The butyrylcholinesterase knockout mouse as a model for human butyrylcholinesterase deficiency

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Abbreviation: BChE, butyrylcholinesterase; AChE, acetylcholinesterase; +/-, wild type; +/- heterozygote; -/-, nullizygote;

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Abstract

Butyrylcholinesterase (BChE) is an important enzyme for metabolism of ester drugs. Many humans have partial or complete BChE deficiency due to genetic variation. Our goal was to create a mouse model of BChE deficiency to allow testing of drug toxicity. For this purpose we created the BChE knockout mouse by gene targeted deletion of a portion of the BCHE gene (accession # M99492). The BChE-/- mouse had no BChE activity in plasma, but had low residual butyrylthiocholine hydrolase activity in all other tissues attributed to carboxylesterase ES-10. The BChE-/- mouse had a normal phenotype except when challenged with drugs. Nicotinic receptor function as indicated by response to nicotine appeared to be normal in BChE-/- mice, but muscarinic receptor function as measured by response to oxotremorine and pilocarpine was altered. Heart rate, blood pressure, and respiration measured in a Vevo imager, were similar in BChE+/+ and BChE-/- mice. Like BChE-/- humans, the BChE-/- mouse responded to succinylcholine with prolonged respiratory arrest. Bambuterol was not toxic to BChE-/- mice, suggesting it is safe in BChE-/- humans. Challenge with 150 mg/kg pilocarpine IP, a muscarinic agonist, or with 50 mg/kg butyrylcholine IP, induced tonic-clonic convulsions and death in BChE-/- mice. This suggests that butyrylcholine, like pilocarpine, binds to muscarinic receptors. In conclusion, the BChE-/- mouse is a suitable model for human BChE deficiency.
Introduction

The butyrylcholinesterase (BChE) knockout mouse, recently developed in our laboratory (Li et al., 2006), has no BChE enzyme and therefore provides a model for BChE deficiency in humans. Since BChE has a role in drug ester hydrolysis, it is expected that the mouse will aid in identifying drugs that are not harmful to BChE deficient humans, as well as drugs and chemicals especially toxic to BChE deficient humans. The BChE-/- mouse may also be useful for identifying a physiological function for BChE (Duysen et al., 2007).

Genetic variants of human BChE are common. The K variant, Ala539Thr, is carried on one allele by 1 out of 4 persons (Bartels et al., 1992). Homozygous K variants have a 33% reduction in BChE activity. People who carry the K variant allele, as well as other BChE deficiency alleles, are at reduced risk of developing Alzheimer's disease and the progress of their cognitive decline is slower than that of people with wild-type BChE (O'Brien et al., 2003; Holmes et al., 2005). The genetic variants responsible for succinylcholine apnea are less frequent than the K variant. In European and American populations 1 in 3000 persons is homozygous for the atypical variant, Asp70Gly, while 1 in 25 is a heterozygous carrier. The homozygous silent BChE variant, with 0 to 10% of normal BChE activity in plasma, has a frequency of 1:100,000 in European and American populations, while heterozygotes occur at a frequency of 1 in 160. Selected populations such as the Vysya of India and the Inuit of Alaska are homozygous for silent BChE at a frequency of 1 in 50 (Manoharan et al., 2006).

BChE deficient humans are healthy (Manoharan et al., 2007). Their BChE deficiency is revealed in the operating room when short acting muscle relaxants,
succinylcholine or mivacurium, are injected iv for endotracheal intubation, tonsillectomy, or electroshock therapy (Lockridge, 1990; Ostergaard et al., 2000). Humans with atypical or silent BChE are paralyzed for 2 h after a dose that paralyzes the average individual for 3-5 min. People with atypical or silent BChE do not hydrolyze succinylcholine in blood so that a huge overdose reaches the receptors. One of our goals was to determine whether BChE-/- mice have the same unusual response to succinylcholine as BChE-/- humans.

Bambuterol is an oral bronchodilator prescribed for treatment of asthma, bronchitis, and emphysema. It is a dicarbamate prodrug of terbutaline (Tunek and Svensson, 1988). Bambuterol is slowly converted to terbutaline by the action of BChE. Bambuterol is a poor substrate for BChE, but a very good inhibitor of BChE with an inhibition constant of 2.7 x 10^-9 M (Tunek and Svensson, 1988). Bambuterol does not inhibit AChE at clinical doses. It has been reported that a 30 mg dose of bambuterol in adult humans inhibits 90% of the plasma BChE and that the bambuterol-induced BChE deficiency causes succinylcholine apnea (Bang et al., 1990). AChE-/- mice die when they are treated with bambuterol, suggesting that inhibition of BChE is responsible for their death (Xie et al., 2000; Chatonnet et al., 2003). Since no drug is completely specific in vivo it was possible that other enzymes were also involved in this response. The BChE-/- mouse would be expected to be resistant to bambuterol toxicity, if bambuterol were a specific inhibitor of BChE.

Muscarinic and nicotinic receptor levels are down regulated in AChE deficient mice (Li et al., 2003; Volpicelli-Daley et al., 2003; Adler et al., 2004). In this study receptor function in BChE-/- mice was tested using the nicotinic
receptor agonist nicotine, and the muscarinic receptor agonists oxotremorine and pilocarpine.

Since butyrylcholine is a good substrate for BChE, but a very poor substrate for AChE, it was of interest to determine whether butyrylcholine would have an effect on BChE-/- mice.

In this report we describe the phenotype of the BChE knockout mouse. BChE-/- animals, like BChE deficient humans, are unusually sensitive to succinylcholine. Bambuterol had no toxic effects on the BChE-/- mouse even at high doses. Pilocarpine and butyrylcholine were lethal to BChE-/- but not to BChE+/+ mice.
Methods

Reagents. Bambuterol ([3-(dimethylcarbamoyloxy)-5-[1-hydroxy-2-(tert-butylamino) ethyl] phenyl] N,N-dimethylcarbamate) was a gift from Astra Draco AB, Lund, Sweden. Pilocarpine hydrochloride ((3S, 4R)-3-Ethylidihydro-4-[(1-methyl-1H-imidazol-5-yl) methyl]-2(3-H)-furanone-hydrochloride) was purchased from MP Biomedicals, Irvine, California. Benzil (1,2-diphenylethanenedione), Succinylcholine chloride dihydrate (Bis(trimethylammonioethyl) succinate chloride), ethopropazine (10-[2-(Diethylamino) propyl] phenothiazine hydrochloride), acetylthiocholine iodide ((2-Mercaptoethyl)trimethylammonium iodide acetate), butyrylthiocholine iodide ((2-Mercaptoethyl)trimethylammonium iodide butyrate), p-nitrophenylacetate (Acetic acid 4-nitrophenyl ester), eserine (1,2,3,3a,8a-hexahydro-1,3a, 8-trimethyl-, methylcarbamate (ester), (3aS,8aR)-pyrrolo(2,3-b)indol-5-ol), nicotine ((S)-3-(1-Methyl-2-pyrroli-dinyl)pyridine), oxotremorine (1-(4-(1-Pyrrolidinyl)-2-butynyl)-2-pyrroloidinone), fasciculin 2 (Sigma F4293) and other chemicals were from Sigma-Aldrich, St. Louis, MO.

Animals. 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. BChE-/- knockout mice were produced by gene-targeting. Out of 30 chimeras only 3 transmitted the BChE-/- allele to progeny. The chimeras were bred to mice in strain 129S1/SvImJ (Jackson Labs) to make BChE-/- