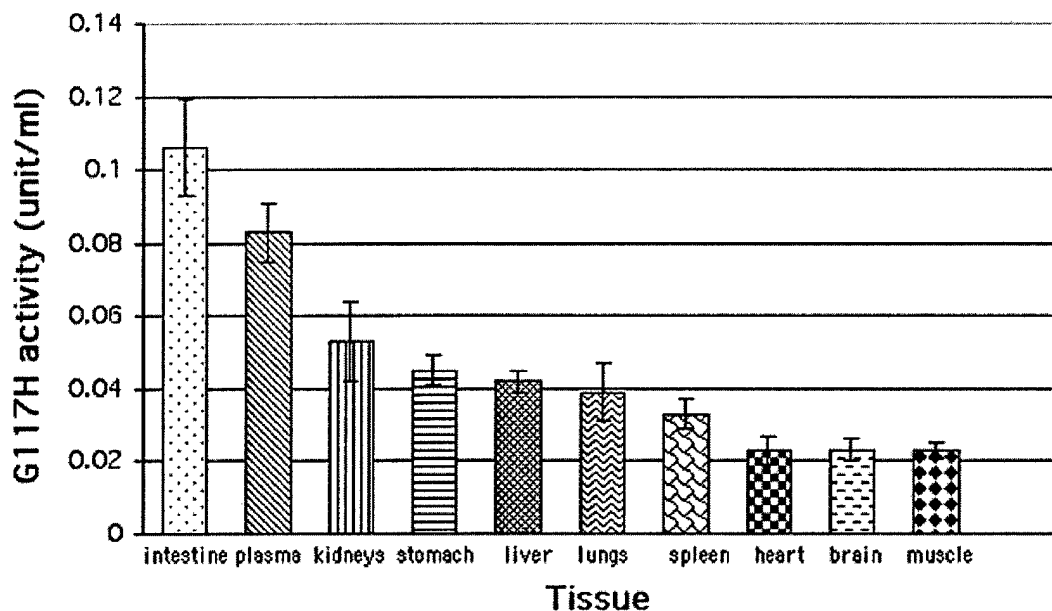


Figure 3.4. Tissue expression of G117H BChE in transgenic line M844T. Mice were perfused with saline to wash blood out of tissues, before tissues were removed. Mice were 3 months old. The mean $\pm$ SD is shown for 4 mice. The G117H activity values have been corrected for 22% inhibition by 1 μ M DFP.

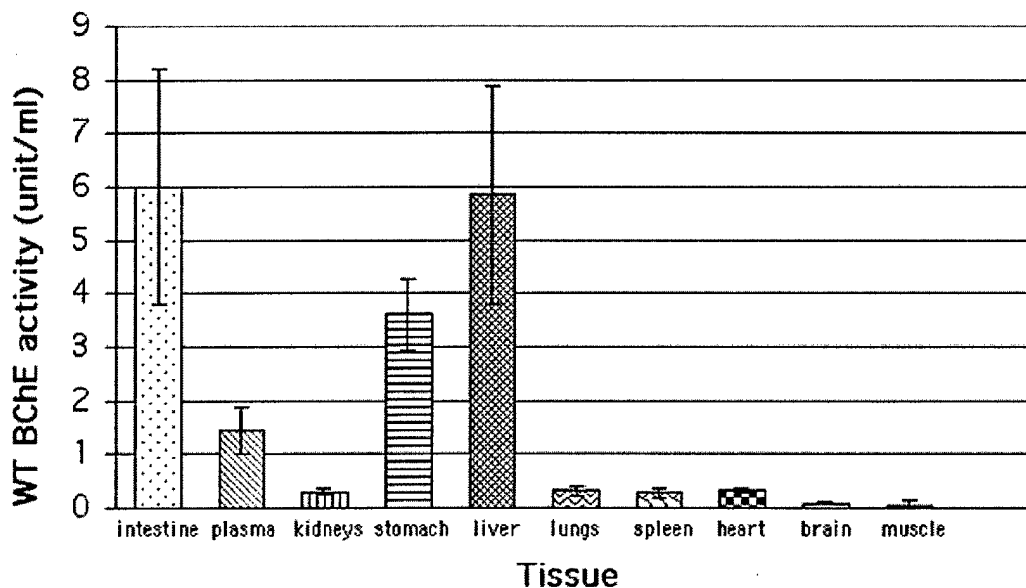


Figure 3.5. Tissue expression of wild-type BChE in transgenic line M844T. The same 4 mice were tested in Figures 3.4 and 3.5.

To determine whether expression of wild-type mouse BChE was affected by human G117H BChE, the BChE activity levels were measured in the high-expressing M816T founder and his F1 progeny. The founder had 1.49 u/ml total BChE and 0.64 u/ml G117H BChE in plasma. The F1 had 3.03 u/ml total BChE and 1.38 u/ml G117H BChE. For comparison, the plasma BChE activity was 0.65 and 1.31 u/ml in two male wild-type mice, and 1.87 in one female wild-type mouse of strain B6SJL/F1Tac. These activity values suggest that expression of wild-type mouse BChE was unaffected by expression of human G117H BChE. The BChE promoter and the ROSA26 promoter acted independently.

The different pattern of expression of G117H BChE and wild-type BChE in transgenic line M844T (Figures 3.4 and 3.5) supports the conclusion that the BChE and ROSA26 promoters acted independently.

Antibody assays to confirm expression of human BChE.

The FLAG epitope at the carboxy terminus of the 574 amino acid human G117H BChE subunit was detected in transgenic mouse plasma, but only in the mouse plasma with the highest level of expression of G117H BChE, founder M816T. A 13 μ l aliquot of mouse plasma containing 4.27 μ g/ml of G117H BChE, for a total of 0.055 μ g, gave a positive signal, but plasma from mice expressing less G117H BChE did not. Control assays with the same BChE-FLAG protein expressed in culture medium could detect as little as 0.009 μ g. This suggests the FLAG epitope had been proteolytically removed from a substantial fraction of the G117H BChE molecules in mouse plasma. (Cousin et al., 1996) have also reported proteolytic cleavage of the FLAG epitope from snake AChE expressed in COS cells.

The monoclonal antibody from Dr. Jacques Grassi (Checler et al., 1990) was 10-20 times more sensitive than the FLAG antibody for detecting human BChE in mouse plasma. Mouse plasma from the F3 generation of M844T had detectable human BChE in an ELISA assay with anti-human BChE Mab7, but was negative in an ELISA assay with anti-FLAG.

Transgenic mice resist OP toxicity

Three out of 8 wild-type mice died after receiving 0.1 mg/kg echothiophate sc, suggesting that this dose was approximately the LD₅₀ dose for wild-type mice. None of the 8 transgenic mice, treated with this level of echothiophate, died.

The wild-type mice had severe signs of toxicity including profuse salivation, lacrimation, whole body tremor, abnormal gait, impaired mobility, flattened posture, no response to being handled, and decrease in body temperature. By contrast transgenic mice had only slight salivation, slight tremor, and no significant decrease in body temperature (Figure 3.6). Transgenic mice showed no impairment in gait, mobility, or posture, and no change in reactivity to being handled.

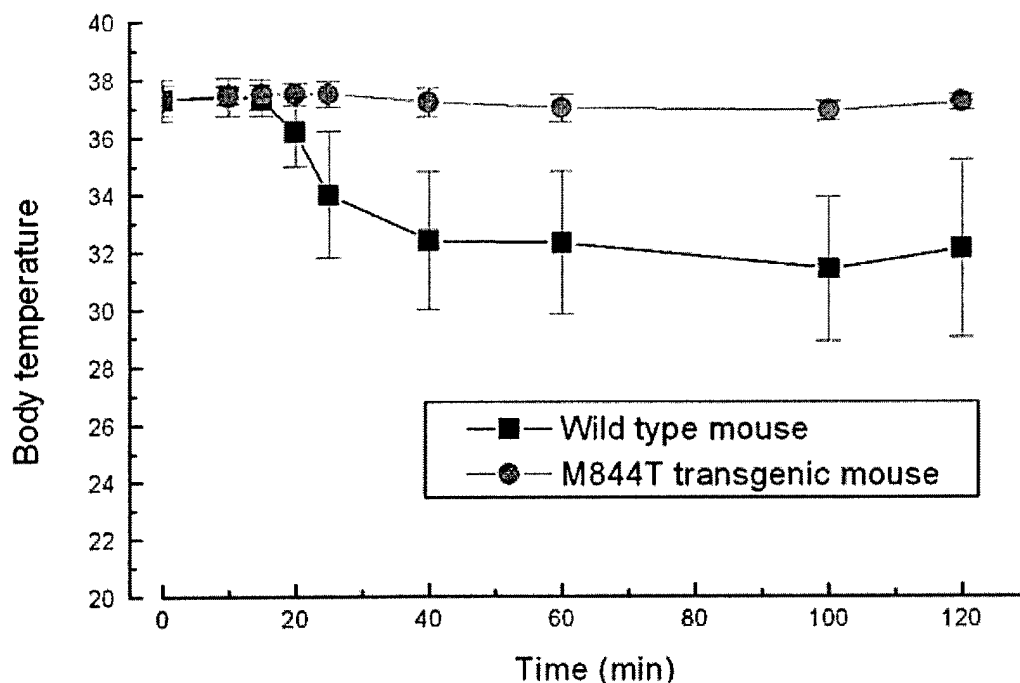


Figure 3.6. Transgenic G117H mice maintain body temperature after OP exposure. Echothiophate 0.1 mg/kg sc caused a 6°C drop in body temperature in wild-type mice but not in transgenic mice. Data are for 5 wild-type and 8 transgenic mice.

The LD₅₀ for transgenic mice has not been measured. Preliminary experiments with 2 mice suggested that a small increase in dose was lethal. One transgenic mouse was tested with 0.12 mg/kg and one with 0.15 mg/kg echothiophate. Both transgenic mice died. It is normal for dose response curves for OP to be very steep.

AChE, BChE, and G117H activity in OP treated mice

The question in these experiments is whether G117H BChE protected AChE and endogenous wild-type BChE from inhibition by OP. Results in Table 3.2 show that AChE and BChE activities decreased to the same extent in transgenic and wild-type mice after treatment with 0.1 mg/kg echothiophate sc.

The highest inhibition was in plasma where BChE was inhibited 77-84% and AChE was inhibited 58% both in transgenic and wild-type mice. In most other tissues BChE and AChE enzymes were inhibited about 50%. The level of inhibition of AChE and BChE in transgenic mice was not less than in wild-type mice. Echothiophate injected into the periphery did not inhibit AChE and BChE in the brain, thus demonstrating that the positively charged echothiophate did not cross the blood-brain barrier.

Table 3.2. AChE, BChE and G117H BChE activity in tissues of echothiophate-treated mice. Wild-type and transgenic mice received 0.1 mg/kg echothiophate sc. Animals were euthanized 2 h later. Units of enzyme activity are micromoles substrate hydrolyzed per minute per gram wet weight of tissue.

Mouse	Tissue	AChE (mean±SD)	BChE (mean±SD)	G117H (mean±SD)
untreated wild-type	Brain	1.01±0.16	0.09±0.01	0
wild-type	Brain	1.05±0.17	0.14±0.05	0
untreated transgenic	Brain	1.08±0.04	0.09±0.01	0.023±0.003
transgenic	Brain	1.04±0.09	0.09±0.01	0.022±0.005
untreated wild-type	Muscle	0.32±0.14	0.16±0.01	0
wild-type	Muscle	0.16±0.03	0.10±0.05	0
untreated transgenic	Muscle	0.31±0.06	0.22±0.14	0.023±0.002
transgenic	Muscle	0.17±0.09	0.13±0.07	0.021±0.002
untreated wild-type	Liver	0.06±0.02	4.09±1.11	0
wild-type	Liver	0.05±0.02	2.80±1.03	0
untreated transgenic	Liver	0.07±0.02	5.84±2.03	0.042±0.003
transgenic	Liver	0.06±0.01	2.70±1.40	0.032±0.017
untreated wild-type	Heart	0.10±0.01	0.38±0.02	0
wild-type	Heart	0.04±0.01	0.12±0.05	0
untreated transgenic	Heart	0.09±0.02	0.32±0.04	0.023±0.004
transgenic	Heart	0.05±0.02	0.11±0.04	0.036±0.007
untreated wild-type	Lungs	0.15±0.09	0.27±0.07	0
wild-type	Lungs	0.10±0.06	0.16±0.02	0
untreated transgenic	Lungs	0.17±0.04	0.32±0.09	0.039±0.008
transgenic	Lungs	0.09±0.04	0.14±0.06	0.040±0.009
untreated wild-type	Plasma	0.45±0.15	1.71±0.56	0
wild-type	Plasma	0.19±0.09	0.28±0.24	0
untreated transgenic	plasma	0.31±0.06	1.44±0.44	0.083±0.008
transgenic	Plasma	0.13±0.03	0.33±0.19	0.080±0.025
untreated wild-type	Intestine	0.18±0.03	9.56±0.44	0
wild-type	Intestine	0.10±0.01	4.25±1.96	0
untreated transgenic	intestine	0.18±0.05	5.99±2.20	0.106±0.013
transgenic	Intestine	0.09±0.02	3.12±1.45	0.090±0.016

The data are for 8 treated transgenic mice (derived from founder M844T), 5 treated wild-type mice that survived this dose of echothiophate (another 3 died at 20 to 40 minutes after injection with echothiophate), 3 untreated wild-type mice, and 4 untreated transgenic mice.

G117H BChE activity was not significantly inhibited by treatment of mice with 0.1 mg/kg echothiophate.

Comparison of AChE and BChE activities in untreated mice shows that most tissues have more BChE than AChE activity. Only brain and muscle have more AChE than BChE activity. This finding for strain B6SJL supports results for strain 129Sv (Li et al., 2000; Duysen et al., 2001).

Catalytic activity of G117H BChE with acetylthiocholine

The above results led us to consider that G117H BChE might be protecting mice from OP toxicity not by catalysis of OP to inactive products, but by hydrolysis of

acetylcholine. The potential of G117H BChE to hydrolyze acetylcholine was estimated by measuring the k_{cat} and K_m values for acetylthiocholine. For comparison, k_{cat} and K_m values were also measured with butyrylthiocholine. Table 3.3 shows that highly purified human G117H BChE binds acetylthiocholine with a K_m value of 1.75 mM and that it hydrolyzes acetylthiocholine at a rate of 12,000 μ moles per min per μ mole active site. Butyrylthiocholine is a better substrate for G117H BChE in terms of binding affinity and catalytic rate than acetylthiocholine. Both the K_m and the catalytic rate for butyrylthiocholine with G117H, in Table 3.3, are more than twice the values previously reported for this reaction. The differences can be attributed to difficulties in reproducibly defining excess substrate activation for G117H. The current data could not be fit to a substrate activation scheme. Previous data (Lockridge et al., 1997) showed a small inflection, which fit to a substrate activation scheme, but required that K_{ss} (at 400 μ M) be only 2.7-fold larger than K_m (at 150 μ M). In the current report, the K_m value (420 μ M) is approximately the same as the earlier K_{ss} value and the k_{cat} value (18,300 min^{-1}) is approximately the same as the earlier $b k_{cat}$.

Table 3.3. Binding affinity and catalytic rate for purified human G117H BChE

substrate	K_m , mM	k_{cat} , min^{-1}
Acetylthiocholine	1.75 ± 0.09	12,000
Butyrylthiocholine	0.42 ± 0.02	18,300

Literature values for G117H BChE: (Lockridge et al., 1997) K_m for butyrylthiocholine 0.15 mM, k_{cat} 9000 min^{-1} , K_{ss} 0.4 mM, and $b k_{cat}$ 15,000 min^{-1} . Literature values for wild-type human BChE: (Lockridge et al., 1997) K_m for butyrylthiocholine 0.02 mM, k_{cat} 24,000 to 30,000 min^{-1} , $b k_{cat}$ 90,000 min^{-1} . The b value in $b k_{cat}$ reflects the fold increase in activity under substrate activation conditions.

Discussion

Characteristics of the G117H BChE transgenic mouse

This is the first transgenic mouse that expresses human BChE. It is also the first mammal with hereditary OP resistance. Transgenic mouse line M844T has stably transmitted the human BCHE gene for 6 generations. Three stable G117H BChE transgenic lines expressed 0.5 $\mu\text{g/ml}$ (6th generation of M844T), 0.7 $\mu\text{g/ml}$ (5th generation of M837T), and 0.5 $\mu\text{g/ml}$ (3rd generation of M853T) G117H BChE enzyme per ml plasma. This is 25-30% of the wild-type BChE in the same animals. All tissues tested expressed G117H BChE enzyme activity.

The transgenic mice are fertile and have no apparent ill effects from overexpression of BChE. Litter sizes are normal at 7 to 9 pups per litter. Normal health was expected because expression of excess BChE in humans has no deleterious effect. People with the Cynthiana variant of BChE have 2 to 4 times the normal amount of BChE and are resistant to the muscle relaxant succinylcholine, but have no other phenotype (Neitlich, 1966; Yoshida and Motulsky, 1969; Delbruck and Henkel, 1979; Hada et al., 1985; Yao and Savarese, 1997).

An important unusual feature of the G117H transgenic mouse is its resistance to OP toxicity. At this time it is unclear how G117H BChE protects mice from OP toxicity.

Biochemical characteristics of human G117H BChE

The human G117H BChE is a genetically engineered mutant designed to be an OP hydrolase. Millard et al. (Millard et al., 1995) applied biochemical principles for the mechanism of dephosphorylation to a model of the crystal structure of BChE and succeeded in converting an enzyme that had no OP hydrolase activity into an enzyme with OP hydrolase activity. Gly 117 forms part of the oxyanion hole that stabilizes the transition state intermediate. Gly117 is very near the active site serine that becomes phosphorylated by OP. In wild-type BChE the phosphorylated serine is a stable adduct and the BChE is irreversibly inhibited. By contrast, the G117H mutant has a histidine in the right location to activate a water molecule for attack on the phosphorylated serine, thus releasing the OP from the serine, and freeing the BChE for a second round of catalysis.

The human G117H BChE enzyme was produced in Chinese Hamster Ovary cells by recombinant DNA techniques, and purified from culture medium (Millard et al., 1995; Lockridge et al., 1997). The G117H BChE was found to hydrolyze the nerve agents sarin, soman, and VX, the anti-glaucoma drug echothiophate, and the active metabolite of the insecticide parathion, namely paraoxon. The catalytic rate constant for hydrolysis of echothiophate was about 1 per min, a rate 10,000 fold lower than the catalytic rate constant for hydrolysis of butyrylthiocholine. The binding affinity for butyrylthiocholine measured as the K_m value, was poorer for G117H BChE ($K_m = 150 \mu\text{M}$) compared to wild-type BChE ($K_m = 22 \mu\text{M}$). The maximum rate of hydrolysis of butyrylthiocholine under substrate activation conditions was $15,000 \text{ min}^{-1}$ for G117H BChE and $90,000 \text{ min}^{-1}$ for wild-type BChE. G117H BChE hydrolyzed acetylthiocholine ($K_m = 1.7 \text{ mM}$, $k_{\text{cat}} = 12,000 \text{ min}^{-1}$), and by implication, acetylcholine. The binding affinity of G117H BChE for echothiophate ($K_m = 74 \mu\text{M}$) was similar to the binding affinity of G117H BChE for butyrylthiocholine ($K_m = 150 \mu\text{M}$). These biochemical characteristics of G117H BChE made it a suitable candidate for testing protection from OP toxicity in transgenic mice.

A side reaction that destroys activity of G117H BChE is the partial dealkylation reaction of the BChE-OP complex, called "aging". The half-time for aging is 5.5 hours for echothiophate (Lockridge et al., 1997).

Mechanism of protection

The transgenic G117H BChE mouse is resistant to OP toxicity. Two possible ways in which G117H BChE could be protecting the mouse are by hydrolyzing OP or by hydrolyzing acetylcholine after AChE has been inhibited. We reasoned that if OP hydrolysis were the mechanism, then AChE and BChE should be less inhibited in the transgenic mouse. This was not found. The experimental result was that AChE and BChE were inhibited to similar extents in echothiophate-treated transgenic and wild-type mice. Therefore, OP hydrolysis does not seem to be the mechanism of protection in the transgenic mouse. The purified G117H enzyme was found to hydrolyze echothiophate at the rate of 0.75 min^{-1} (Lockridge et al., 1997). This rate may be too slow to destroy a significant amount of OP before the OP inhibits AChE and BChE.

An alternative possibility to consider is that protection is explained by the resistance of G117H BChE to inhibition and the ability of G117H BChE to hydrolyze acetylcholine. It is known that inhibition of AChE causes accumulation of excess acetylcholine, and excess acetylcholine leads to death. The G117H BChE with its widespread distribution might be present in or near nerve synapses, where it could aid in metabolism of excess acetylcholine. Additional work needs to be done to support this hypothesis. There is no doubt that G117H BChE is resistant to inhibition by OP and that G117H is capable of hydrolyzing acetylcholine. What is not known is whether G117H BChE is found in locations where it could dispose of synaptic acetylcholine. Another issue to resolve is the observation that an LD₅₀ dose of echothiophate inhibits tissue AChE 50%, but after 50% inhibition a tissue, for example muscle, still has more AChE activity than G117H activity. Why should 0.02 units/g of G117H BChE contribute significantly to acetylcholine hydrolysis in muscle that still has 0.16 units/g of AChE activity?

A protective mechanism that relies on resistance to inhibition by OP rather than on ability to hydrolyze OP has examples in nature. OP-resistant AChE has been identified in the azinphos-methyl-resistant strain of the Colorado potato beetle (Zhu and Clark, 1997), the paraoxon-resistant strains of house flies (Oppenoorth, 1982), the malaoxon-resistant grain borer (Guedes et al., 1997), and the dimethoate-resistant olive fruit fly (Vontas et al., 2001). The OP resistance is explained by mutations that decrease the binding affinity of AChE for OP.

There are two examples in nature of OP resistance due to a mutation that conferred OP hydrolase activity. These examples are the diazinon-resistant sheep blowfly and house fly (Newcomb et al., 1997; Claudianos et al., 1999) where the carboxylesterase E3 protein has a mutation that changes Gly 137 to Asp. Position 137 in the sheep blowfly is homologous to position 117 in human BChE. This carboxylesterase mutant and the G117H BChE mutant have similar properties in that both enzymes are resistant to inhibition by OP and can hydrolyze OP. Carboxylesterase is not found in nerve synapses and does not hydrolyze acetylcholine. The mechanism of OP resistance in the sheep blowfly could be similar to the mechanism of OP resistance in the G117H BChE transgenic mouse, though details of the mechanism are unknown.

More results related to

Tasks 3.1, 3.2, 3.3, 3.4, 3.5, 3.6

Screening assays for cholinesterases resistant to inhibition by organophosphorus toxicants

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Abstract

Methods to measure resistance to inhibition by organophosphorus toxicants (OP) for mutants of butyrylcholinesterase (EC 3.1.1.8 BChE) and acetylcholinesterase (EC 3.1.1.7 AChE) enzymes were devised. Wild-type cholinesterases were completely inhibited by 0.1 mM echothiophate or 0.001 mM diisopropylfluorophosphate, but human BChE mutants G117H, G117D, L286H, W231H and snake AChE mutant HFQT retained activity. Tissues containing a mixture of cholinesterases could be assayed for amount of G117H BChE. For example, the serum of transgenic mice expressing human G117H BChE contained 0.5 µg/ml human G117H BChE, 2 µg/ml wild-type mouse BChE, and 0.06 µg/ml wild-type mouse AChE. The oligomeric structure of G117H BChE in the serum of transgenic mice was determined by nondenaturing gel electrophoresis followed by staining for butyrylthiocholine hydrolysis activity in the presence of 0.1 mM echothiophate. Greater than 95% of the human G117H BChE in transgenic mouse serum was a tetramer. To visualize the distribution of G117H BChE in tissues of transgenic mice, sections of small intestine were treated with echothiophate and then stained for BChE activity. Both wild-type and G117H BChE were in the epithelial cells of the villi. These assays can be used to identify OP-resistant cholinesterases in culture medium and in animal tissues.

Introduction

There is a need to protect people from the toxic effects of organophosphorus nerve agents and organophosphorus pesticides (OP). Pretreatment with wild-type human BChE, horse BChE, or bovine AChE gives complete protection against 2 LD₅₀ doses of the nerve agents sarin, soman, and VX (Wolfe et al., 1987; Raveh et al., 1989; Ashani et al., 1991; Broomfield et al., 1991; Doctor et al., 1991; Maxwell et al., 1992; Wolfe et al., 1992; Brandeis et al., 1993; Maxwell et al., 1993; Raveh et al., 1993; Castro et al., 1994; Genovese and Doctor, 1995; Raveh et al., 1997; Allon et al., 1998; Matzke et al., 1999). Animals have no toxic signs and no side effects. Monkeys pre-treated with cholinesterase continue to perform well in a serial probe task despite being treated with lethal doses of nerve agent (Broomfield et al., 1991). It is estimated that a 70 kg person will require about 200 mg of purified human BChE to be protected against 2 LD₅₀ doses

of nerve agent. This high amount of BChE is needed because BChE is a stoichiometric scavenger, requiring one molecule of BChE monomer to inactivate one molecule of OP. If BChE or AChE could be converted to an OP hydrolase the dose of BChE needed for protection could be lower. This goal was partially achieved with the G117H mutant of human BChE. G117H BChE hydrolyzed all OP tested including the nerve agents sarin and VX, the anti-glaucoma drug echothiophate, and the active metabolite of the insecticide parathion, namely paraoxon (Millard et al., 1995; Lockridge et al., 1997). The double mutant G117H/E197Q hydrolyzed soman because it resisted inactivation by the dealkylation process called "aging" (Millard et al., 1998). The limitation for G117H BChE was the slow rate of OP hydrolysis, k_{cat} of about 1 per min. Additional mutants of BChE and AChE have been made and expressed in stable CHO cell lines. A screening assay was needed to test these mutants for resistance to inhibition by OP. This report describes the assays developed to screen mutants of human BChE and snake AChE (*Bungarus fasciatus*) expressed in cell culture.

Transgenic mice carrying the gene for human G117H BChE were made (Wang et al., 2004). A breeding program to establish stable transgenic lines expressing maximum levels of G117H BChE activity relied on a screening assay capable of quantifying G117H BChE activity in mouse plasma.

Materials and methods

BChE mutants

Mutants of human BChE were made by PCR with Pfu polymerase and cloned into the mammalian expression plasmid pGS. Stably transfected CHO-K1 cells (American Type Culture Collection, No. CCL61) were selected in 50 μ M methionine sulfoximine (Lockridge et al., 1997). BChE was secreted into serum free medium (Ultraculture 12-725B, BioWhittaker). BChE was purified by affinity chromatography on procainamide-Sepharose and concentrated in an Amicon stirred cell with a PM10 membrane as previously described (Lockridge et al., 1997).

AChE mutant

A snake AChE mutant containing 3 amino acid substitutions in the active site gorge was designed by modeling the snake AChE structure to make it similar to the human G117H BChE structure. The G117H BChE structure was built from the structure of wild-type human BChE (Nicolet et al., 2003). The sequence of residues 122-125 of the snake *Bungarus fasciatus* AChE is GFYS (Cousin et al., 1996). The sequence of wild-type human BChE is GFQT for homologous residues 117-120 (Lockridge et al., 1987). The snake AChE was mutated to HFQT at residues 122-125 to mimic human G117H BChE. A stable CHO cell line that secreted this snake AChE mutant into serum-free culture medium was made.

Transgenic mice

Transgenic mice expressing human G117H BChE under the control of the ROSA26 promoter were made as described (Wang et al., 2004). The mice were in strain B6SJL (Taconic). The ROSA26 promoter directs expression of the transgene in most tissue types (Zambrowicz et al., 1997).

96-well plate assay

Cholinesterase activity was measured in the absence and presence of an OP to identify mutants resistant to inhibition by OP. In the absence of OP, activity was measured after a 10 minute preincubation of enzyme with 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB, from Sigma) in buffer to allow free sulfhydryls to react. Each well contained 165 μ l of 0.1 M potassium phosphate pH 7.0, 5 μ l of 20 mM DTNB, and 10 μ l of cholinesterase enzyme. The substrate turnover reaction was started by adding 20 μ l of 10 mM butyrylthiocholine iodide or acetylthiocholine iodide to make a final concentration of 1 mM substrate. In the presence of OP, substrate turnover was measured after a 10 minute preincubation of enzyme with buffer, DTNB, and 0.1 mM echothiophate. Echothiophate iodide was from Wyeth-Ayerst, Rouses Point, NY. Each well contained 155 μ l buffer, 5 μ l of 20 mM DTNB, 10 μ l of 2 mM echothiophate, and 10 μ l of cholinesterase enzyme. The substrate turnover reaction was started by adding 20 μ l of 10 mM substrate. Increase in absorbance was recorded at 412 nm for 15 min in a Molecular Devices SpectraMax 190 plate reader.

Assay in the Gilford spectrophotometer

One cuvette was used to measure both the uninhibited and OP-inhibited rates of substrate hydrolysis in a temperature-controlled single-beam Gilford spectrophotometer interfaced via a MacLab data recorder (ADInstruments) to a Macintosh computer. First, 1 mM butyrylthiocholine and 0.5 mM DTNB were incubated in 2.0 ml of 0.1 M potassium phosphate buffer, pH 7.0, at 25°C. The change in optical density with time was followed at 412 nm to establish the background rate for spontaneous reduction of DTNB. The molar extinction coefficient at 412 nm for the yellow product was 13,600 M⁻¹cm⁻¹ (Ellman et al., 1961). Next, an aliquot of the enzyme preparation was added to the cuvette and the uninhibited, linear rate for steady-state turnover of butyrylthiocholine was determined. The amount of enzyme was adjusted so that the final optical density at the end of the inhibition phase did not exceed 1.0. Finally, echothiophate iodide was added to a final concentration of 0.1 mM. The activity remaining 10 min after addition of echothiophate was attributed to enzyme resistant to OP inhibition. This assay was used for recombinant BChE in cell culture medium, for purified BChE mutants, as well as for G117H BChE in mouse tissues.

Units/ml of G117H activity were converted to μ g/ml using a specific activity of 0.15 units/ μ g for pure G117H BChE (Lockridge et al., 1997). The G117H u/ml and μ g/ml values were corrected for the 30% inhibition of G117H BChE by 0.1 mM echothiophate. This inhibition value was determined with highly purified G117H BChE. Activity of wild-type mouse BChE in units/ml was converted to μ g/ml using a specific activity of 0.7 units/ μ g. Activity for wild-type mouse AChE was converted using a specific activity of 5 units/ μ g.

Nondenaturing gel electrophoresis

To visualize tetramers, dimers, and monomers of BChE, mouse plasma samples were loaded onto a nondenaturing 4-30% polyacrylamide gradient gel (Hoefer Scientific, presently owned by Pharmacia) and electrophoresed for 24 hours at 120 volts at 4°C. 3 μ l of mouse plasma were loaded per lane. The gel was stained for BChE activity by the

method of Karnovsky and Roots (Karnovsky and Roots, 1964). The staining solution contained 180 ml of 0.2 M maleic acid adjusted to pH 6.0 with NaOH, 15 ml of 0.1 M sodium citrate, 30 ml of 0.030 M CuSO₄, 30 ml water, 30 ml of 0.005 M potassium ferricyanide, and 0.180 g of butyrylthiocholine iodide. To reveal G117H BChE activity, the gel was preincubated in 0.1 mM echothiophate for 30 min in the staining solution, before addition of butyrylthiocholine iodide. The gel was incubated 2 to 5 hours at room temperature with gentle shaking.

Tissue sections stained for BChE activity

Mice were cardially perfused to wash out blood before tissues were removed. Small intestine was cut on a cryostat into 30 micron sections. The frozen sections were transferred to slides precoated with gelatin and stained for BChE activity by the method of Mesulam et al. (Mesulam et al., 2002a) in the presence of 5 mM butyrylthiocholine for 20 h. To stain for G117H BChE activity, slides were preincubated for 30 min in 0.1 mM echothiophate before addition of butyrylthiocholine. Color was developed in sodium sulfide solution for 20 sec for uninhibited sections, and for 20 min for sections treated with echothiophate. Uninhibited intestine developed a very dark stain even after just 20 sec in sodium sulfide. Therefore, a second staining method was also used, that of Karnovsky and Roots (Karnovsky and Roots, 1964). Images of stained sections were photographed on a Nikon Optiphot-2 microscope with a Hitachi HV-C20 camera and transferred to a computer with Optimas 5.23 software.

Results

96-well plate assay

Echothiophate iodide was chosen because it is a stable solid at room temperature, it is soluble in water and is stable in water, and it inhibits both AChE and BChE.

Diisopropylfluorophosphate was also effective in these assays. Figure 3.1.1 shows that wild-type BChE as well as the BChE mutants E197Q and A328W were completely inhibited by 0.1 mM echothiophate. In contrast, the BChE mutants G117H, G117D, L286H and W231H retained 76%, 79%, 2% and 2% activity after 10 min exposure to 0.1 mM echothiophate. Figure 3.1.1 also shows that the snake AChE mutant HFQT retained 100% of its activity in the presence of echothiophate, whereas wild-type bovine AChE was completely inhibited.

BChE mutants G117H, G117D, L286H, and W231H and the HFQT mutant of AChE were designed to be OP hydrolases. It was therefore anticipated that they would be resistant to OP inhibition. The residues that were mutated were within 7 Angstroms of the active site serine. Gly117 is in the oxyanion hole. Leu 286 and Trp231 are part of the acyl-binding pocket.

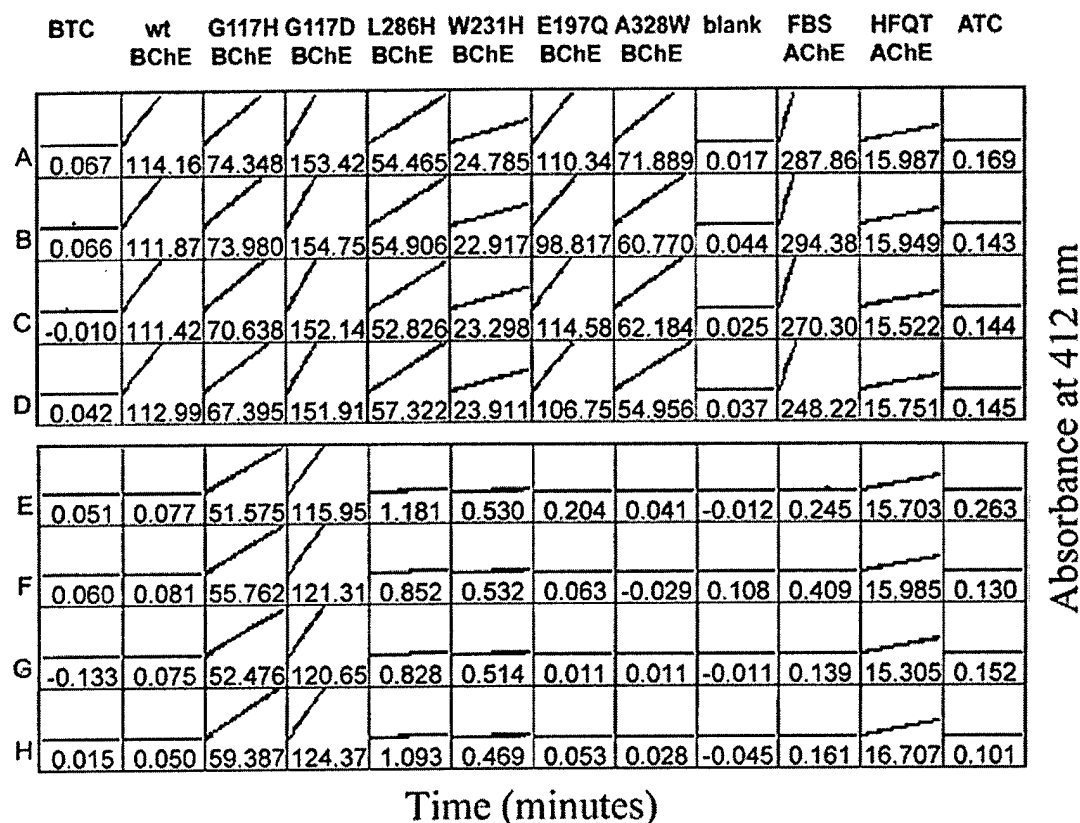


Figure 3.1.1. 96-well plate assay for OP resistance. Column 1 is the reagent blank for butyrylthiocholine, buffer, and DTNB. Columns 2 – 8 contain human BChE wild-type and mutants. Column 9 is empty. Column 10 contains wild-type fetal bovine AChE. Column 11 contains the snake AChE triple mutant HFQT. Column 12 is the reagent blank with acetylthiocholine. Rows A, B, C, D show the rate of hydrolysis of substrate in the absence of inhibitor. Rows E, F, G, H show the rate of hydrolysis of substrate in the presence of 0.1 mM echothiophate. Each reaction was assayed in quadruplicate. The numbers are the slopes in milli OD per minute. The time scale is 0 to 15 min. The absorbance scale is 0 to 1.

Gilford spectrophotometer

Assays in the Gilford spectrophotometer were useful when there were few samples to test. Figure 3.2.2 shows activity traces for mouse plasma from a wild-type mouse and for plasma from a transgenic mouse. The wild-type mouse plasma contained endogenous mouse BChE, while the transgenic mouse plasma contained both wild-type mouse BChE and human G117H BChE. The two plasma samples could not be distinguished with a standard BChE activity assay as shown in the initial traces in Figure 3.2.2, before echothiophate addition. However, they were clearly different in their response to echothiophate. The plasma from the transgenic mouse still had significant BChE activity in the presence of 0.1 mM echothiophate, whereas no BChE activity remained in the wild-type mouse plasma. The residual BChE activity in the transgenic mouse was due to the activity of G117H BChE.

Wild-type BChE in tissues other than plasma was not completely inhibited by 0.1 mM echothiophate. A high background rate was observed in kidney and liver. However, 1 μ M DFP gave complete inhibition of wild-type BChE in tissue extracts after a 10 min reaction period. Therefore G117H activity in tissue extracts was measured after treating for 10 min with 1 μ M DFP.

Highly purified human G117H BChE was tested for resistance to inhibition by OP. Echothiophate at 0.1 mM inhibited G117H BChE 30%, while 1 μ M DFP inhibited G117H BChE 22%, after 10 min exposure. The human G117H BChE used for these assays was a recombinant BChE produced by CHO cells and purified from culture medium (Lockridge et al., 1997).

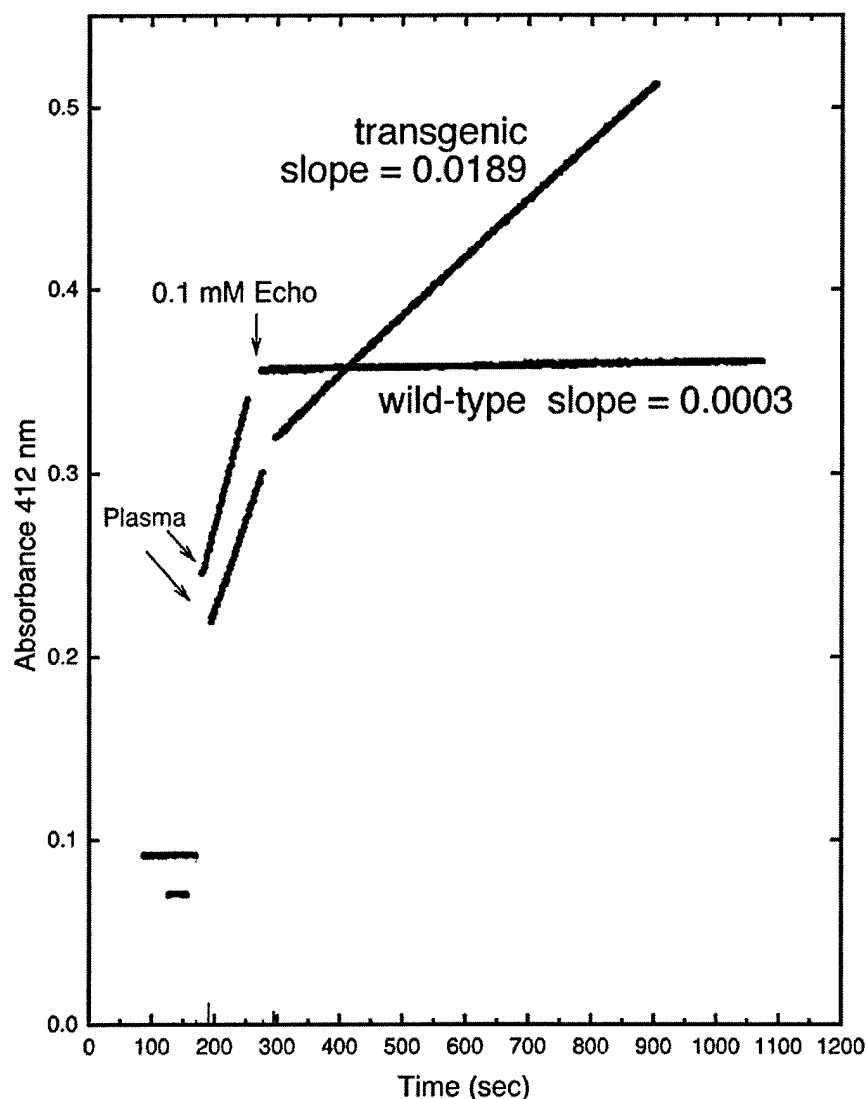


Figure 3.2.2. Measurement of G117H BChE activity in the Gilford spectrophotometer. Hydrolysis of 1 mM butyrylthiocholine by 6 μ l mouse plasma in a 2 ml reaction volume was measured initially in the absence of inhibitor. After about 100 seconds of reaction, 10 μ l of 20

mM echothiophate was added to give a final concentration of 0.1 mM. The echothiophate completely inhibited BChE activity in wild-type plasma, but only partially inhibited BChE activity in transgenic mouse plasma. The echothiophate resistant activity was attributed to G117H BChE. Transgenic mouse plasma contains both wild-type mouse BChE and human G117H BChE; the amount of each type of BChE was calculated from the slopes. Plasma from transgenic mouse M816T had a slope of 0.0609 per min before echothiophate, and a slope of 0.0189 per min after echothiophate addition. This represented 1.2 $\mu\text{g/ml}$ wild-type BChE and 4.3 $\mu\text{g/ml}$ G117H BChE. Wild-type mouse plasma had a slope of 0.0805 per min before echothiophate and a slope of 0.0003 per min after echothiophate. This represents 2.7 $\mu\text{g/ml}$ wild-type BChE in wild-type mouse plasma.

Nondenaturing PAGE stained for G117H BChE activity

Another assay for OP resistant BChE was electrophoresis on a nondenaturing polyacrylamide gel followed by staining for BChE activity. The left side of Figure 3.3.3 shows that human and mouse serum had similar patterns of BChE activity. The most intense band was a tetramer. Weak dimer and monomer bands were present. The right side of Figure 3.3.3 shows that preincubation of the gel with 0.1 mM echothiophate completely inhibited BChE activity in human and wild-type mouse serum, but not in serum from the transgenic mouse. The G117H BChE retained activity in the presence of echothiophate.



Figure 3.3.3. Nondenaturing PAGE assay for G117H BChE in mouse serum. A nondenaturing 4-30% polyacrylamide gradient gel was stained for BChE activity with butyrylthiocholine by the method of Karnovsky and Roots (Karnovsky and Roots, 1964). The gel on the left was stained in the absence of inhibitor. The gel on the right was stained after preincubation in 0.1 mM echothiophate. The wild-type mouse BChE and human BChE completely disappeared after echothiophate incubation, but the G117H BChE tetramers in M816T mouse serum retained strong activity.

Mouse BChE has 574 amino acids and 7 N-linked carbohydrate chains per subunit (Rachinsky et al., 1990) for a subunit molecular weight of 81,000. Human BChE has 574 amino acids, plus 8 amino acids from the FLAG epitope, and 9 N-linked carbohydrate chains, for a subunit molecular weight of 85,000 (Lockridge et al., 1987). These small differences in molecular weight did not affect the migration of the BChE tetramers; human and mouse tetramers had similar mobilities. The echothiophate resistant tetramer had two bands in Figure 3.3.3. It is unknown whether these two bands represent glycoforms, or possibly tetramers made up of mouse and human BChE subunits.

Phenotyping transgenic mice

The assay described in Figure 3.2.2 was used to identify expression levels of G117H BChE in transgenic mice. Plasma samples from all pups in a litter were tested for G117H BChE activity by assaying the rate of hydrolysis of 1 mM butyrylthiocholine in the presence of 0.1 mM echothiophate. The F1 generation resulted from the mating of a founder mouse with a wild-type mouse. The F2 generation was from brother-sister mating of animals with the highest G117H BChE activity in the F1 generation. The F3 generation was from brother-sister matings of the F2 generation, using animals with the highest G117H activity.

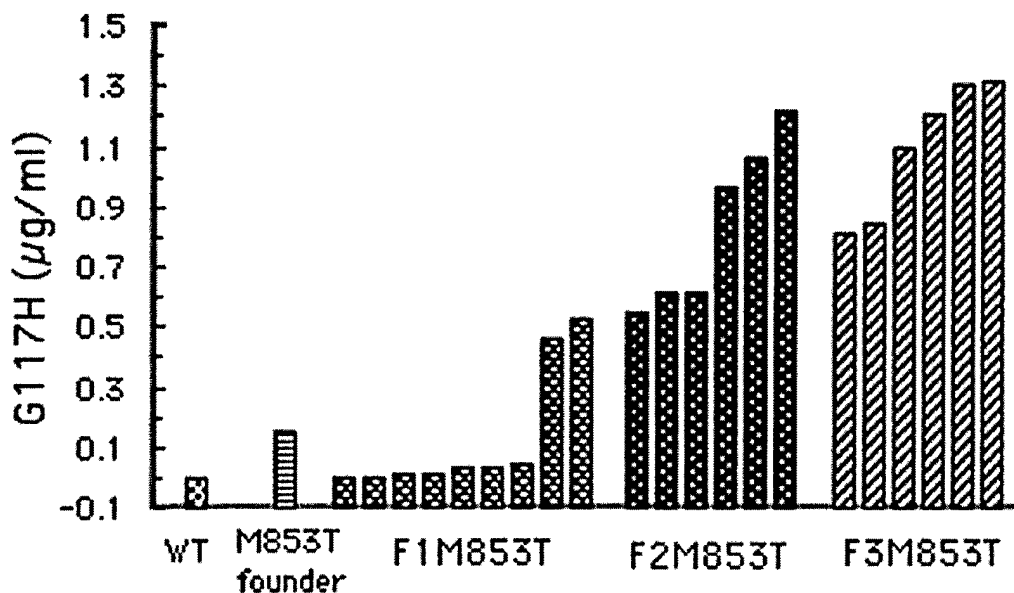
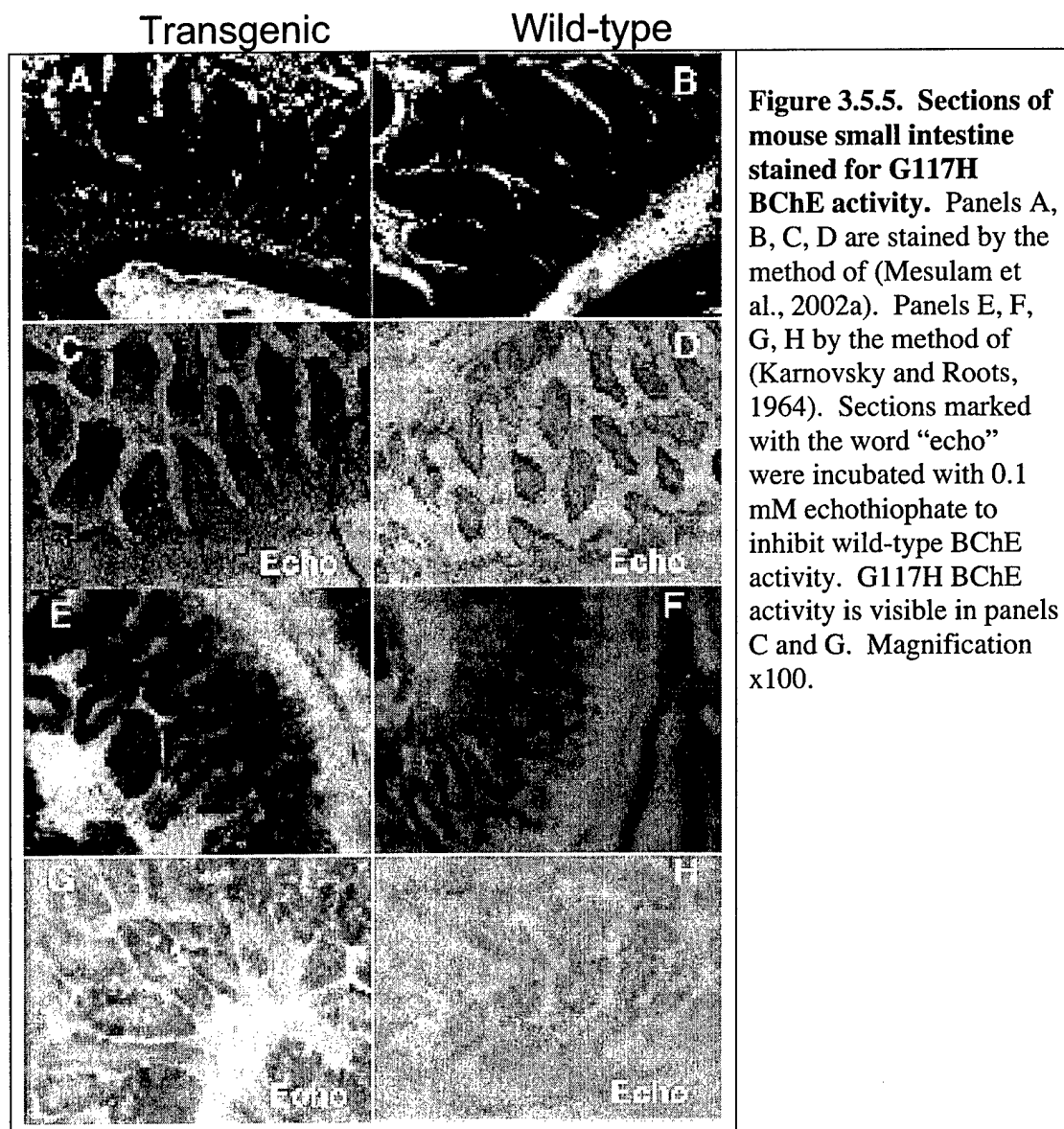


Figure 3.4.4. Screening mice for OP resistant G117H BChE activity. Transgenic founder mouse M853T was positive for the human G117H gene by PCR and had 0.15 µg/ml G117H BChE in plasma. The F1 generation from the mating of M853T founder with a wild-type mouse had 9 pups, two of which had 0.5 µg/ml G117H. These two were bred to produce the F2 generation. The two mice with the highest G117H activity in the F2 generation were bred to produce the F3 generation.

As can be seen in Figure 3.4.4, founder transgenic mouse M853T had low G117H BChE activity in his plasma. The 9 pups in the F1 generation had zero to 0.5 µg/ml G117H BChE. All 6 pups in the F2 generation had significant G117H BChE activity. The G117H BChE activity increased even further in the F3 generation. The level of G117H protein in the F3 generation reached a high of 1.3 µg/ml, which is close to the wild-type BChE level of 2 µg/ml in plasma.

Tissue sections

Sections of small intestine gave a very dark stain for BChE activity in the absence of echothiophate (Figure 3.5.5A and 5B), a result consistent with the high amount of BChE activity in mouse intestine (Sine et al., 1988; Li et al., 2000). To allow visualization of structures in the intestine, sections were also stained by a less sensitive method (Figure 3.5.5E and 5F). In uninhibited sections the wild-type BChE activity was present in the epithelial cells of the villi (Figure 3.5.5E and 5F). In sections inhibited with echothiophate, the G117H BChE activity was also in the epithelial cells of the villi (Figure 3.5.5C and 5G). The contrast between transgenic and wild-type sections is best seen in Figures 3.5.5C and 5D where significant BChE staining is still present in the transgenic mouse (5C) but not in the wild-type mouse (5D).



Discussion

Methods for determining OP resistance

No mammal has been found with hereditary OP resistance acquired either by natural selection after exposure to OP, or by genetic engineering. The single exception is the transgenic mouse in this report. The G117H BChE transgenic mouse is not intoxicated by a dose of echothiophate, 0.1 mg/kg sc, that is lethal to wild-type mice (Wang et al., 2004). Most published methods for measuring OP resistance have been developed for studies on insects. Resistance to OP insecticides has been reported in the fruit fly,

house fly, aphids, mosquitoes, Colorado potato beetle, German cockroach, greenbug, lesser grain borer, and sheep blowfly. A microtiter plate assay comparing AChE activity in the presence and absence of insecticides was used by Moores et al. (Moores et al., 1988) to establish resistance profiles for individual *Musca domestica*. Oppenoorth (Oppenoorth, 1982) treated house-fly extracts with paraoxon and then measured residual AChE activity. The methods in these two publications most closely resemble the methods in the present report.

A laboratory that has access to specialized equipment could automate the method for measuring cholinesterase activity (Lassiter et al., 2003). Vertebrate animals show species differences in susceptibility to OP toxicity (Fulton and Key, 2001; Worek et al., 2002), but these differences are innate to the species and have not been shown to be an adaptation to OP exposure. Only insects have adapted to OP by mutating their genes.

OP resistant AChE

The HFQT mutant is the first mammalian AChE with OP resistance. Previous attempts to mimic the G117H BChE mutation by changing residues in the oxyanion hole and acyl binding pocket of human AChE have led to an AChE with no catalytic activity. The crystal structure of human BChE (Nicolet et al., 2003) allowed a better understanding of what residues needed to be changed in AChE to accommodate a histidine side chain at position 117 (position 122 in snake AChE).

Many insects have been reported to have OP resistant AChE (Fournier and Mutero, 1994). Their OP resistance is attributed to decreased binding affinity for OP or to overexpression of AChE. No insect AChE with OP hydrolase activity is known. In many cases the OP-resistant insect AChE is resistant to specific OP rather than to the entire class of OP insecticides. For example the AChE of the Colorado potato beetle is resistant to azinphos-methyl but not to paraoxon (Zhu and Clark, 1995).

OP hydrolase activity as a mechanism of OP resistance

The assays in this report do not identify the mechanism of OP resistance. An enzyme that resists inhibition by OP could have reduced binding affinity for the OP, or the ability to hydrolyze OP, or both. To date there are only four examples where reduced binding affinity and OP hydrolase activity are present in the same enzyme: the human G117H BChE and G117H/E197Q BChE mutants, the sheep blowfly G117D carboxylesterase mutant, and the house-fly G117D carboxylesterase mutant (Millard et al., 1995; Lockridge et al., 1997; Newcomb et al., 1997; Millard et al., 1998; Claudianos et al., 1999).

A search for mutants capable of hydrolyzing OP could begin by screening for OP resistance. OP hydrolase activity would be tested later when larger amounts of enzyme are available. Lockridge et al. (Lockridge et al., 1997) used the Ellman assay to demonstrate hydrolysis of echothiophate, and measured absorbance increase at 400 nm to show hydrolysis of paraoxon by G117H BChE. Millard et al. (Millard et al., 1995; Millard et al., 1998) measured residual sarin, soman and VX to show that G117H BChE consumed these nerve agents. Hydrolysis of a radiolabeled OP, C14-chlorfenvinphos, was used to demonstrate OP hydrolase activity in the sheep blowfly *Lucilia cuprina* and *Musca domestica* mutants (Campbell et al., 1997; Claudianos et al., 1999).

Tetramers of human G117H BChE in mouse plasma

An unanswered question is whether tetramers of plasma butyrylcholinesterase are composed simply of 4 identical subunits or whether they include a tetramer organizing peptide. A proline rich organizing peptide is required for assembly of recombinant butyrylcholinesterase and acetylcholinesterase into tetramers (Bon and Massoulie, 1997; Simon et al., 1998; Altamirano and Lockridge, 1999; Kronman et al., 2000). The tetramer organizing peptide, PRAD, derives from the N-terminus of the collagen tail (Bon et al., 1997; Krejci et al., 1997). A different proline rich membrane anchor, PRiMA, organizes AChE and BChE into tetramers and serves to anchor the tetramers to brain and muscle membranes (Perrier et al., 2002). To date no evidence has been obtained to support the presence of a tetramer organizing peptide within the native BChE tetramer from serum. However, the possibility has not been ruled out.

The transgenic G117H mouse contains the gene for human BChE but does not contain the gene for human PRAD or human PRiMA. The human G117H BChE in mouse plasma was a tetramer. This shows that mouse liver, where serum BChE is produced, has the necessary machinery for assembling human BChE subunits into tetramers and exporting the tetramers into mouse blood. If a proline rich peptide is present in the tetramer, it has to be the mouse version.

Transgenic mice expressing OP resistant BChE

The transgenic mice expressing G117H BChE under the control of the ROSA26 promoter are the first transgenic mice to express human BChE activity. The transgenic mice made by Beerli et al. (Beerli et al., 1994) had no detectable human BChE activity. The assays for OP resistance developed in the present report allowed us to identify mice that expressed human G117H BChE and to quantify the level of expression of the transgene.

Task 4. Gene therapy with AChE.

4.1 Human AChE cDNA will be cloned into a shuttle vector. The linearized shuttle vector and pAdEasy-1 will be cotransfected into bacteria to allow homologous recombination. Colonies resistant to kanamycin will be screened by restriction endonuclease digestion.

4.2 The adenoviral vector containing human AChE, pAd-ACHE, will be linearized and transfected into 293 cells. Virus production will be visualized by fluorescence of green fluorescence protein and by measuring AChE activity.

4.3 Viral stocks will be amplified in 293 cells to obtain 10^{11} to 10^{12} plaque forming units. The virus will be purified in preparation for injection into mice.

4.4 Mice will be injected intravenously with various doses of adenoviral vector. The site of localization of the adenovirus will be determined. Expression levels of AChE will be determined. The duration of expression of AChE will be measured.

Tasks 4.1, 4.2, 4.3, and 4.4

Delivery of human acetylcholinesterase by adeno-associated virus to the acetylcholinesterase knockout mouse

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Prepared for submission to BioMedCentral

Abstract

Background

The purpose of this work was to provide a gene delivery system that expressed acetylcholinesterase (AChE) for prolonged periods. An adeno-associated virus expressing human AChE was constructed by cotransfecting three plasmids into HEK 293T cells. The purified virus expressed 0.17 μg AChE per 1 million viral particles in culture medium in 23 hours, or 0.8 units/ml. The virus was injected into muscle of adult AChE knockout mice and into the brains of 3-6 week old AChE knockout mice.

Results

Intramuscular injection yielded plasma AChE levels approaching 50% of the AChE activity of wild-type mouse plasma. The highest AChE activity was found on day 3 post-injection. AChE activity declined thereafter to a constant 7% of normal. The decreased level was explained by partial clearance of AChE from plasma by anti-human AChE antibodies. Intrastratial injection resulted in AChE expression near the needle track. No

antibodies were detected in animals treated intrastrially. Motor coordination was improved. The lifespan of intrastrially-treated AChE knockout mice was prolonged.

Conclusion

Human AChE was expressed in mouse plasma for up to 5 months. Gene-therapy to supply AChE to the striatum prolonged the life of mice genetically deficient in AChE, by reducing their susceptibility to spontaneous seizures. This supports the hypothesis that their seizures are induced by excess acetylcholine.

Background

AChE is present in most tissues and is abundant in brain and muscle. Its function at cholinergic nerve synapses is to terminate nerve impulse transmission by hydrolyzing the neurotransmitter acetylcholine. AChE has no known function in plasma though it is present in mouse plasma at significant concentrations (Li et al., 2000). AChE has several isoforms, the most abundant of which is the soluble tetramer encoded by exons 2, 3, 4, and 6. The splice form encoded by exons 2, 3, 4, and 6 is found in brain in a soluble form as well as anchored to membrane through the PRiMA protein (Perrier et al., 2002). This splice form is also found in muscle as a soluble form as well as anchored to the basal lamina in the neuromuscular junction through the collagen subunit COLQ (Krejci et al., 1997; Feng et al., 1999). The AChE form encoded by exons 2, 3, 4, and 6 was expressed in this report in an adeno-associated virus vector.

The AChE knockout mouse has no AChE enzyme activity and no AChE protein in any tissue. Despite the complete absence of this important component of the cholinergic nervous system, mice are born alive (Xie et al., 2000). They are kept alive to adulthood by feeding a liquid diet (Duysen et al., 2002c). They have weak muscles, uncoordinated movements, and they die from spontaneous seizures at an average age of 4 months. Our goal was to determine whether their phenotype could be improved by gene therapy with adeno-associated virus expressing AChE.

Materials and Methods

Vector construction. Plasmid pAAV/hAChE for expression of human AChE was constructed. Briefly, 1.9 kb of human AChE cDNA, encoding 31 amino acids in the signal peptide, 583 amino acids of the full-length AChE subunit (accession number NM 015831), and 8 amino acids of the FLAG epitope was cloned into the pAAV-MCS plasmid (catalog #240071 AAV Helper-Free System, Stratagene, La Jolla, CA). The vector contains the CMV promoter and beta-globin intron for high-level expression in mammalian cells. XL10-Gold[®] ultracompetent cells (catalog #200314, Stratagene) were used to amplify plasmid pAAV/hAChE. It was essential to use XL10-Gold cells to avoid unwanted recombination through the inverted terminal repeat sequences. The pAAV/hAChE plasmid was sequenced to verify the integrity of the inverted terminal repeats and to confirm the AChE sequence. Exons 2, 3, 4, and 6 encoding the most abundant splice variant of AChE, the T-form, were present. The plasmid has a total of 6512 base pairs. Figure 4.1.

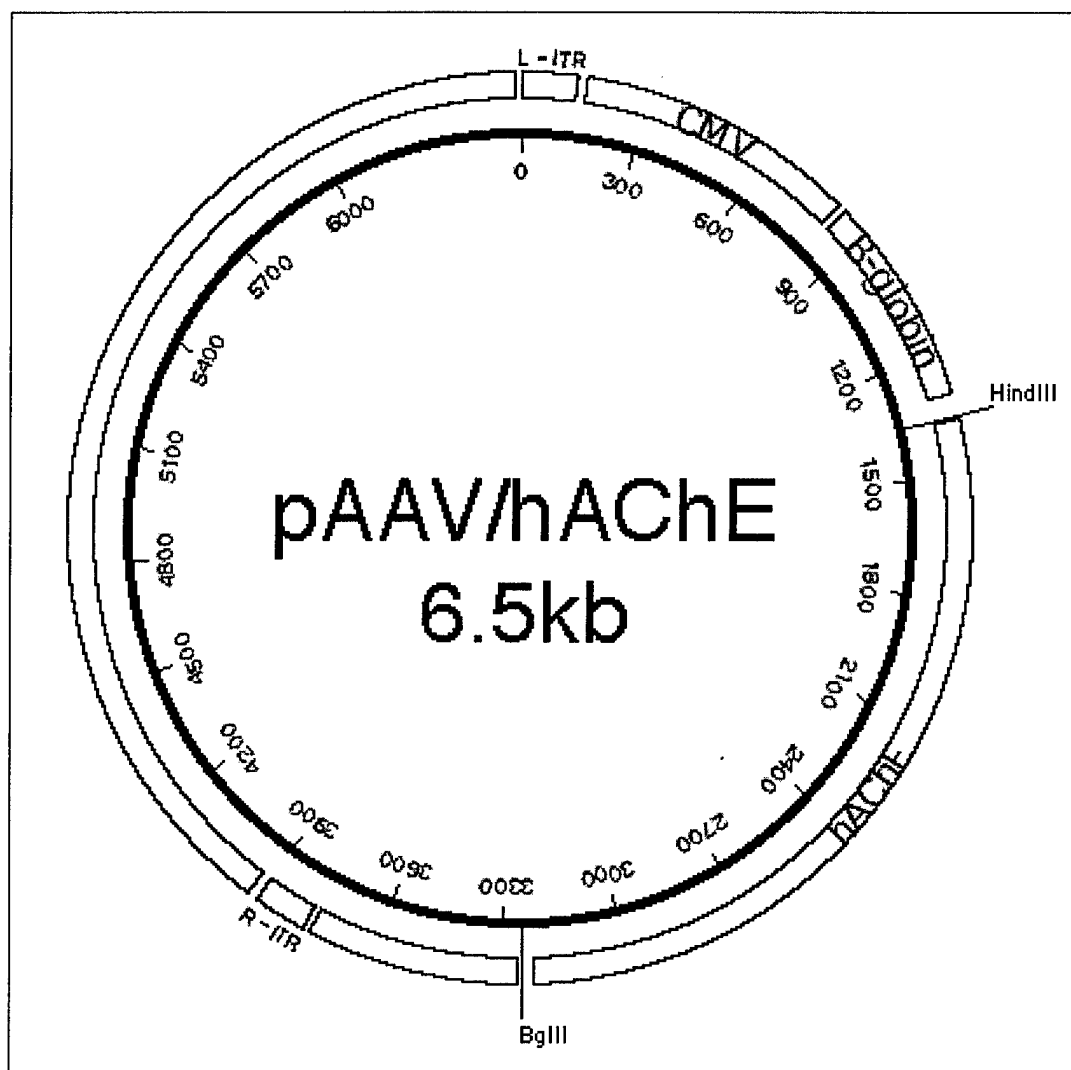


Figure 4.1. Schematic diagram of the plasmid encoding human AChE and the inverted terminal repeats of adeno-associated virus. The CMV promoter is followed by the β -globin intron and exons 2, 3, 4, and 6 of human AChE. A FLAG epitope is at the 3' end of AChE.

Virus preparation. rAAV virus of serotype-2 was produced and purified using procedures established in Samulski's laboratory (Xiao et al., 1998; Haberman et al., 1999; Amiss and Samulski, 2001). Viral particles were prepared by adenovirus-free cotransfection of HEK 293T cells with three plasmids: plasmid pAAV/hAChE, plasmid pHelper (carrying adenovirus derived genes) and plasmid pAAV-RC (carrying AAV-2 replication and capsid genes). These three plasmids together supplied all the trans-acting factors required for AAV replication and packaging in HEK 293T cells. Plasmids AAV-MCS, pHelper, and pAAV-RC were from Stratagene.

Cells were grown in twenty 15-cm dishes in 25 ml of DMEM/10% FBS per dish. Three hours before cotransfection, when cells were 70-80% confluent, the medium was replaced with Iscove's Modified Dulbecco's Medium (IMDM) containing 10% FBS to maintain a constant pH during transfection. Cells were cotransfected with 30 µg of DNA per plate (10 µg of each plasmid) by the calcium phosphate coprecipitation method. The IMDM/10% FBS was replaced with DMEM/2% FBS seven to eight hours after transfection. Incubation was continued for another 64 hours before medium and cells were harvested for purification of virus.

Purification of adeno-associated virus. Culture medium and cells were harvested 72 hours post-transfection (Amiss and Samulski, 2001). Virus was released from the cells by three cycles of freezing and thawing, and by sonication of cell debris. 500 ml of viral suspension was partially purified and concentrated by ammonium sulfate precipitation. 25 ml of viral suspension was overlaid on a cesium chloride gradient (Haberman et al., 1999) and centrifuged for 48 h at 288,000 x g (41,000 rpm in a Beckman SW-41 Ti rotor). The tube was punctured 1 cm from the bottom and 0.5 ml fractions collected. Fractions were frozen at -80°C. After determination of viral titer, the virus was desalted and concentrated in Amicon Ultra centrifugal filter device 100,000 molecular weight cut-off (catalog #UFC910002, Millipore Corp., Bedford, MA). The 1-3 ml of purified virus in phosphate buffered saline was filter sterilized (catalog #UFC30GV25, Durapore 0.22 µm, Millipore), divided into 300 µl aliquots, and frozen at -80°C. The desalted virus was titered again expression of AChE enzyme activity.

A second batch of virus was produced and purified at the Vector Core Lab of the University of North Carolina Gene Therapy Center using the same pAAV/hAChE plasmid. Virus was made by adenovirus-free triple cotransfection of HEK 293 cells and purified by iodixanol gradient centrifugation followed by heparin column chromatography.

Viral titer. Two methods were used to determine viral titer. The dot blot method quantified viral DNA by comparing the intensity of labeling with a P32 labeled, 947 bp probe for human AChE against known quantities of plasmid pAAV/hAChE.

A second method measured viral infectivity and AChE expression. HEK 293T cells were plated in a 6-well plate in DMEM with 10% heat-inactivated FBS (Gibco #16140-071), at 3 ml culture medium per well. Heat-inactivated FBS was used because heat destroys the activity of bovine AChE in FBS. When cells were 70-80% confluent, they were infected with 1 to 10 µl of purified viral particles. Culture medium was harvested 23 hours later. Enzymatic AChE activity in the culture medium was measured by the Ellman (Ellman et al., 1961) method adapted to a 96-well plate. 10 µl of culture medium was assayed in 200 µl reaction mixture containing 0.1 M potassium phosphate pH 7.0, 0.5 mM DTNB, and 1 mM acetylthiocholine. The reaction was started by adding acetylthiocholine. Absorbance increase at 412 nm was read in a microtiter plate reader (Molecular Devices SpectraMax 190) for 20 minutes. The change in absorbance per min was converted to µmoles acetylthiocholine hydrolyzed per min by using the molar extinction coefficient 6,800 M⁻¹ for the 0.5 cm pathlength. Units per ml of AChE activity were converted to µg AChE protein per ml using the

specific activity of 5,200 units/mg for pure AChE (Rosenberry and Scoggin, 1984). Control assays measured AChE activity in culture medium from uninfected cells.

AChE knockout mice. Animal work was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques when applicable. Mice with no AChE activity in any tissue were the subjects for gene therapy trials. AChE^{-/-} mice were made by gene-targeting (Xie et al., 2000) and raised to adulthood on a liquid diet of Ensure (Duysen et al., 2002c). AChE^{-/-} mice do not breed. Therefore, the colony is maintained by breeding heterozygotes. The AChE^{-/-} mice have a strain 129Sv genetic background. Mice of both sexes were treated with rAAV/hAChE.

AAV delivery. 50 μ l of rAAV/hAChE virus (titer 510 μ g AChE/ml in the expression assay) was injected into each hind leg tibialis anterior muscle of adult AChE^{-/-} mice, for a total of 100 μ l virus per mouse. 12 adult (2 to 3 months old) AChE^{-/-} mice were injected intramuscularly.

10 AChE^{-/-} mice (ages 17-44 days) were anesthetized with 20-30 μ l/g Avertin i.p. Some animals required isoflurane inhalation because the depth of anesthesia as determined by the tail pinch test was inadequate with Avertin alone. The striatum was injected bilaterally with 2.5 μ l of rAAV/hAChE (titer 510 μ g AChE/ml). The striatum of an additional 6 AChE^{-/-} mice (all 20 days old) was injected with phosphate buffered saline. Mice completely recovered from anesthesia within one hour. During the recovery period they were placed on a heating pad. Mice were weighed and their temperature measured daily after surgery to detect infection.

Collection of blood. Blood samples were collected from the saphenous vein in the hind leg into heparinized capillary tubes. Plasma was separated from red blood cells by centrifugation.

Tissue extraction. 3 adult mice were euthanized 3 days after being injected i.m. with rAAV/hAChE. Mice were perfused transcardially with phosphate buffered saline to wash out the blood. Perfused tissues were homogenized in 10 volumes of ice-cold 50 mM potassium phosphate pH 7.4, 0.5% Tween 20 in a Polytron (Brinkmann Instruments) for 10 seconds. Cell debris was removed by centrifugation at 12,000 rpm in a microfuge at 4°C. Plasma and tissue extracts were assayed for AChE activity.

Ellman assay for AChE activity. 4 μ l plasma was preincubated with 0.5 μ M bambuterol in 2 ml of 0.1 M potassium phosphate pH 7.0, 0.5 mM DTNB for 20 min to inhibit butyrylcholinesterase (BChE). Bambuterol hydrochloride was a gift from Dr. Leif Svensson, Astra Draco, Lund, Sweden (Tunek and Svensson, 1988). Assay of AChE activity was begun by adding acetylthiocholine to a final concentration of 1 mM. Units of activity are μ moles acetylthiocholine hydrolyzed per min. Units of activity were calculated using the extinction coefficient 13,600 M⁻¹ cm⁻¹ (Ellman et al., 1961).

Radiometric assay for AChE activity. The Johnson and Russell (Johnson and Russell, 1975) method was used. 5 μ l of plasma diluted to 80.5 μ l with phosphate buffer, or 80.5 μ l of tissue extract was preincubated with 0.5 μ M bambuterol for 30 min to inhibit BChE activity. This resulted in 97% inhibition of BChE activity. The reaction was started by adding C14-acetylcholine to a final concentration of 1 mM. Each reaction contained 0.05 microCuries (Catalog #1711103, ICN Radiochemicals, Costa Mesa, CA) in a total volume of 0.1 ml. The reaction was stopped after 50 minutes by adding 0.1 ml of stopping mixture that brought the pH to 2.5. The ^{14} C -acetate was extracted into 4 ml of scintillation cocktail and counted. The scintillation cocktail was prepared by mixing 8 ml of rpi scintillator (#111023 ppo-dimethyl-popop concentrated liquid scintillator, Research Products International, Mount Prospect, IL) with 33.3 ml of iso-amyl alcohol and 159 ml toluene.

A set of positive and negative controls was included in each assay. The positive controls were 1, 5, and 10 μ l of wild-type plasma (n=4). The negative controls were 5 μ l of plasma from untreated AChE^{-/-} mice (n=5).

Activity stained gel to visualize AChE. Nondenaturing 0.75 mm thick, 4-30% gradient polyacrylamide gels were prepared in a Hoefer apparatus. 3 μ l plasma was treated with 1 mM iso-OMPA for 60 min before being loaded on the gel. Iso-OMPA is a specific BChE inhibitor. The upper buffer contained 600 ml of 0.021 M Tris base, 0.023 M glycine pH 9.0; the lower buffer contained 4.5 L of 0.06 M TrisCl pH 8.1. Electrophoresis was at 4°C constant voltage for 5600 volt hours. Gels were stained for AChE activity in the presence of 1.7 mM acetylthiocholine iodide (Karnovsky and Roots, 1964).

Antibody detection. To detect serum antibodies reactive with human AChE, an enzyme-linked immunosorbent assay (ELISA) was performed using Nunc-Immuno Module (Nalge Nunc International) immunoassay plates coated with purified human AChE. Each well of a 96-well plate was coated with 1 μ g of purified human AChE diluted to 200 μ l with buffer. Human AChE was produced by expression of AChE in CHO cells. The secreted AChE was purified from culture medium on procainamide-Sepharose affinity gel. Plates were blocked with 3% bovine serum albumin in phosphate buffered saline, 0.05% Tween-20 overnight at 4°C. 2 μ l and 4 μ l of mouse plasma or tissue extract diluted to 200 μ l were added to each well and incubated for 3 hours at room temperature. Plates were washed three times with phosphate buffered saline, 0.05% Tween-20 and then incubated with goat anti-mouse IgG conjugated to horse radish peroxidase (1:2000 dilution) overnight at 4°C. Plates were washed three times with phosphate buffered saline, 0.05% Tween-20 and then developed to reveal bound horseradish peroxidase. 0.4 mg/ml o-phenylenediamine dihydrochloride (Sigma) was dissolved in 0.05 M phosphate-citrate pH 5.0. Immediately before use, 10 ml of the 0.4 mg/ml substrate solution was mixed with 4 μ l of 30% H₂O₂. 200 μ l of this mixture was added to each well. After 5 minutes, the reaction was stopped by adding 50 μ l of 3 M H₂SO₄. The absorbance at 492 nm was read on a microtiter plate reader.

Positive control antibodies were Mab304 (Chemicon International, Inc., Temecula, CA), a monoclonal generated against human red cell AChE (Fambrough et al., 1982) and Mab123 a gift from Dr. Steven Brimijoin, Mayo clinic, raised against

human red cell AChE (Brimijoin et al., 1983). Positive control antibodies were diluted 1:100 – 1:2500. Negative controls included 200 µl of diluted plasma from uninjected AChE-/- mice, 200 µl from uninjected wild-type mice, 200 µl of phosphate buffered saline containing 0.05% Tween-20, and uncoated wells.

Brain sections. Mice were euthanized with CO₂, and then perfused transcardially with 30 ml PBS followed by a 40 ml mixture of 2% paraformaldehyde and 2% glutaraldehyde in PBS. The brain was removed and stored in 15% sucrose in PBS at 4°C over night. Brains frozen on dry ice were sectioned on a cryostat into 40 micron sections and placed on Superfrost/Plus slides (Fisher Scientific, #12-550-15).

Staining brain sections for AChE activity. Brain sections were stained for AChE activity by the method of Tago et al. (Tago et al., 1992). Frozen sections were rinsed 3 times in 0.1 M maleate buffer pH 6.0 and then placed into 0.1 M maleate buffer pH 6.0 containing 5 mM potassium ferricyanide, 30 mM cupric sulfate, 50 mM sodium citrate, and 1 mM iso-OMPA. A 25 min preincubation with iso-OMPA at room temperature served to inhibit BChE activity. Color was developed by adding 0.286g acetylthiocholine iodide to 60 ml buffer to make 18 mM acetylthiocholine. Slides were stained for 10 min. Longer incubation times made the background dark. No enhancement was performed. Slides were washed in 50 mM TrisCl pH 7.4 and stored in this buffer until photographed on a Nikon Optiphot-2 microscope with a Hitachi HV-C20 camera with Optimas 5.23 software.

Inverted screen test. The inverted screen test was used to measure muscle coordination. Mice were placed on a horizontal screen. Then the screen was rotated 90° and the time the mice remained on the screen was measured. The test was videotaped to facilitate measurement of time. Each mouse was tested three times per session on days 3, 14, 19 and 28 after virus injection. The longest time was chosen.

Climbing test. Animals were placed on a screen set at a 45° angle, with the head pointed toward the floor. The time it took the mouse to turn around and climb to the top of the 15 cm screen was measured. Each mouse was tested 3 times and the results were averaged.

Other behavioral tests. Many of the behavioral tests are from McDaniel and Moser (McDaniel and Moser, 1993). The following behaviors were observed and quantified in control and AAV treated AChE-/- mice: body posture, body tremor, vocalization, mobility, distance traveled in an open field, rearing, gait, arousal, repetitive movements, involuntary movements, tail pinch response, click response, touch response, geotaxis, beam walking, learning maze, pupil response, landing foot splay, righting reflex, eye blink in response to touch, awareness of cliff, cross extensor response. Surface body temperature and body weight were measured.

Statistics. An independent samples T-test assuming equal variances was run on tests results using the Excel program in Microsoft Office. P values below 0.05 were considered significant.

Results

Comparison of viral titer by two methods. Virus prepared in our laboratory, rAAV/hAChE, and by the Vector Core facility at the University of North Carolina Gene Therapy Center, rAAV/hAChE (UNC) was titered by dot blot and by viral infectivity and expression. The rAAV/hAChE had a titer of 3×10^9 viral particles per ml in the dot blot assay and produced 510 μg AChE/ml in the expression assay. The value 510 μg AChE/ml was calculated from the experimental observation that 1×10^6 viral particles expressed 0.17 μg AChE per ml culture medium in 23 hours (0.88 units/ml). The rAAV/hAChE(UNC) had a titer of 1×10^{12} viral particles per ml in the dot blot assay and produced 20 μg AChE/ml in the expression assay. The value of 20 μg AChE/ml was calculated from the observation that 1×10^6 viral particles expressed 0.00002 μg AChE per ml culture medium in 23 hours. The rAAV/hAChE expressed 8,500 times more AChE enzyme per viral particle than the rAAV/hAChE (UNC). This result shows that the dot blot viral titer was not a reliable predictor of AChE enzyme expression.

Virally expressed human AChE in plasma of adult AChE^{-/-} mice. 12 adult AChE^{-/-} mice (2-3 months old) were injected i.m. with 100 μl of rAAV/hAChE. The viral titer was 510 μg AChE per ml, as measured in the expression assay. Blood was collected on days 0, 3, 6, 9, 14, and 30 post-injection from 7 animals, on day 150 from 2 animals, and on days 185-240 from 5 animals. The plasma was tested for AChE activity in the Ellman assay (Ellman et al., 1961). Figure 4.2 shows that AChE^{-/-} plasma had no AChE activity before injection of virus but acquired 0.27 ± 0.16 units/ml of AChE activity on day 3 after rAAV/hAChE delivery. This level of AChE activity is 47% of the activity in wild-type mouse plasma. Wild-type mice have 0.57 ± 0.06 units/ml AChE activity in plasma. AChE activity dropped significantly by day 6 to 0.039 units/ml and persisted unchanged for 30 days. Two mice tested on day 150 were found to express a low level of AChE, suggesting that the virus continued expressing AChE for at least 150 days.

The presence of AChE activity in plasma of AChE^{-/-} mice treated i.m. with rAAV/hAChE was confirmed by Karnovsky and Roots activity staining of a nondenaturing polyacrylamide gel (Karnovsky and Roots, 1964). The gel (Figure 4.3) separates AChE from butyrylcholinesterase (BChE) and therefore makes clear that the acetylthiocholine hydrolysis activity comes from the virally expressed AChE and not from BChE. The AChE^{-/-} mouse has no AChE activity, so all AChE activity must have come from the gene therapy vector.

The finding of tetramers of AChE in the virus-treated knockout mice shows that the virally expressed human AChE assembled into tetramers. No exogenous administration of a polyproline rich peptide was required to get assembly into tetramers.

Two adult AChE^{-/-} mice (133 days old) received rAAA/hAChE(UNC) i.m., but this viral preparation yielded no AChE expression.

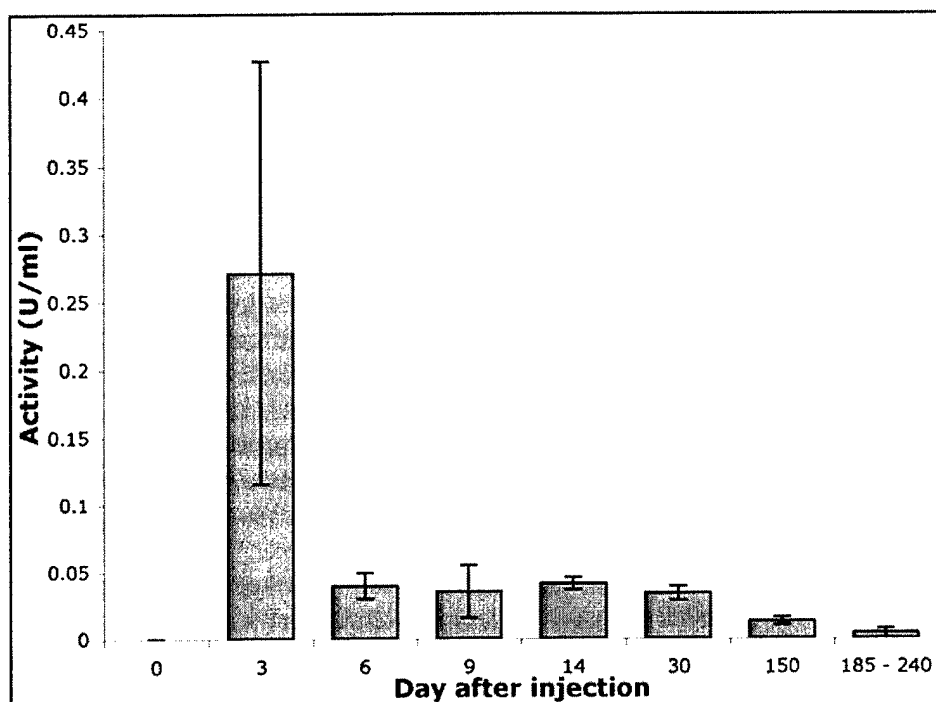


Figure 4.2. AChE activity in plasma of AChE^{-/-} mice after rAAV/hAChE delivery i.m. Plasma from 7 adult AChE^{-/-} mice was tested for AChE activity in the Ellman assay on days 0, 3, 6, 9, 14, 30, 150, and 185-240 post-injection. Day 0 values show zero AChE activity. AChE^{-/-} mice were 2-3 months old when they received the virus. Untreated wild-type plasma has 0.57 units/ml of AChE activity.

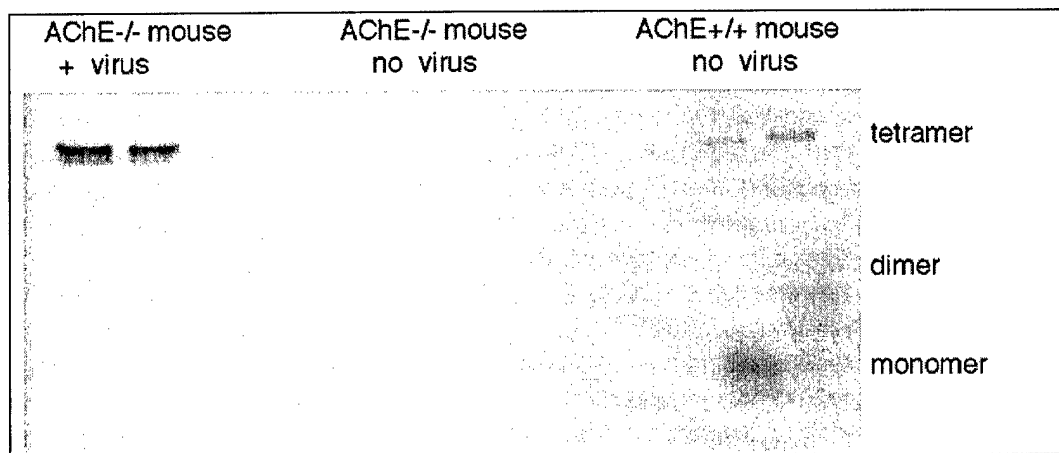


Figure 4.3. Nondenaturing gel to show AChE activity in virus-treated AChE^{-/-} mice. Plasma from 2 AChE^{-/-} mice treated with rAAV/hAChE, 3 untreated AChE^{-/-} mice, and 2 untreated wild-type mice was subjected to gel electrophoreses to separate AChE from BChE. The BChE activity was inhibited with iso-OMPA. AChE tetramers were visible in plasma of virus-treated AChE^{-/-} mice and in untreated wild-type mice. No AChE activity was present in untreated AChE^{-/-} mice. Wild-type mice had monomers, dimers, and tetramers of AChE.

Virally expressed human AChE in tissues. Three of the 12 adult AChE^{-/-} mice treated with rAAV/hAChE intramuscularly and 3 uninjected AChE^{-/-} mice were euthanized on day 3 post-injection. Tissue extracts from perfused mice were tested for AChE activity with the radiometric assay. Low AChE activity, less than 0.01 units/g, was found in kidney, spleen, heart, and stomach of mice that had been treated with virus. Activity in muscle near the site of injection was as high as 0.25 units/g on day 2, but as low as 0.008 units/g on day 7 after injection. Tissues tested for AChE activity a week or more after i.m. injection of virus, showed no significant AChE activity.

Histochemical staining for AChE activity in brains of AChE^{-/-} mice treated with rAAV/hAChE intrastrially revealed AChE activity in the striatum. The activity in brain persisted for months. Figure 4.4.

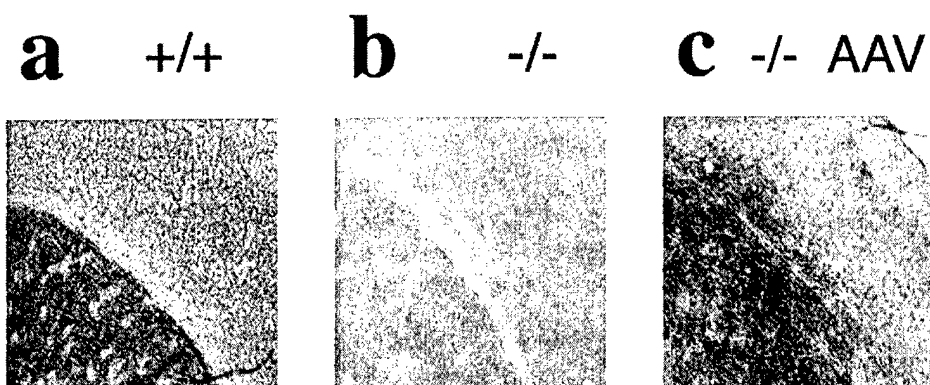


Figure 4.4. Brain sections stained for AChE activity. a) AChE activity in wild-type striatum and cortex; the striatum has more AChE activity than the cortex. b) absence of AChE activity in uninjected AChE^{-/-} brain. c) AChE activity in AChE^{-/-} brain injected with virus; AChE activity is mainly in the striatum (site of injection) with some diffusion into the corpus callosum and cortex. This mouse had received virus 4 weeks earlier. Magnification X40.

Production of antibodies against human AChE. Expression of AChE activity in plasma was found to be highest on day 3 post-injection i.m. and to decrease thereafter. The decrease in the level of human AChE activity led us to test the possibility that the AChE^{-/-} mouse had produced antibodies against human AChE. Mice were bled on days 0, 3, 6, 9, 14, 30, 150, and 185-240 post-injection and the plasma was tested for the presence of anti-human AChE antibodies. The ELISA assay results in Figure 4.5 show that antibody against human AChE was not present on day 0, but was present on all other days tested. The antibody level increased up to 30 days post-injection, suggesting that AChE continued to be expressed and continued to stimulate the immune system. Antibody level was still high on day 150 post-injection but had decreased by days 185-240. The presence of antibodies to human AChE explains the low level of AChE activity in mice treated with virus intramuscularly.

Two adult heterozygous AChE^{+/-} mice were injected with virus i.m. to see whether the presence of endogenous mouse AChE would prevent an immune response to human AChE. Mice were bled on day 5 post-injection and tested for anti-human

AChE antibody and for AChE activity. The adult AChE^{+/-} mice also formed antibodies against human AChE. No increase in AChE activity was found in the Ellman assay.

No antibody against human AChE was found in plasma of AChE^{-/-} mice when the virus was injected into the brain. Plasma was collected from animals on days 19 and 20 after intrastriatal injection. None of the plasma samples (n = 10) had antibodies to human AChE and none had AChE activity.

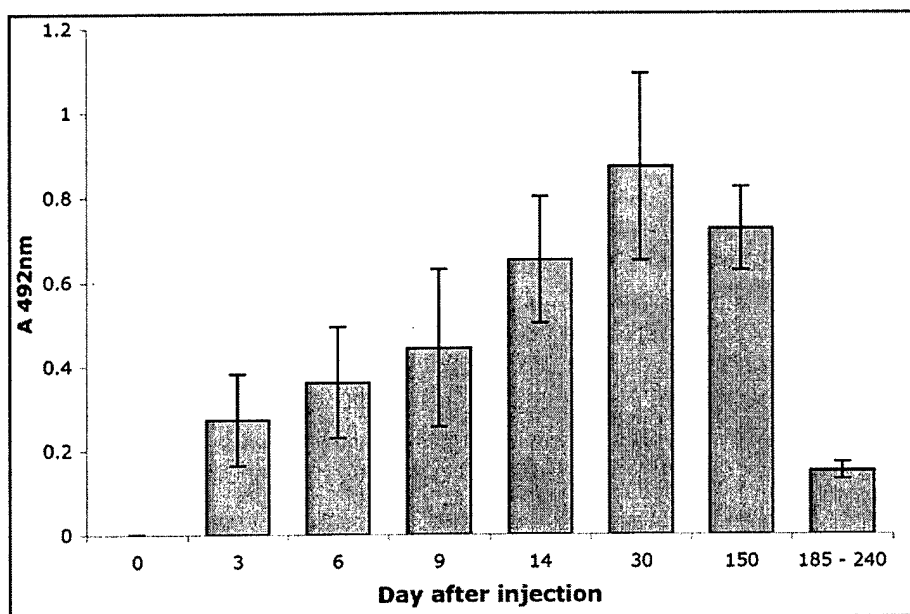


Figure 4.5. Antibody against human AChE after i.m delivery of virus into adult AChE^{-/-} mice. Plasma from 7 adult AChE^{-/-} mice injected intramuscularly with rAAV/hAChE was tested for the presence of anti-human AChE antibodies by ELISA. Absorbance at 492 nm is proportional to the amount of antibody.

Phenotype after i.m. injection. The adeno-associated virus injected intramuscularly into adult AChE^{-/-} mice had an effect on lifespan but not on behavior. The mice continued to show whole body tremor, abnormal body posture, splayed hind legs, twitching hind legs when the mouse was held by the investigator, and inability to eat solid food. No changes in the rate of gain in body weight, and no changes in body temperature were found. They still vocalized during cage changing. Their climbing and rearing activity continued to be lower than in normal littermates. They did not acquire grip strength, but fell off an inverted screen within seconds. They displayed no sexual activity and no animals became pregnant.

Phenotype after intrastriatal injection. Most behavioral tests showed no differences between virus treated and untreated knockout mice. However, two tests of motor coordination did show a difference. Gene therapy with rAAV/hAChE by intrastriatal injection improved motor coordination in AChE^{-/-} mice as measured on the inverted screen test. Before injection, no AChE^{-/-} mouse was able to stay on the screen, tilted at 90°, for more than 7 seconds. After intrastriatal injection the mice treated with virus gained the ability to stay on the screen for at least 20 seconds (range 20-60 sec) while mice injected with PBS never acquired this staying power (see Figure 4.6).

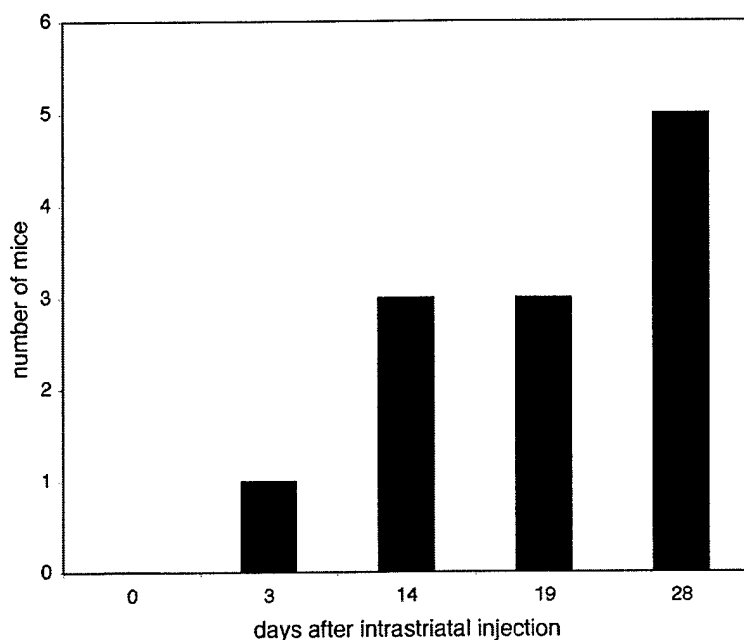


Figure 4.6. Inverted screen. Mice were placed on the screen and then the screen was rotated 90°. The time until mice fell off was measured. The number of mice that stayed on the screen 20 seconds or longer is reported. On day 28, 100% of the AChE^{-/-} mice treated intrastratially with rAAV/hAChE (n=5) stayed on the screen. None of the control AChE^{-/-} mice, treated intrastratially with PBS, stayed on the screen for 20 sec (n=6).

Wild-type mice stayed on the screen indefinitely. Intrastratial injection of virus had no deleterious effect on muscle coordination of wild-type mice.

The climbing test, measured as the length of time it took a mouse to turn around and climb to the top of a tilted screen, confirmed the conclusion that intrastratial delivery of rAAV/hAChE to AChE^{-/-} mice improved their motor coordination. Uninjected, or PBS injected, mice turned to face head up but they were unable to climb to the top within the 60 second cut-off time. The mice did not fall off the 45° screen but stayed near the bottom. In contrast, all treated mice climbed to the top in about 15 seconds. Figure 4.7.

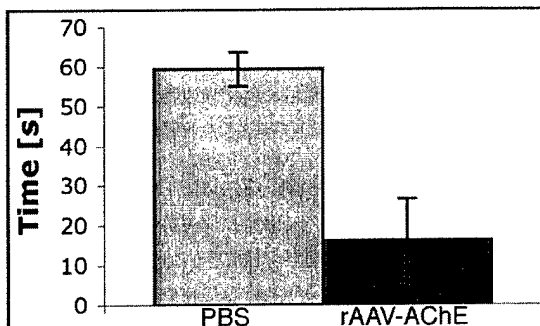


Figure 4.7. Climbing screen tilted 45 degrees. AChE^{-/-} mice (n=7) 4 weeks after injection of virus into the striatum were 60 days old at the time of the test. Control AChE^{-/-} mice (n=30) received PBS. Mice were placed head down on a screen tilted 45°. The time to turn around and climb to the top of the screen was measured. The statistical significance for the difference between the two groups is $p < 3 \times 10^{-9}$.

Survival after intrastriatal injection. Mice that received rAAV/hAChE in the striatum live longer. See Figure 4.8. AChE^{-/-} mice were 17 to 43 days old at the time of intrastriatal injection of virus. Of the 21 mice that were treated with virus, 16 were still alive on day 100 after injection. The saline injected animals died off more rapidly, so that only 50% were alive on day 100.

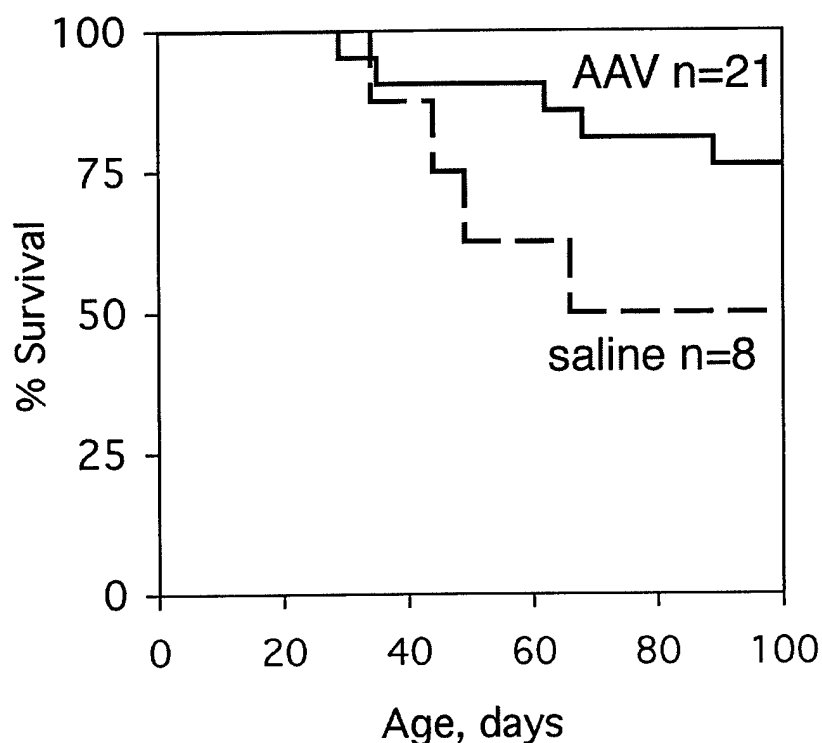


Figure 4.8. Survival after intrastriatal injection of rAAV/hAChE. AChE^{-/-} mice were 17-43 days old at the time of treatment. 21 AChE^{-/-} mice were injected with virus and 8 with saline.

Discussion

AChE activity. This is the first gene therapy vector and gene therapy protocol to report expression of human AChE. Adeno-associated virus gave long-term expression of human AChE. AChE activity was detected in tissues distant from the injection site. For comparison, AAV delivery of the α -gal A gene in Fabry mice led to the expression of α -gal A protein in all peripheral tissues (Park et al., 2003). These results contrast with reports where expression of β -glucuronidase was seen only near the site of injection of the virus (Elliger et al., 1999).

Antibodies. Exposure to organophosphorus pesticides is linked to immune system suppression (Repetto and Baliga, 1997). For example, parathion suppresses T-cell proliferative response and blocks both humoral and cell-mediated immune responses in vitro. Parathion has also been shown to reduce host resistance to viral and bacterial infection in live animals. A mechanism linking organophosphorus pesticides to immunotoxicity could involve inhibition of AChE since AChE is exquisitely sensitive to inhibition by organophosphorus agents, and since the consequence of AChE inhibition is a buildup of acetylcholine. The cholinergic anti-inflammatory pathway uses acetylcholine to reduce release of proinflammatory cytokines (Borovikova et al., 2000; Tracey et al., 2001)

If AChE inhibition leads to immunotoxicity, then it was possible that absence of AChE in AChE knockout mice could also result in immunotoxicity. We had previously reported that AChE^{-/-} mice, but not AChE^{+/-} and ^{+/+} mice, had an unusual response to infection by *Helicobacter hepaticus* (Duysen et al., 2002b). The AChE^{-/-} mice had severe bloating of the gastrointestinal tract. The esophagus, stomach, small intestine, large intestine, and colon were expanded several times their normal size by gas. Uninfected AChE^{-/-} mice in strain 129Sv had no bloating. The observation that AChE^{-/-} mice produced antibodies against human AChE provided evidence that the antibody producing machinery in these mutant mice was functioning.

Motor coordination. The brain was selected to receive rAAV/hAChE for two reasons: to avoid antibody production, and to increase the possibility that treatment with virus would improve the phenotype of AChE^{-/-} mice. The striatum has the highest number of cholinergic neurons in the brain, suggesting that this region would benefit most from expression of AChE. The striatum is involved in movement coordination, body posture, and tremor. The AChE^{-/-} mice have problems with movement coordination, body posture and tremor. It was found that expression of AChE in striatum had a positive effect on motor coordination, but not on body posture and tremor.

Lifespan prolonged. Untreated AChE^{-/-} mice die at an early age from seizures. A peak number of deaths are observed for animals in the age range 19-40 days. 33% of all AChE^{-/-} mice convulse and die before day 40. The average age of death in this age group is 27 ±5.6 days. Animals that survive this period generally live to about 120 days. A few AChE^{-/-} mice have lived as long as two years. Administration of rAAV/hAChE appears to be increasing the lifespan of AChE^{-/-} mice. The virally expressed AChE may have reduced the concentration of acetylcholine, and thus protected from acetylcholine induced seizures. Excess acetylcholine is known to initiate a cascade of reactions that lead to seizures and death (McDonough and Shih, 1997).

Conclusions

Gene-therapy with adeno-associated virus expressing human AChE prolongs the life of AChE knockout mice by reducing their susceptibility to seizures. The exogenously supplied AChE is hypothesized to reduce the concentration of acetylcholine and therefore to protect from acetylcholine-induced seizures.

More results related to

Tasks 4.1, 4.2, 4.3, and 4.4

prepared for submission to Neuroscience

Brain of acetylcholinesterase knockout mouse: normal thalamocortical projections, but 95% loss of striatal D1 dopamine receptors, and indicators of abnormal neuronal activity

Anna Hrabovska, Jeffrey D. Sanders, Ellen G. Duysen, Richard T. Robertson, L. Charles Murrin, Oksana Lockridge

Abstract

The acetylcholinesterase (AChE) knockout mouse has no AChE activity and no AChE protein. This makes it a suitable subject for determining roles that AChE may have in brain development and organization. Anterograde axonal tracing with a fluorescent carbocyanine dye demonstrated that the areal and laminar patterns of projections from the thalamus to the visual, auditory, and somatosensory cortical areas appear normal in AChE^{-/-} brains. These data indicate that the impaired vision and hearing characteristic of AChE^{-/-} mice are not explained by disturbances in fiber connectivity. While the AChE^{-/-} brain has normal choline acetyltransferase and butyrylcholinesterase expression levels, it has 50% fewer muscarinic receptors. An even more drastic change was found in dopamine receptor levels. Binding of [³H]SCH23390 to striatal membranes showed that D1-like dopaminergic receptors were decreased by 95% in AChE^{-/-} striatum, while binding affinity was unchanged. This may indicate a compensatory down regulation of D1 receptors to maintain synaptic balance in the striatum. Synaptic activity was assessed by imaging brains for activity-regulated cytoskeleton-associated (Arc) mRNA. In wild-type brains, Arc expression was high in the cortex, but in the AChE^{-/-} brain Arc was high in the striatum and piriform cortex. The AChE^{-/-} mouse is a model for brain responses to chronic treatment with anticholinesterase drugs, and indicates changes in molecular and synaptic characteristics, but not in fundamental morphological features. The adaptations in the AChE^{-/-} brain may be an extreme version of adaptations that occur in Alzheimer and Parkinson's disease patients chronically treated with anticholinesterase drugs.

Introduction

Acetylcholinesterase (EC 3.1.1.7) terminates nerve impulse transmission at cholinergic synapses by hydrolyzing the neurotransmitter acetylcholine. Because acute inhibition of AChE typically leads to death, it is remarkable that the AChE knockout mouse is viable (Xie et al., 2000; Duysen et al., 2002c; Mesulam et al., 2002b; Li et al., 2003). Aside from the acute effects of AChE inhibition on cholinergic transmission,

chronic absence of AChE might be expected to affect brain development and organization. Absence of AChE would be expected to lead to an excess of acetylcholine, and the presence of acetylcholine has been shown to influence neuronal maturation, including axon outgrowth and connectivity (Nguyen et al., 2001; Berger-Sweeney, 2003; Hohmann, 2003). Further, in addition to its well-known function in hydrolysis of acetylcholine, AChE is thought to have a role in neurite outgrowth, morphogenesis, and cell adhesion in a variety of regions of the nervous system, including the retina, spinal nerves brainstem, and thalamocortical projections. The deficits in hearing, vision, and motor behavior that characterize the AChE knockout mouse could result from an excess of acetylcholine or an absence of AChE at one or more sites along the relevant neural pathways. The AChE knockout mouse, having no AChE activity and no AChE protein, provides an opportunity to test for evidence of a morphogenic function of AChE. If AChE has an essential role in morphogenesis, then AChE^{-/-} mice may have a disordered connectivity for axons projecting from the thalamus to the sensory cortex.

Because either the absence of AChE or the possible excess of acetylcholine could have consequences on neural systems other than the central cholinergic systems, it is important to determine whether other neurotransmitter systems might be affected in the AChE knockout mouse. Dopaminergic neurons in the striatum play a key role in the control of motor function and it is important to note that muscarinic receptor subtypes are coexpressed with dopamine receptors on striatal projection neurons. Complex interactions between these two neurotransmitter receptor systems are critical for the proper regulation of motor control. The motor deficits observed in Parkinson's patients are thought to reflect, in part, an imbalance between muscarinic cholinergic and dopaminergic tone in the striatum. AChE^{-/-} mice have severely reduced levels of muscarinic receptors (Bernard et al., 2003; Li et al., 2003; Volpicelli-Daley et al., 2003) and they have body tremor. This led to the hypothesis that AChE^{-/-} mice have an imbalance in their dopaminergic system. To test this hypothesis, the quantity and the affinity of D1 dopaminergic receptors in the striatum were examined and compared between wild type and AChE knockout animals.

Perhaps the most critical issue to be addressed relates to how alterations in brain organization might be expressed in brain function. A convenient indicator of general neural activity is the expression of the immediate early gene, *Arc* (activity-regulated cytoskeleton-associated) mRNA. *Arc* has been shown to be a marker for active synapses (Steward and Worley, 2001). Following exposure to a novel environment or to a strong electrical or drug stimulant, *Arc* mRNA and protein increase transiently in the specific neurons that were stimulated. Differences in *Arc* levels between wild type and AChE knockout mice may provide important clues for understanding the consequences of absence of AChE or the excess of acetylcholine.

Methods

AChE knockout mice. Animal work was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. Formal approval to conduct the experiments was obtained from the animal subjects

review board. AChE^{-/-} mice with no AChE activity and no AChE protein in any tissue were made by gene-targeting (Xie et al., 2000) and raised to adulthood on a liquid diet of Ensure (Duysen et al., 2002c). The AChE^{-/-} mice have a strain 129Sv genetic background.

Axon tracing. Animals 13-14 days old were narcotized with CO₂, and transcardially perfused with 15-20 ml of phosphate buffered saline (PBS), followed by cold 4% paraformaldehyde in PBS. Brains were removed and stored in 4% paraformaldehyde in PBS. There were 3 AChE^{-/-}, 5 AChE^{+/-}, and 4 AChE^{+/+} brains.

A mid-sagittal cut divided the fixed brains into two hemispheres. Small crystals of the fluorescent neuronal tracer 1,1'-dioctyldecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes D-3911, Eugene, OR) were placed in the dorsal thalamus of the fixed brains, using an approach from the exposed midline. Small glass pipettes were used to manipulate the crystals under a dissecting microscope (Kageyama and Robertson, 1993) and the DiI crystals were targeted to the ventrobasal nucleus of the thalamus. Hemispheres with DiI placements were stored in PBS for several weeks at 37°C. Sections at 100µm thickness were cut using a Vibratome. Approximately half the brains were sectioned in the transverse plane; in the other brains the cerebral cortex was separated from the rest of the brain and sections were cut tangential to the pial surface. After the cortex was removed in those cases, the diencephalons was cut in the transverse plane to identify the DiI placement site. Wet-mounted sections were examined under rhodamine epifluorescence illumination at excitation wavelength 547 nm.

Dopamine receptor. D1-like receptors in the striatum of AChE^{-/-} and ^{+/+} mice were quantified by radioligand binding. Adult male AChE^{-/-} mice (n=5) and adult male AChE^{+/+} mice (n=4) were euthanized by asphyxiation with carbon dioxide at ages 3, 4.5, and 8 months. The striatum was dissected, homogenized with a Teflon glass homogenizer in 20 volumes of ice cold 50 mM TrisCl, pH 7.4, containing 2 mM EGTA and 10% sucrose. Cell debris was removed by centrifugation at 800xg for 5 min (1900 rpm in a microfuge). The supernatant was centrifuged at 49,000xg for 20 min (20,000 rpm in Sorvall rotor SS34). The pellet was washed twice in 50 mM TrisCl, pH 7.4, and suspended in 50 mM TrisCl, pH 7.4. After the protein concentration was determined with the BCA method (Pierce), the suspension was frozen at -70°C.

The ligand [³H]SCH23390, specific for D1 dopamine receptors (Hyttel, 1983; Billard et al., 1984) was from Perkin-Elmer Life Science (NET-930, 3.2 TBq/mmol in 0.025 ml ethanol; 86.00 Ci/mmol). The binding reaction contained 120 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 50 mM TrisCl, pH 7.4, 10 µM mesulergine to block serotonin receptors, 0.5 to 15 nM [³H]SCH23390, and 0.4 mg protein in a total reaction volume of 250 µl. Non-specific binding was measured in the presence of 10 µM cis-flupentixol to block D1 receptor binding. The reactions were incubated for 30 min at 30°C without radioligand to allow binding of cis-flupentixol to D1 receptor. Then the radioligand was added and the tubes were incubated an additional 30 min at 30°C. The reaction mixtures were filtered through Whatman GF/B glass fiber filters to wash out unbound radioligand. The filters were washed three times with 4 ml of ice-cold 50 mM Tris HCl, pH 7.4, then placed in scintillation vials, covered with scintillation cocktail and

stored overnight in the dark to minimize chemiluminescence, before being counted the next day. Non-specific binding was subtracted from total binding to yield specific binding.

For determination of the dissociation constant, K_d , and the number of receptors, B_{max} , the data were fit to equation 1 using SigmaPlot software (Jandel Scientific).

$$\text{Equation 1: } \text{Bound} = (B_{max} \times \text{Free}) / (K_d + \text{Free})$$

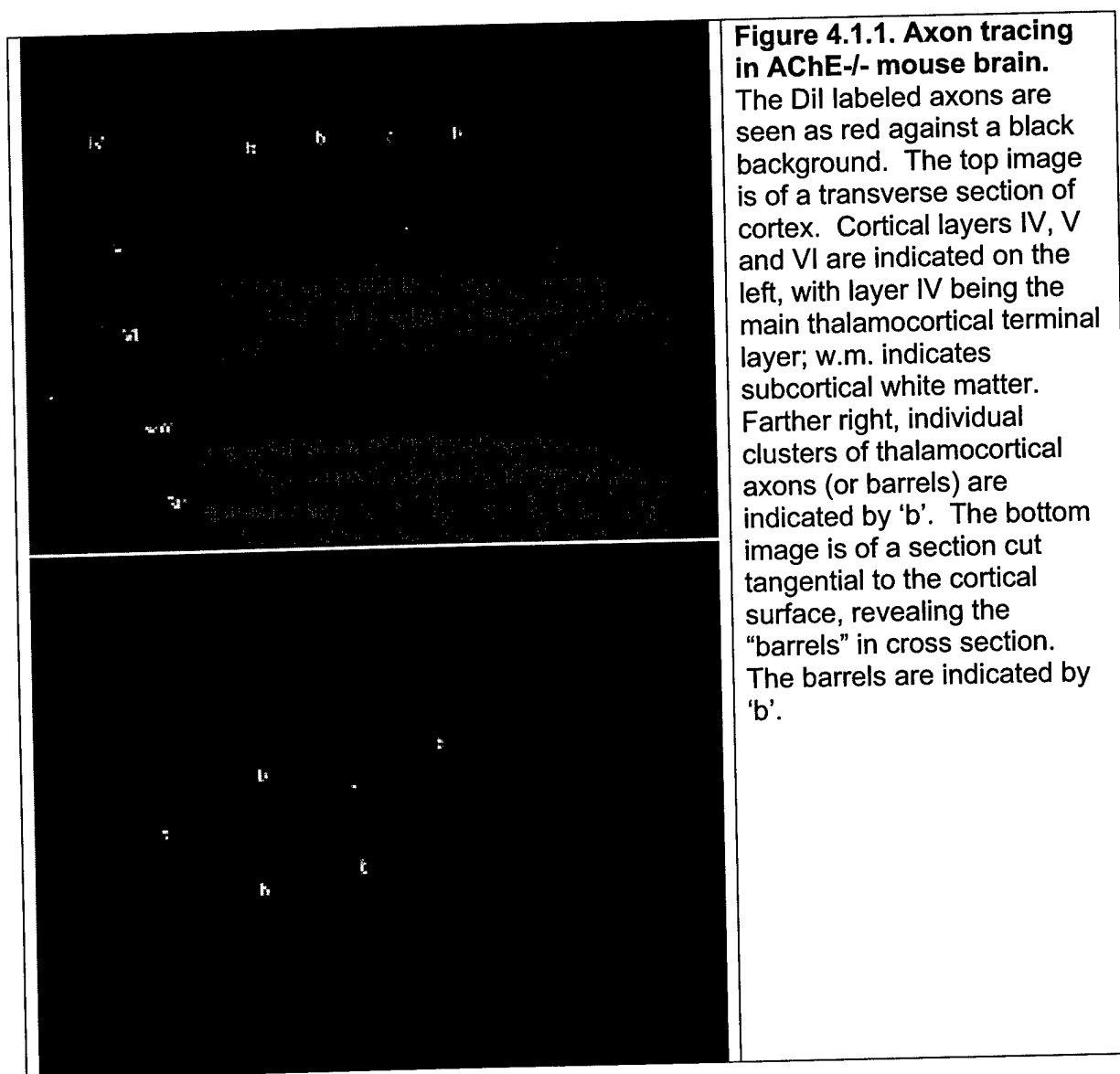
where Bound is proportional to the radioactivity specifically bound to D1; and Free is the concentration of radioligand.

In situ hybridization for Arc mRNA. Sections at thickness of 16 μm were cut on a cryostat from fresh frozen brains of 50-60 day old AChE +/+ and -/- mice and were thaw-mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were fixed in ice cold 4% paraformaldehyde in concentration of radioligand. 1 X PBS, pH 7.4, for 5 min. They were then washed in 1 X PBS for 1 min, 75% ethanol for 2 min and stored in 100% ethanol at 4C. Prior to hybridization slides were allowed to dry at room temperature. The 45mer oligonucleotide probe targeting Arc mRNA had the sequence 5'-CTTGGTTGCCCATCCTCACCTGGCACCCAAGACTGGTATTGCTGA-3'. A Blast search of Genbank revealed that this sequence did not have significant homology with other known sequences. Probes were 3' end labeled with [^{35}S]dATP (1200 Ci/mmol, NEN) using terminal deoxyribonucleotidyl transferase (3' end labeling system, NEN). The labeled probe was separated from unincorporated radionucleotide on a Biospin-6 chromatography column (BioRad) by centrifugation at 2500 rpm for 5 minutes. 150 mL hybridization buffer (50% formamide, 4 x SSC, 8% dextran sulfate, 1 x Denhardt's, 500 ug/mL salmon sperm DNA, 270 ug/mL yeast tRNA, 0.1 mM dithiothreitol) was applied per slide with 1×10^6 cpm of labeled probe. Control hybridizations were performed by including 10X unlabelled probe in the hybridization reaction. Slides were coverslipped, sealed with D.P.X. and placed overnight in a 1 x SSC humidified Tupperware container at 42°C. The next day coverslips were removed in warm 1 X SSC and slides were washed 4 x 15 min in 1 x SSC at 55°C. After washing, slides were briefly dipped in room temperature water to remove salts and in 70% ethanol to facilitate drying. Slides were placed on film (Biomax, Kodak) for 2-3 weeks. The film was developed by standard procedures. Autoradiograms were analyzed using the MCID imaging system (Imaging Research, Inc., St. Catherine's, Ontario).

Results

Axon tracing. Inspection of sections through the diencephalons revealed that the Dil placements were largely confined within the ventrobasal nucleus of the thalamus, but in several cases the Dil placement also involved the lateral or medial geniculate bodies (or their efferent axons). Prominently labeled axons exited the dorsal thalamus and extended through the internal capsule to reach the neocortex. Within the somatosensory regions of neocortex, Dil labeled thalamocortical axons were detected in

cortical layer IV and the deeper part of layer III, and formed the well described "barrel" pattern (Woolsey and Van der Loos, 1970; Erzurumlu and Jhaveri, 1990) characteristic of this cortical area (Figure 4.1.1). Prominent Dil labeling also was detected in layers V and VI, where it displayed an anterograde terminal appearance as well as a few retrograde labeled pyramidal cells in layer VI. In hemispheres in which the Dil placement involved the lateral or medial geniculate bodies, labeled thalamocortical axons also were detected in primary visual and auditory cortical areas (data not presented), where they were found primarily in cortical layer IV and deep layer III. The areal distributions and overall intensities of the cortical labeling varied with the location and size of the Dil placement in thalamus. However, the laminar patterns of Dil labeling in the three primary sensory cortical areas (and the "barrel" formations in somatosensory cortex) of the AChE-/- mice were no different from the patterns of Dil labeled thalamocortical terminals in the wild-type controls, or in the heterozygotes.



Dopamine D1 receptor results. The binding of the D1 dopamine receptor ligand [^3H] SCH23390 to striatal membranes isolated from wild-type and AChE $^{-/-}$ brains was measured. Table 4.1.1 shows that the dissociation constant was similar for wild-type and AChE $^{-/-}$, the K_d value being 2.9 ± 0.5 nM for wild-type and 3.1 ± 2 nM for AChE $^{-/-}$. However, the B_{max} value, which reflects the number of receptors, was 95% lower in AChE $^{-/-}$ striatum; $B_{\text{max}} = 550 \pm 29$ fmol/mg for wild-type, and 19 ± 13 fmol/mg for AChE $^{-/-}$ striatum.

Table 4.1.1. D1 dopamine receptor binding in striatal membranes

Species	K_d , nM	B_{max} , fmol/mg	reference
AChE $^{+/+}$ mouse	2.9 ± 0.5	550 ± 29	This work
AChE $^{-/-}$ mouse	3.1 ± 2	19 ± 3	This work
D1 $^{+/+}$ mouse	0.4	1700	Drago et al., 1994
D1 $^{+/-}$ mouse	0.38	700	Drago et al., 1994
Rat	0.53	69	Billard et al., 1984
Rat	1.5	86	Mansour et al., 1990
Rat	1.5	643	Cai et al., 2002
Human	1.2	132	Mattila et al., 2001

The ligand is [^3H]SCH23390.

Arc. The wild-type brain has intense Arc mRNA staining in the cortex and only low levels in the striatum (Figure 4.1.2). In contrast the AChE $^{-/-}$ brain has little Arc in the cortex but substantial Arc in the striatum. This is a striking difference between wild type and AChE $^{-/-}$ mice. The data indicate that synaptic activity is far greater in the cortex of wild-type mice compared to activity in the striatum, as reflected by expression of Arc mRNA. The situation is the opposite in AChE $^{-/-}$ mice, which have relatively low levels of synaptic activity in the cortex compared to wild type animals and significantly greater activity in striatum compared to wild types, again as reflected by Arc expression. There is very strong expression of Arc mRNA in the piriform cortex of AChE $^{-/-}$ brain.

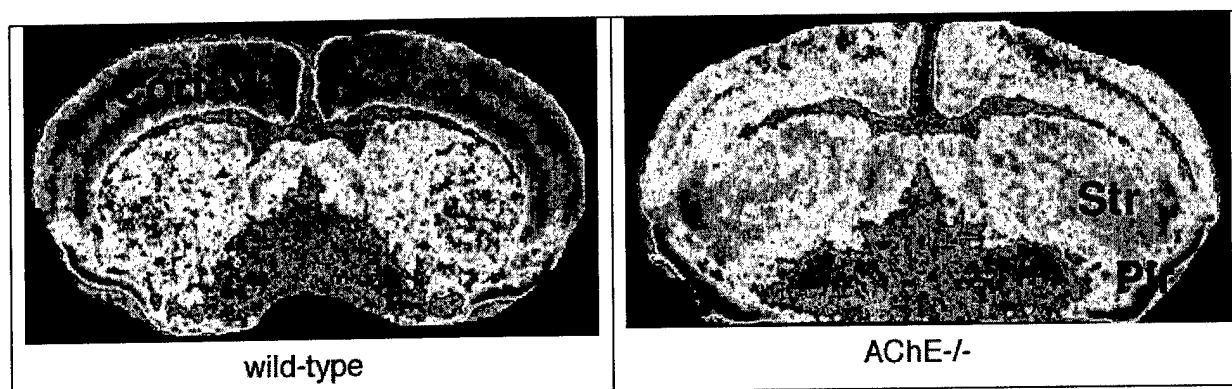


Figure 4.1.2. Arc mRNA in brain sections. The hottest colors (red) indicate the greatest level of mRNA localization while the coolest colors (blue, violet) indicate the lowest levels. The wild-type brain stains for Arc in the cortex. The AChE $^{-/-}$ brain stains for Arc in the striatum and piriform cortex.

Discussion

Morphogenic role of AChE. Connections between the thalamus and respective sensory cortical areas are formed within the first three weeks after the birth of a rat or mouse. These connections are important for the animal to sense its environment. Transient expression of AChE activity by thalamocortical neurons in rats correlates with the period of time when these neurons send their axons into layer IV of cerebral cortex and form synaptic connections with cortical neurons (Robertson, 1987; Robertson et al., 1989). This observation led to the hypothesis that AChE has a role in morphogenesis (Robertson and Yu, 1993). While similar transient expression of AChE activity has been reported for humans and other primates (Kostovic and Rakic, 1984), this phenomenon appears to vary between species (Robertson et al., 1989; Sendemir et al., 1996). The finding that mice with no AChE protein form normal thalamocortical projections shows that the presence of AChE is not essential for the ingrowth of thalamic axons and formation of normal terminal field patterns. Further, because the formation of barrels in somatosensory cortex is sensitive to disturbances all along the somatosensory pathway (Killackey et al., 1995), from the peripheral receptors through the brainstem and thalamic relay nuclei, the presence of normal barrels in the AChE $-/-$ mice indicates that neither absence of AChE nor surplus of acetylcholine has a demonstrable negative effect on the formation of these pathways. This is of particular interest because cholinergic systems had been shown to affect neural processing at several points along sensory pathways (Mooney et al., 2004).

Though AChE knockout mice appear to have normal thalamocortical connections to the sensory cortex, they do not have normal sensory functions. (Bytyqi et al., 2004) have found abnormalities in the retina of AChE $-/-$ mice that progress to complete loss of the photoreceptor layer by 3 months of age. Auditory function is also abnormal in AChE $-/-$ mice (McGee et al., 2001). The loss of vision and hearing cannot be attributed to disordered thalamocortical connections, because the present work has found the connections, at least during the developmental period, to be normal.

The possibility of a morphogenic role for AChE remains unresolved. The brain has remarkable abilities to compensate for loss of a structure or, in this case, loss of a protein. Redundancy is common, although it is of interest that the "nonspecific" butyrylcholinesterase is not upregulated in the AChE $-/-$ animals (Li et al., 2000; Mesulam et al., 2002b). Further, absence of a demonstrable negative effect on thalamocortical morphogenesis does not necessarily impact other suggestions of non-cholinergic roles for AChE in other systems (Greenfield, 1984; Kristt, 1989; Layer and Willbold, 1995; Sharma et al., 2001).

D1 dopamine receptor. The huge reduction in D1-like dopamine receptor level in AChE $-/-$ striatum suggests that the striatal neurons of the AChE $-/-$ mouse may have degenerated. However, light microscopic examination by Mesulam et al. (Mesulam et al., 2002b) revealed no structural differences between wild-type and AChE $-/-$ brains. Furthermore, the D1 receptor knockout mouse (Drago et al., 1994) had intact striatal neurons despite the complete absence of D1 dopamine receptors. This showed that D1 receptor integrity is not required for survival of striatal neurons.

In Parkinson's disease the changes in dopamine receptor level are much smaller. Mattila et al (Mattila et al., 2001) found reduced numbers of D1 and D2 dopamine receptors in Parkinson's patients who had not been treated with neuroleptics. The D1 receptors were reduced 26% in the caudate nucleus and 17% in the putamen. By contrast, patients treated with neuroleptics had normal levels of dopamine receptors.

Arc mRNA. In wild-type brain, expression of the immediate early gene, Arc, is enriched in cortex (Link et al., 1995) and hippocampus and appears to be regulated by excitatory neural transmission, particularly by activity at NMDA receptors (Lyford et al., 1995). It also has been shown that seizures induce expression of Arc mRNA (Steward and Worley, 2001). As a result, the strong expression of Arc mRNA in the piriform cortex of AChE^{-/-} brain is particularly interesting because this brain region has been implicated in epileptogenic activity (Schwabe et al., 2000), as have NMDA receptors. This suggests there is increased glutamatergic activity in piriform cortex of AChE^{-/-} mice and this may explain, at least in part, the high incidence of seizures seen in these animals.

Adaptations to absence of AChE. The complete absence of AChE activity in the AChE^{-/-} mouse is assumed to result in excess acetylcholine. Excess acetylcholine is lethal when the acetylcholine level rises abruptly, as after acute inhibition of AChE by an organophosphorus nerve agent or pesticide. In contrast, low levels of AChE inhibitor administered chronically result in tolerance. A tolerant rat survives a dose of organophosphorus toxicant that is lethal to other rats (Costa et al., 1982). The AChE^{-/-} mouse is a model for an organophosphate tolerant animal, in the sense that both animals have undergone metabolic changes that allow them to survive in the presence of excess acetylcholine.

The adaptations that allow the AChE^{-/-} mouse and the organophosphate tolerant rat to survive include decreased muscarinic receptor levels in the brain and in peripheral tissues (Schiller, 1979; Costa et al., 1982; Myslivecek et al., 1996; Bernard et al., 2003; Li et al., 2003; Volpicelli-Daley et al., 2003). To maintain neurotransmitter balance, the AChE^{-/-} brain has decreased dopamine receptors. The beta-adrenergic receptors in cardiac atria of organophosphate tolerant rats are decreased 50% (Myslivecek et al., 1996), but are unchanged in AChE^{-/-} heart (Teplicky et al., 2004).

The AChE^{-/-} brain is normal in several regards. Its structure, as viewed under light microscopy, is normal (Xie et al., 2000; Mesulam et al., 2002b). The distribution and density of choline acetyltransferase and of butyrylcholinesterase are normal (Mesulam et al., 2002b). Cholinergic and cholinceptive neurons are normal in size, density, and distribution in AChE^{-/-} brain. The thalamocortical projections are normal.

An understanding of the mechanism by which the AChE^{-/-} mouse has adapted to absence of AChE is relevant to understanding tolerance to anticholinesterase drugs. Alzheimer and Parkinson's disease patients treated chronically with anticholinesterase drugs may undergo similar adaptations.

More results related to

Tasks 4.1, 4.2, 4.3, and 4.4

Lifespan of acetylcholinesterase knockout mice is prolonged by intrathecal injection of adeno-associated virus expressing acetylcholinesterase

Bin Li, Ellen G. Duysen, Oksana Lockridge

Abstract

The purpose of this work was to deliver AChE by recombinant adeno-associated virus (rAAV) to mice deficient in AChE, and to observe the effect of AChE expression on phenotype. In contrast to our earlier report, here we used mouse AChE rather than human AChE. Another difference was the addition of a protein transduction domain to the C-terminal of mouse AChE. Three routes of administration of the virus were tested: intramuscular, intrathecal, and intrastriatal. Intramuscular injection of rAAV/mAChE into 14-48 day old mice resulted in high-level expression of AChE in muscle. Virus-treated AChE^{-/-} mice had an average of 8.5 units/g AChE activity in muscle (n=5), whereas untreated AChE^{-/-} mice had zero AChE activity. Virus-treated AChE^{+/-} mice averaged 10.75 units/g AChE activity in muscle (n=5), a value 10-fold above endogenous. AChE activity was found only in muscle, near the site of injection, suggesting that the virus did not migrate to other tissues. Production of antibody against mouse AChE was tested by ELISA. Antibody against mouse AChE was detected in virus treated AChE^{-/-} mice, but not in AChE^{+/-} mice. Despite the presence of anti-mouse AChE antibody, AChE activity was expressed in muscle for at least 3 months. Expression of AChE in muscle of AChE^{-/-} mice did not improve their muscle strength, a result consistent with the findings of others that the collagen-tailed form of AChE anchored at the neuromuscular junction is the form required for proper muscle function. Intrathecal injection of rAAV into 2 day-old AChE^{-/-} mice resulted in expression of AChE in the spinal cord and brain. Intrastriatal injection of rAAV into 2- day old mice resulted in expression of AChE in the striatum. AChE expression in the central nervous system prolonged the lifespan of AChE^{-/-} mice by reducing their susceptibility to seizure-induced death.

Introduction

Acetylcholinesterase (AChE EC 3.1.1.7) has a clear role in nerve impulse transmission, where it hydrolyzes the neurotransmitter, acetylcholine. Mice with no AChE enzyme in any tissue are born alive and survive to adulthood (Xie et al., 2000; Duysen et al., 2002c). Their muscles are weak but functional. Their most important clinical pathology

is susceptibility to seizures. They survive many episodes of seizure activity before dying at an average age of 120 days.

The AChE $-/-$ mouse with its distinct phenotype is a good model for determining the effect of prolonged administration of AChE. Our goal was to deliver active AChE enzyme to AChE deficient mice using a gene therapy vector capable of expressing AChE continuously for several months.

In previous work we reported the effects of expressing human AChE in an adeno-associated virus vector. We found low-level expression in mouse tissues due to the formation of antibodies against human AChE. In the present work, we expressed mouse AChE rather than human AChE in mouse tissues.

Methods

Vector construction: 2.1 kb of mouse AChE cDNA containing HA and TAT tags at the C-terminus was cloned into the pAAV-MCS plasmid (catalog #240071, AAV Helper-Free System, Stratagene, La Jolla, CA). Mouse cDNA was a gift from Palmer Taylor, University of California, San Diego (Genbank accession number X56518). The HA tag is a 9 amino acid epitope from the human influenza hemagglutinin protein, with the sequence YPYDVPDYA. The TAT tag is an 11 amino acid protein transduction domain with the sequence YGRKKRRQRRR (Schwarze et al., 2000). XL10-Gold[®] ultracompetent cells (catalog #200314, Stratagene) were used to amplify plasmid pAAV/mAChE. It was essential to use XL10-Gold cells to avoid unwanted recombination through the inverted terminal repeat sequences. Exons 2, 3, 4, and 6 encoding the most abundant splice variant of AChE, the T-form, were present in rAAV/mAChE; this form makes soluble tetrameric AChE in plasma. A second vector encoding the read-through form of mouse AChE contained exons 2, 3, 4, and intron 5; this form makes monomeric AChE and is called read-through AChE in this report. The read through AChE also had the HA and TAT tags at the C-terminus.

Virus preparation: Viral particles of serotype-2 were prepared by adenovirus-free cotransfection of HEK 293T cells with three plasmids: pAAV/AChE, pHelper (carrying adenovirus derived genes), and pAAV-RC (carrying AAV-2 replication and capsid genes). These three plasmids together supplied all the transacting factors required for AAV replication and packaging in HEK 293T cells (Xiao et al., 1998; Haberman et al., 1999; Amiss and Samulski, 2001). Plasmids pAAV-MCS, pHelper, and pAAV-RC were from Stratagene. HEK 293T cells gave higher yields of virus than HEK 293 cells or AAV-cells from Stratagene.

Purification of adeno-associated virus: Culture medium and cells were harvested 72 hours post-transfection from forty 150 mm dishes. Each dish had 20 ml of DMEM, 2% FBS for a total of 800 ml culture medium. Virus was released from the cells by three cycles of freezing and thawing. Virus was purified by PEG8000/NaCl precipitation, chloroform extraction, and ultrafiltration (Wu et al., 2001; Grimm et al., 2003). In brief, the thawed cells were centrifuged at 2000xg to remove cell debris (3,500 rpm in a Sorvall rotor). Undesired protein was precipitated by adding calcium chloride to 25 mM.

The mixture was incubated 1 h on ice, and centrifuged at 2000xg for 30 min. The supernatant was adjusted to 0.65 M NaCl by the addition of solid NaCl. After the sodium chloride was completely dissolved, solid PEG8000 was added to a final concentration of 10%. This step precipitated the virus. The mixture was incubated at 4°C overnight. A light yellow viral pellet was obtained by centrifuging the mixture at 12,000 rpm (20,000xg) in a Sorvall rotor for 20 min. The viral pellet was dissolved in 100 ml PBS. DNase and RNase were added to a final concentration of 1 microgram per ml each. Digestion was for 1 h at 37 C. An equal volume of chloroform was added, the mixture was shaken, and centrifuged to separate the layers. The upper, aqueous layer contained the virus. The aqueous layer was extracted a second time with chloroform. The aqueous layer was reduced in volume to 1 ml in an Amicon Ultra-15 centrifugal filter device with a 100 kDa cutoff (Amicon catalog # UFC9 100 08).

Viral titer: HEK 293T cells were plated in a 24-well plate with 1 ml culture medium per well containing 10% heat-inactivated FBS. When cells were 70-80% confluent, they were infected with 1 µl of purified virus. Culture medium was harvested 23 hours later. AChE enzyme activity in the culture medium was measured. The full-length AChE, rAAV/mAChE, gave an average titer of 0.9 (range 0.7 to 1.2) units AChE activity per ml culture medium after infection of cells with 1 µl of purified virus. The read-through AChE gave higher activity, averaging 1.7 units per ml culture medium. These AChE activity values correspond to 0.19 and 0.36 µg AChE enzyme per 1 million viral particles. In a dot blot assay the number of viral particles was 3×10^9 per ml. Thus the titer can be expressed as 570 µg AChE/ml for the purified rAAV/mAChE, and 1080 µg AChE/ml for the purified read-through AChE virus.

Mice: Animal work was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques when applicable. The AChE knockout colony is maintained at the University of Nebraska Medical Center by breeding heterozygotes. The genetic background of the animals is strain 129Sv.

AAV delivery: Intramuscular injection. 10 µl of rAAV/mAChE virus was injected into the tibialis anterior muscle of each hind leg of 14-day-old AChE^{-/-} mice (n=4). 100µl of virus was injected by the same method into adult AChE^{-/-} (n=1) and AChE^{+/-} mice (n=5).

Intrathecal injection. 10 to 20 µl rAAV/mAChE virus was injected intrathecally into 2-day-old AChE^{-/-} mice. 26 mice received rAAV/mAChE, 11 received saline.

Intrastriatal injection. 2.5 µl read-through AChE virus was injected into the striatum of each hemisphere of 2-day-old AChE^{-/-} mice, for a total of 5 µl virus per mouse. 15 mice received read-through AChE virus, and 9 received saline.

Tissue enzyme activity. Tissues from mice were extracted with 50 mM potassium phosphate buffer, pH 7.4, containing 0.5% Tween. After centrifugation to clarify the extract, AChE activity was measured with 1 mM acetylthiocholine by the Ellman method (Ellman et al., 1961) following inhibition of BChE with 0.3 mM tetraisopropylpyrophosphoramidate (iso-OMPA) for 30 min. BChE in liver was inhibited

with 1.0 mM iso-OMPA. Assays were performed in 0.1 M potassium phosphate buffer pH 7.0 in 1 cm cuvettes at 25°C. Units of activity are defined as μ moles acetylthiocholine hydrolyzed per min.

Detection of antibody against mouse AChE. ELISA was performed using 96-well immunoassay plates coated with purified mouse AChE, 0.5 microgram AChE per well. Purified mouse AChE was a gift from Zoran Radic, University of California, San Diego. Test plasma was allowed to bind to the AChE. Excess plasma was washed away, and the primary antibody was hybridized with goat anti-mouse IgG conjugated to horse radish peroxidase. Peroxidase activity was detected by reaction with o-phenylenediamine dihydrochloride at 450 nm.

Detection of the HA epitope. The HA epitope at the C-terminus of AChE was measured by coating a 96-well plate with anti-HA antibody (Boehringer-Mannheim, renamed Roche Applied Science), followed by incubation with tissue homogenate or plasma. Bound AChE activity was measured spectrophotometrically at 412 nm in a Molecular Devices microplate reader.

Neutralizing antibody assay to detect antibodies to viral proteins. Mouse serum and dilutions of mouse serum were incubated with purified virus at 37°C for 1 or 2 hours. The virus-serum mixture was added to 293T cells at a dose equivalent to 1 μ l virus (before dilution) per well in a 24-well plate. The culture medium was assayed for AChE activity 23 hours later. In this method, antibody to viral proteins is assumed to neutralize the virus, so that the virus is unable to infect 293T cells. Lower infectivity is reflected by lower AChE activity in the culture medium.

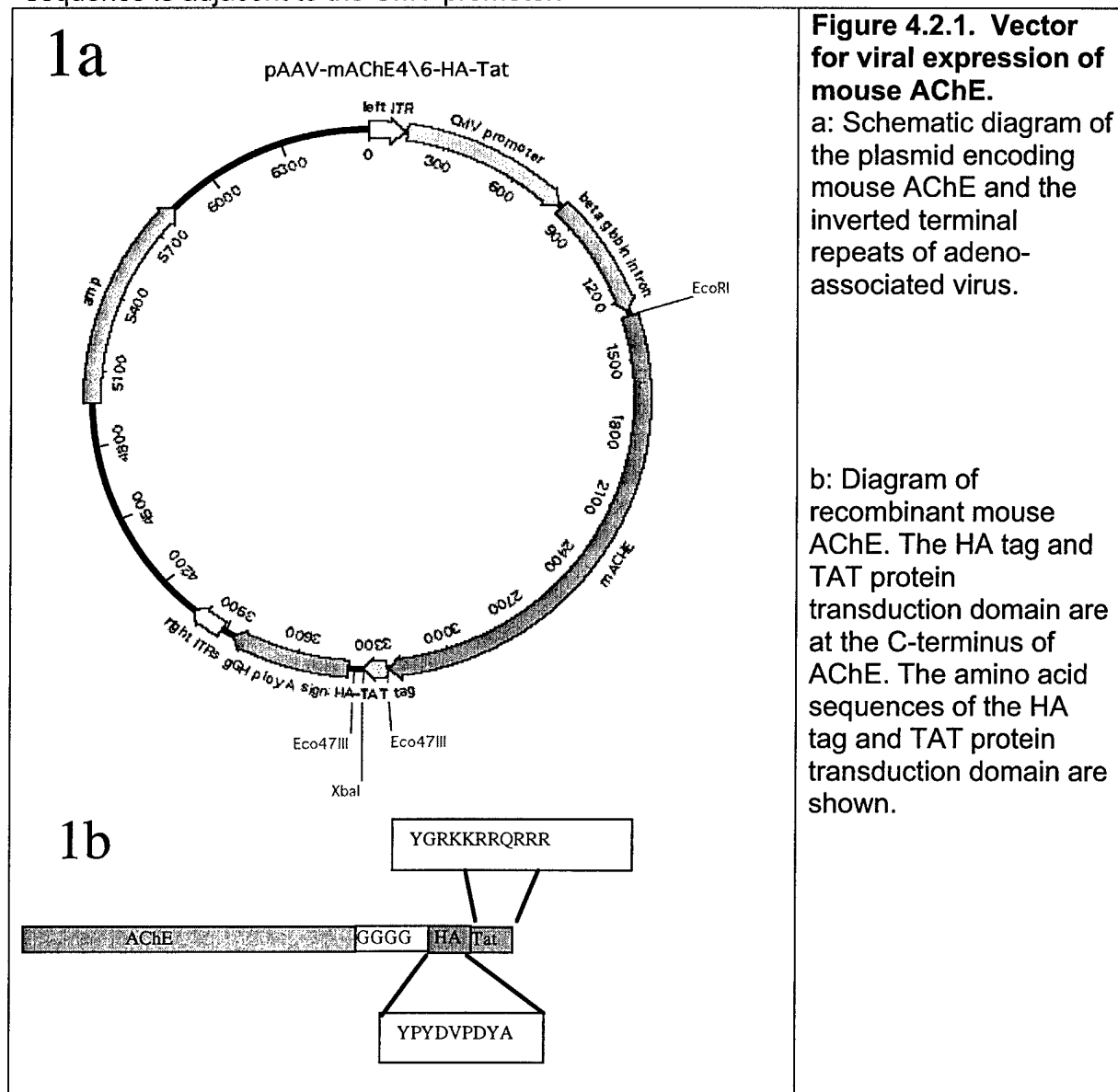
Brain sections. Mice were euthanized with CO₂, and then perfused transcardially with 30 ml PBS. The brain was removed and frozen on dry ice. It was sectioned on a cryostat into 30 micron sections and placed on Superfrost/Plus slides (Fisher Scientific, #12-550-15).

Brain sections were stained for AChE activity by the method of (Karnovsky and Roots, 1964). Frozen sections were placed into 0.1 M maleate buffer pH 6.0 containing 5 mM potassium ferricyanide, 30 mM cupric sulfate, 50 mM sodium citrate, and 0.1 mM iso-OMPA. A 30 min preincubation with iso-OMPA at room temperature served to inhibit BChE activity. Color was developed by adding acetylthiocholine iodide to 60 ml buffer to make 2 mM acetylthiocholine. Slides were stained for 18 h. Slides were washed ten times with water to stop the reaction, and fixed in 4% formalin in PBS for 15 min. Slides were dipped 5 times in 80% ethanol, 5x in 95% ethanol and 5x in 100% ethanol to dehydrate the tissues. They were photographed on a Nikon Optiphot-2 microscope with a Hitachi HV-C20 camera with Optimas 5.23 software.

Phenotype observations. Grip strength was measured on an inverted screen by measuring the time a mouse fell off. Body weight was measured every 2 days. Body temperature was measured with a surface thermometer.

Results

Adeno-associated virus. The plasmid for expression of mouse AChE in an AAV helper-free system is shown in Figure 4.2.1. The CMV promoter controls expression of AChE. The beta-globin intron lies between the CMV promoter and the mouse AChE cDNA. An epitope tag, HA, and a protein transduction domain, TAT, are at the C-terminus of mouse AChE cDNA. Stop codons are followed by a polyA addition region and a viral inverted terminal repeat sequence. A second inverted terminal repeat sequence is adjacent to the CMV promoter.



Injection of rAAV/mAChE into muscle. Mice treated intramuscularly in the hind leg with the virus were analyzed for AChE activity 2 weeks to 2.5 months after injection.

Hind leg muscle, front leg muscle, diaphragm muscle, heart, brain, liver, lung, intestine, and plasma were tested for AChE activity. The AChE activity in hind leg muscle was so high that it was easily measured with the Ellman assay. Activity in heterozygote mouse muscle was 10-fold above endogenous AChE activity. Activity in muscle of AChE knockout mice was almost as high as in the muscle of AChE^{+/+} mice. See Figure 4.2.2.

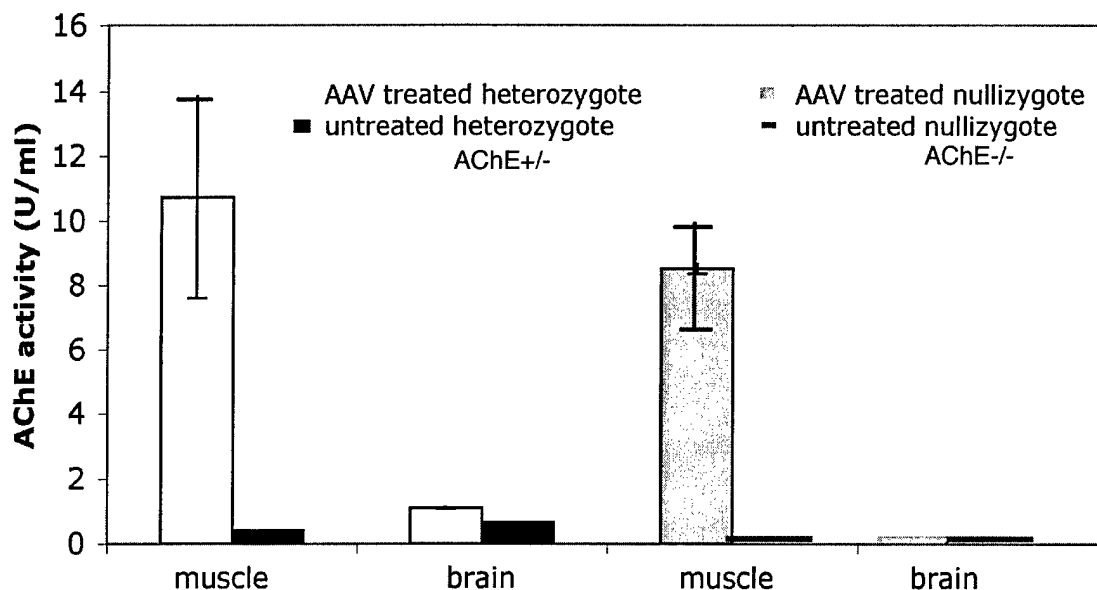


Figure 4.2.2. AChE activity in muscle of AChE^{+/+} and AChE^{-/-} mice after i.m. delivery of rAAV/mAChE. AChE^{+/+} mice had an average of 10.75 units/g AChE activity in muscle, a value 10-fold above endogenous (n = 5). AChE^{+/+} mice were 48-days-old on the day of AAV treatment; they were euthanized two weeks later. Virus-treated AChE^{-/-} mice had an average of 8.5 units/g AChE activity in muscle, in contrast to untreated AChE^{-/-} mice, which had zero AChE activity. AChE^{-/-} mice were 14 days old on the day of AAV treatment; their AChE activity was tested 10-77 days after im injection. Brain did not acquire AChE activity as a result of virus injection into muscle.

The brain did not acquire virally expressed AChE when the virus was injected into muscle. This was best demonstrated in the AChE^{-/-} mouse where the absence of background AChE activity allowed a clear interpretation. There was no evidence of retrograde transport of the virus through the motor neuron into the spinal cord and brain. Experiments with rAAV/mouse AChE and rAAV/human AChE were consistent on this point. Injection of virus into the muscle yielded no detectable AChE activity in mouse brain or spinal cord. A second method to test for retrograde transport of virus was PCR. When spinal cord homogenate was tested for viral DNA by PCR, no viral genes were found. However, im injected hind leg muscle gave a positive result in the PCR assay. These results contrast with work from another laboratory where AAV delivered in the muscle yielded expression of the gene product, insulin growth factor I, in brain (Kaspar et al., 2003).

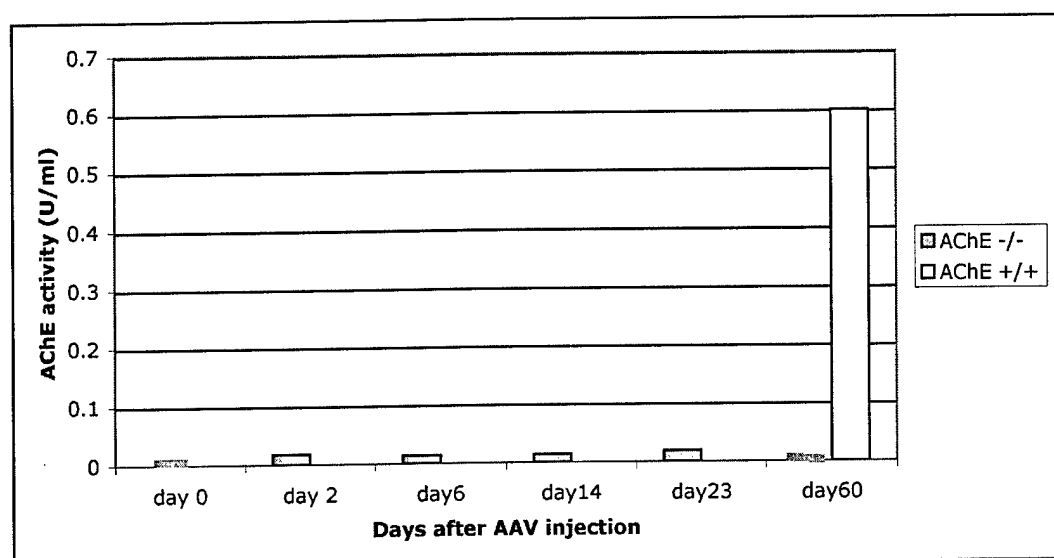


Figure 4.2.3. Intramuscular injection of rAAV/mAChE into adult AChE^{-/-} mice resulted in expression of AChE in plasma at levels approaching 3% of the AChE activity in wild type mouse serum.

After intramuscular injection of rAAV/mAChE into adult AChE^{-/-} mice the AChE activity in plasma increased from zero to 0.018 u/ml, which is about 3% of the AChE activity in plasma from wild-type mice (Figure 4.2.3.). AChE activity continued to be expressed at this level for 23 days after injection. By day 60, there was no detectable AChE activity in plasma of AChE^{-/-} mice.

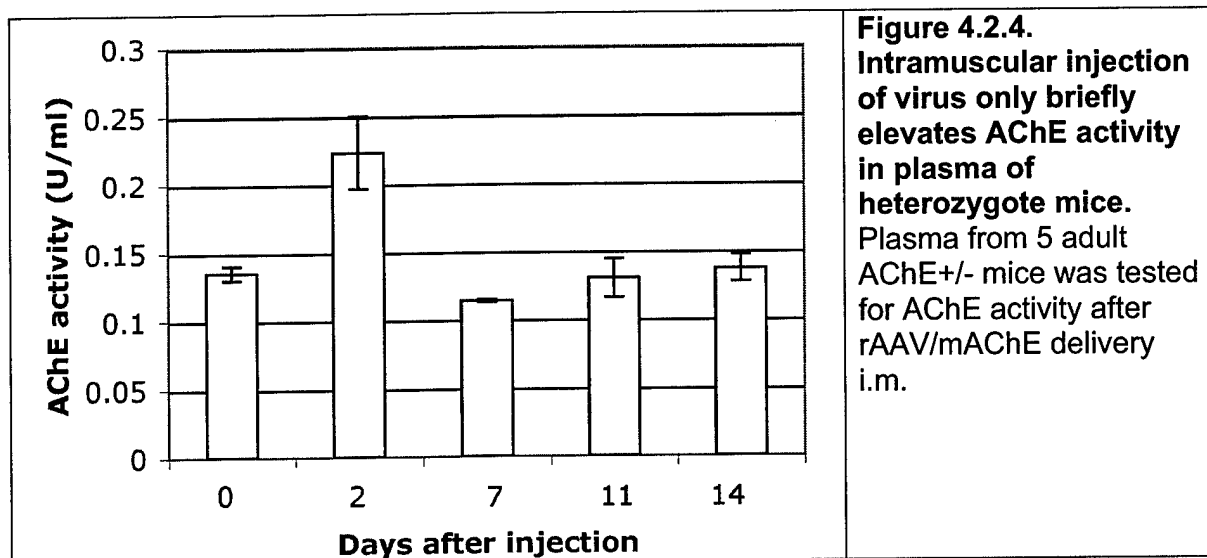


Figure 4.2.4. Intramuscular injection of virus only briefly elevates AChE activity in plasma of heterozygote mice. Plasma from 5 adult AChE^{+/-} mice was tested for AChE activity after rAAV/mAChE delivery i.m.

In AChE^{+/-} mice, the AChE activity in plasma increased from 0.13 to 0.22 but only for a brief period. The activity increased on day 2 post-injection but returned to pre-injection levels thereafter. See Figure 4.2.4.

No tissue other than muscle and plasma acquired AChE activity after im injection of rAAV/mAChE. Expression in muscle was limited to the hind leg muscle where the virus had been injected. The front leg muscle and the diaphragm muscle had no AChE activity. A single injection of virus in the hind leg muscle gave high levels of AChE activity for at least 3 months.

Antibody against mouse AChE and against viral proteins. Since AChE^{-/-} mice have not been exposed to AChE protein during development, their immune system might recognize mouse AChE as foreign. To test this hypothesis, plasma from AChE^{-/-} mice treated with rAAV/mAChE im was tested for the presence of anti-mouse AChE antibodies by ELISA. Figure 4.2.5 shows that virus-treated mice had no antibodies against mouse AChE on day 3, but did have antibodies on days 14 and 23 after treatment. In contrast, AChE^{+/-} mice did not produce antibodies against mouse AChE (Figure 4.2.6).

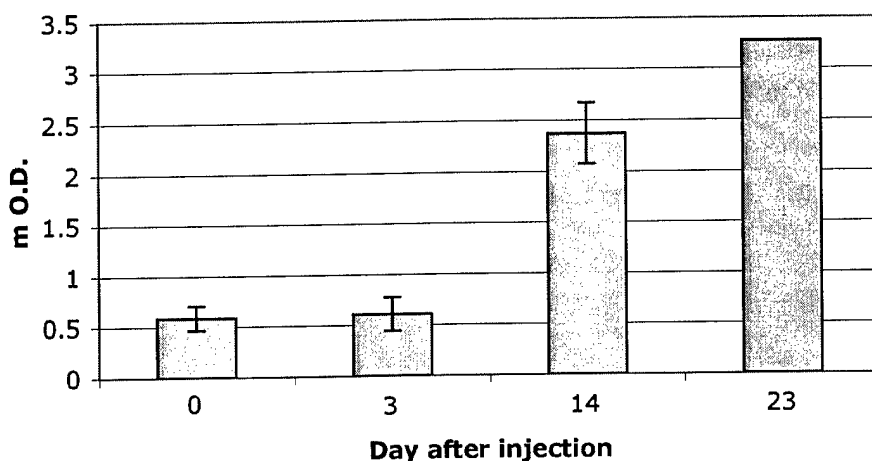


Figure 4.2.5. Antibody against mouse AChE after im delivery of virus into AChE^{-/-} mice. Plasma from AChE^{-/-} mice injected with rAAV/mAChE in hind leg muscle, was tested for the presence of anti-AChE antibodies by ELISA. Antibodies against mouse AChE were found on days 14 and 23 post-treatment. The Y axis reflects the activity of a reporter enzyme conjugated to anti-mouse IgG, in milli optical density at 450 nm.

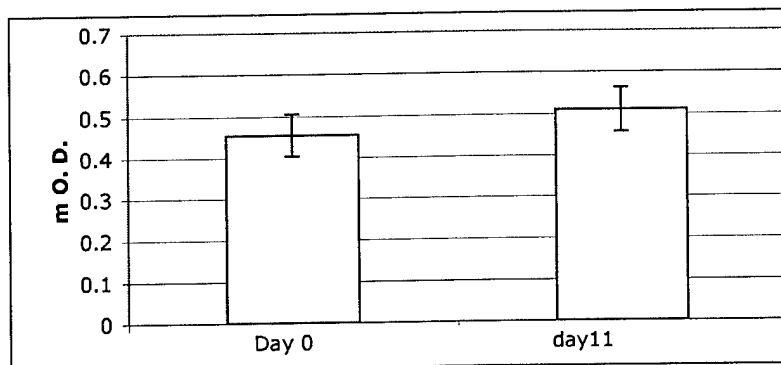


Figure 4.2.6. No antibody against mouse AChE after i.m. delivery of virus into adult heterozygotes. Plasma from five adult AChE^{+/-} mice injected with rAAV/mAChE i.m. was tested for the presence of anti-AChE antibodies by ELISA.

Production of antibodies against viral proteins was tested in a neutralizing assay. Figure 4.2.7 shows that antibodies to viral proteins were detected by day 7 after intramuscular injection of virus. The expression of AChE continued to be very high despite the presence of anti-virus antibodies.

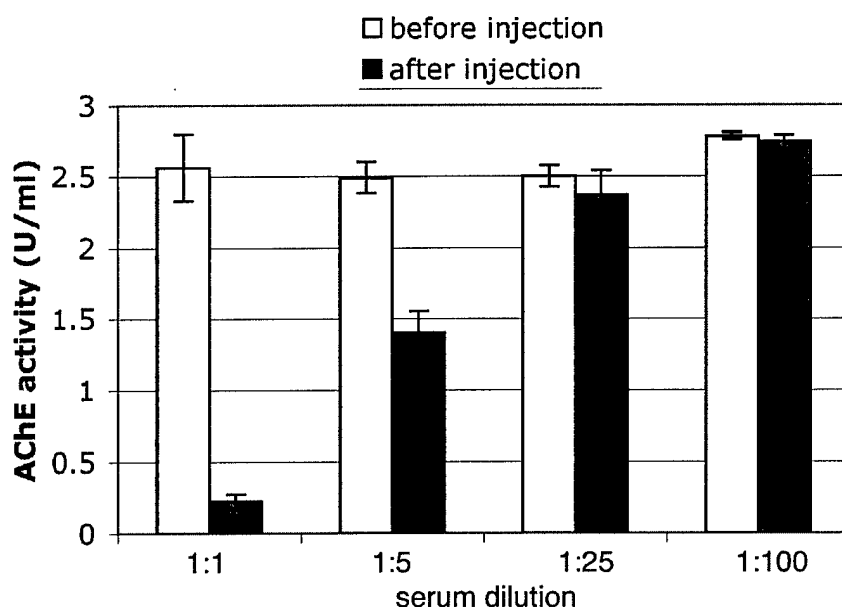


Figure 4.2.7. Neutralizing antibody against AAV protein. Plasma from five adult AChE^{+/±} mice injected with rAAV/mAChE i.m. was tested for virus-specific antibodies by a neutralization assay. Seven days elapsed between treatment with virus and antibody testing. The AChE^{+/±} mice made antibodies against viral proteins but not against AChE. Plasma from the same mice was tested for anti-AChE and anti-viral protein antibodies.

HA antibody. The HA antibody gave a positive result only when AChE activity was high, as in muscle homogenate. When AChE activity was low, as in plasma, the HA tag was undetectable. Proteolysis from the C-terminus of AChE to remove the HA tag may explain the insensitivity of the assay.

Injection of rAAV/mAChE into the spinal cord. It was of interest to determine whether virus injected intrathecally would result in AChE expression in the brain. Two-day old mice were used for this experiment because the spinal cord is easily seen and manipulated at this age. Special Hamilton syringes, with a removable 30-gauge needle were used for injection of 10 to 20 μ l virus per pup. A total of 37 AChE^{-/-} pups were treated intrathecally: 26 received virus and 11 received saline solution. AChE activity was measured in spinal cord and brain in 7 animals that had died. Figure 4.2.8 shows that significant AChE activity was detected in the spinal cord and brain as a result of treatment with virus. The spinal cord had an average of 0.18 and the brain had an average of 0.08 units AChE activity per gram wet weight. AChE activity was expressed in the spinal cord up to 5 months after injection of virus.

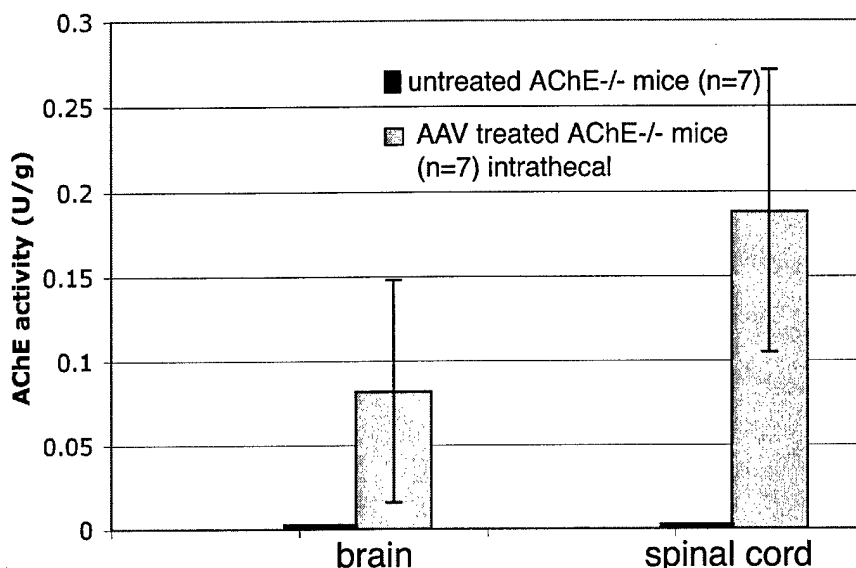


Figure 4.2.8. AChE activity in the spinal cord and brain of 7 AChE-/- mice after delivery of rAAV/mAChE intrathecally. Untreated animals have zero AChE activity. Injection of virus into the spinal cord results in AChE activity in spinal cord and brain. For comparison, AChE activity in spinal cord of untreated wild-type mice was 3.5 ± 0.5 u/ml.

Continued expression of AChE. A single injection of AAV intrathecally into 2-day old mice, yielded active AChE enzyme in the spinal cord and brain for at least 5 months. AChE activity in the spinal cord varied about 30 fold (range 0.02 to 0.74 u/g) between 11 animals, but this variation did not correlate with the time interval between injection and testing. There was no evidence that AChE activity levels in the spinal cord decreased with time.

A similar conclusion was reached for intrastriatal injections of AAV into 2-day old mice. Whole brain homogenates varied about 3.5 fold in AChE activity (range 0.02 to 0.07 u/g) between 6 animals, but there was no indication that expression levels of AChE decreased for 3 months after injection of virus.

Survival after intrathecal injection. Of the 26 AChE-/- mice treated with rAAV/mAChE when they were 2 days old, 4 died within a day due to maternal neglect. These 4 were not counted in the survival curve shown in Figure 4.2.9. None of the 11 saline treated AChE-/- animals died of maternal neglect. Figure 4.2.9 shows that treatment with virus intrathecally was beneficial to AChE-/- mice. On day 150, 55% of the virus treated mice are still alive, while only 27% of the saline treated animals are alive.

A critical period for AChE-/- mice is the age from 19 to 40 days. Many die in this age range from spontaneous seizures. Out of 100 untreated AChE-/- mice, 33 animals convulsed and died before or on day 40, while 67 animals survived past day 40. The average age of death for the 33 animals was 27 ± 5.6 days. Untreated mice that survive this period live to an average age of 120 days. In Figure 4.2.9 animals treated with

saline had a high death rate at this critical age. In contrast, the virus treated animals had a better rate of survival during this critical early period.

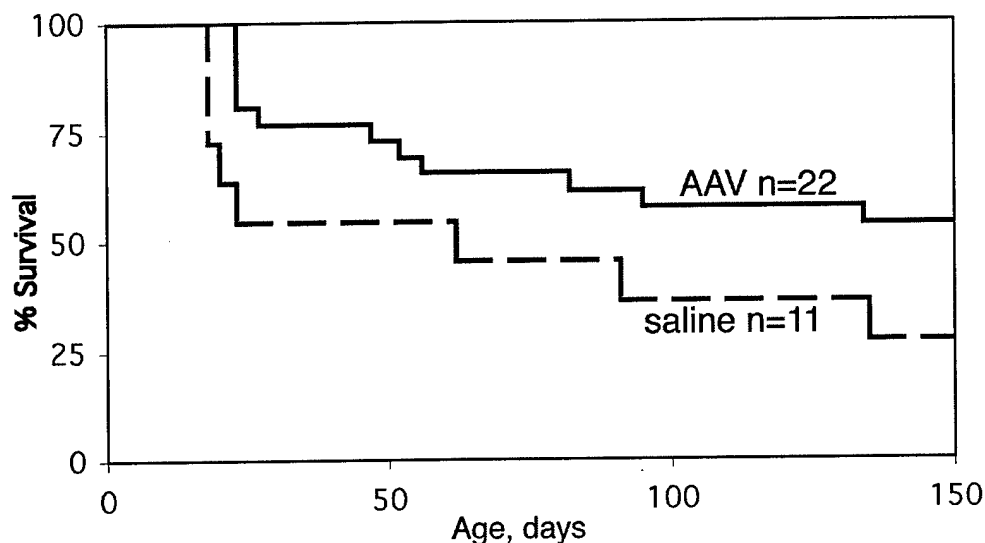


Figure 4.2.9. Survival of AChE^{-/-} mice after intrathecal injection. Mice were 2 days old at the time of injection of rAAV/mAChE or of saline into the spinal cord.

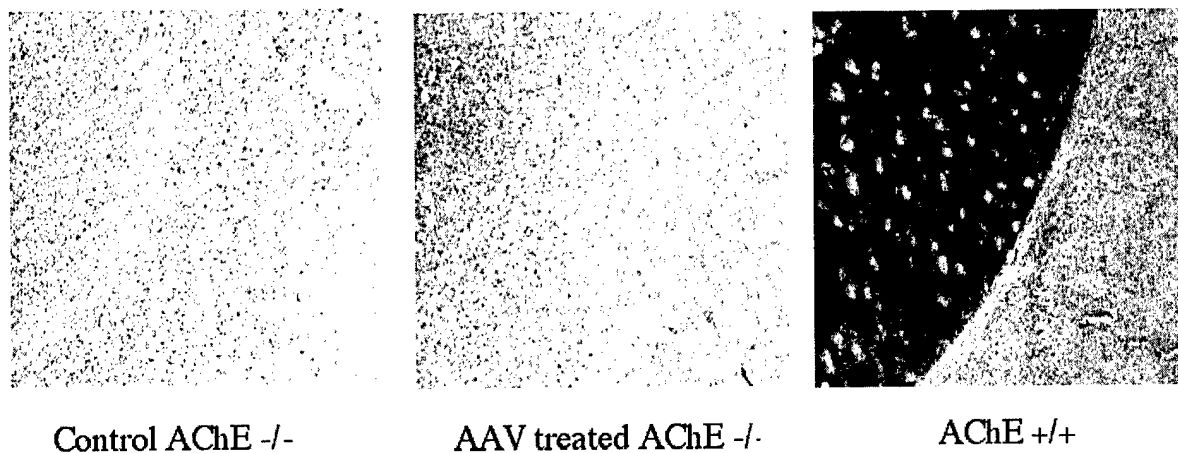


Figure 4.2.10. Brain sections stained for AChE activity. The left panel is a section through the striatum of an untreated AChE^{-/-} mouse; this mouse has zero AChE activity. The middle section, from a virus-treated AChE^{-/-} brain, shows AChE activity in the striatum, one week after treatment with virus. The right panel from an untreated wild-type mouse shows intense AChE activity in the striatum. Magnification, 40x

Injection of AChE into brain. The read-through AChE virus was injected into the striatum of AChE^{-/-} mice (n=15) when they were 2 days old. Control AChE^{-/-} mice (n=9) were injected intrastriatally with saline. AChE enzyme activity was demonstrated

in brain sections of virus treated mice by staining the sections for AChE activity. See Figure 4.2.10.

Survival after intrastriatal injection. 15 AChE^{-/-} mice were injected with the read-through AChE virus intrastriatally two days after they were born. 9 AChE^{-/-} mice received saline. These experiments are still ongoing. The preliminary results show a higher percentage of animals surviving in the virus-treated than in the saline-treated group.

Phenotype changes. Gene therapy of AChE^{-/-} mice by treatment with a virus that delivered AChE enzyme prolonged their lifespan. The virus had to be delivered to the central nervous system to see the beneficial effects of gene therapy. Longer life was the only phenotype change observed.

Virus delivered into the muscle had no effect on muscle strength as measured by lack of grip strength, lack of sexual activity, inability to eat solid food, abnormal gait and abnormal posture. The body weight remained low compared to that of wild-type mice; adult AChE^{-/-} mice weighed 18 g, whereas adult wild-type mice weighed 27-32 g (females) and 30-35 g (males). The body temperature was low by about 0.6°C compared to wild-type mice.

Discussion

Comparison of gene therapy with human AChE and mouse AChE. Hrabovska et al. have previously reported treatment of AChE^{-/-} mice with adeno-associated virus expressing human AChE. In this report the virus expressed mouse AChE. Hrabovska et al. treated adult mice intramuscularly, and young sexually immature mice intrastriatally. In this report 14-day old mice were treated intramuscularly, while 2-day old mice were treated intrathecally and intrastriatally.

There is agreement that injection of AAV/AChE into the central nervous system prolongs the lifespan of AChE^{-/-} mice. Both human AChE and mouse AChE had this beneficial effect.

Another point of agreement is that AChE^{-/-} mice formed antibodies against AChE when the virus was injected intramuscularly. The antibodies against human AChE dramatically reduced AChE activity in plasma and muscle. However, antibodies against mouse AChE were compatible with high expression levels of mouse AChE in the muscle for several months. AChE^{+/-} mice produced antibodies against human AChE, but not against mouse AChE.

Long term expression of AChE following a single injection of virus into the brain was seen with both the human and mouse AChE vectors.

Hrabovska et al saw improvement in motor coordination of AChE^{-/-} mice treated intrastriatally with virus. This change in phenotype was not seen in the present report. The explanation for this difference may be the age at which mice were treated with virus. Hrabovska et al treated 17-43 day old mice; the present report treated 2-day old mice.

Another difference between the two reports is the level of AChE expression after intramuscular injection of virus. Virus expressing human AChE gave low levels of expression, but virus expressing mouse AChE gave very high expression levels for several months.

No effect of protein transduction domain. The 11 residue TAT peptide from HIV-1 has been shown to facilitate passage of proteins across cell membranes. Fusion of the TAT peptide to the C-terminus of beta-glucuronidase in an AAV vector, increased uptake of the enzyme by peripheral tissues by 2-7 fold (Elliger et al., 2002) after iv injection of virus into the tail vein.

In the present report, there was no apparent effect of TAT on distribution of AChE, as only plasma and muscle had AChE activity after im injection of full-length AChE. In the brain, the AChE activity was confined to the needle track after intrastriatal injection of read-through AChE virus. TAT did not cause widespread expression of AChE in brain. It is possible that the lack of TAT effect is due to proteolytic removal of the TAT peptide from the C-terminus since the C-terminus of AChE is susceptible to proteolysis. For example, snake AChE expressed in COS cells was missing the FLAG epitope that had been built into the 3' end of the gene (Cousin et al., 1996); this was interpreted as proteolytic cleavage of the FLAG epitope from the C-terminus. Proteolytic clipping of the AChE C-terminus has no effect on activity. The fully active mouse AChE whose crystal structure was solved had been truncated at the C-terminus to contain 548 amino acids (Marchot et al., 1996). The full-length mouse AChE contains 583 amino acids (Rachinsky et al., 1990). Therefore, removal of the TAT peptide would not be noticed in an AChE activity assay. Tagging the N-terminus of AChE is not feasible because peptides fused to the N-terminus cause loss of AChE activity.

Effect on lifespan. Gene therapy of AChE^{-/-} mice with a viral vector that expressed soluble AChE had a positive effect when the virus was injected into the central nervous system. The lifespan of AChE^{-/-} mice was prolonged by administration of virus to the brain or spinal cord. This result can be explained by hypothesizing that AChE^{-/-} mice have an unusually high amount of acetylcholine in the brain due to their complete lack of AChE enzyme. There is not enough BChE in brain to compensate for the absence of AChE. AChE^{-/-} mice die at an early age because of spontaneous seizures. It is known that excess acetylcholine concentrations trigger a cascade of imbalances in the brain that result in seizures (McDonough and Shih, 1997). Introduction of AChE via gene therapy probably reduces the concentration of acetylcholine, reduces susceptibility to seizures, and therefore prolongs lifespan. This explanation assumes that acetylcholine levels in the brain can be affected by soluble AChE located outside of nerve synapses. Such an assumption is supported by the finding of Descarries that cholinergic innervation in many regions of the adult rat brain is asynaptic (Descarries, 1998). Descarries suggested that transmission in the cerebral cortex, hippocampus, and neostriatum is diffuse, involving acetylcholine receptors on dendritic membranes rather than in synapses. The soluble tetramers and monomers of AChE produced by our viral vectors seem to be affecting neurotransmission even though the AChE is not located in synapses.

No effect on muscles. Treatment of AChE^{-/-} mice intramuscularly with our gene therapy vector had no effect on muscle strength. Absence of effect on muscle strength is in accord with the role of AChE in the neuromuscular junction. Collagen-tailed AChE, anchored in the basal lamina at the neuromuscular junction, participates in nerve-impulse transmission. However, soluble AChE found in abundance in the muscle, but outside of the neuromuscular junction, contributes little to muscle strength. This was demonstrated by COLQ knockout mice that had normal AChE levels in all tissues, but had no collagen-tailed AChE in the neuromuscular junction (Feng et al., 1999). COLQ knockout mice have the same weak muscles as AChE^{-/-} mice (Feng et al., 1999; Minic et al., 2003; Adler et al., 2004). A second example is end-plate AChE deficiency in humans, where a mutation in the collagen-tail is responsible for AChE deficiency in the neuromuscular junction as well as for weakness and abnormal fatigability on exertion (Donger et al., 1998; Ohno et al., 1998). These examples suggest that the soluble AChE produced by our gene therapy vector did not improve muscle strength because the AChE was not in the right location to participate in nerve impulse transmission.

Key research accomplishments

- New methods for measuring resistance to OP toxicity have been developed.
- Three lines of transgenic mice, expressing human butyrylcholinesterase mutant G117H have been established. They express 0.5, 1.5, and 2 µg/ml human BChE in plasma. These mice are resistant to OP toxicity.
- Protection against OP toxicity is achieved with low levels of G117H butyrylcholinesterase in transgenic mice because these mice express G117H butyrylcholinesterase in all tissues. This result shows that tissues besides plasma should be evaluated when administering the therapeutic scavenger protein butyrylcholinesterase.
- Gene therapy with adenoassociated virus expressing acetylcholinesterase prolonged the lifespan of AChE knockout mice. The mechanism of protection is hypothesized to be reduction of acetylcholine levels in brain and consequently a reduction in susceptibility to seizures.

Reportable outcomes

Published manuscripts

Wang Y, Boeck AT, Duysen EG, Van Keuren M, Saunders TL and Lockridge O (2004) Resistance to organophosphorus agent toxicity in transgenic mice expressing the G117H mutant of human butyrylcholinesterase. *Toxicol Appl Pharmacol* **196**:356-366.

Wang Y, Schopfer LM, Duysen EG, Nachon F, Masson P and Lockridge O (2004) Screening assays for cholinesterases resistant to inhibition by organophosphorus toxicants. *Anal Biochem* **329**:131-138.

Schopfer LM, Boeck AT, Broomfield CA, Lockridge O. (2004) Mutants of human butyrylcholinesterase with organophosphate hydrolase activity; evidence that His117 serves as a general base catalyst in the hydrolysis of echothiophate. *J Med Chem Defense* **2**: 1-21 (online publication)

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- Masson P, Goldstein BN, Debouzy JC, Froment MT, Lockridge O and Schopfer LM (2004) Damped oscillatory hysteretic behaviour of butyrylcholinesterase with benzoylcholine as substrate. *Eur J Biochem* **271**:220-234.
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New animals and reagents created for this project

- Transgenic mice resistant to organophosphorus toxicants
- Adenoassociated virus that expresses mouse acetylcholinesterase
- Adenoassociated virus that expresses human acetylcholinesterase

Conclusions. Summary of results to include the implications of the research.

Our goal is to provide new therapeutics, in the form of genes and proteins, for protection against organophosphorus nerve agents.

- The transgenic G117H mouse is resistant to OP toxicity.
 - This shows that the right gene introduced into the chromosome provides protection.
 - The protection is life-long because the gene is a stable component of the genetic make-up of the animal.
 - Relatively low levels of G117H BChE provide protection.
 - These results imply that humans could be protected from nerve agent toxicity by gene therapy with G117H butyrylcholinesterase.
- Gene therapy with adenoassociated virus results in expression of mouse AChE
 - Expression of mouse AChE is prolonged, continuing for at least 5 months.
 - The maximum level of expression of mouse AChE in mouse plasma is about 30-50% of the level of endogenous mouse AChE.
 - The maximum level of expression of mouse AChE in mouse muscle is 1000% of the level of expression of mouse AChE.
 - No antibody against mouse AChE was produced when the virus was delivered to AChE+/- mice.
 - The lifespan of AChE knockout mice was prolonged by gene therapy with

adenoassociated virus.

- These results imply that a gene therapy vector could be used to deliver acetylcholinesterase to provide protection against nerve agent toxicity.

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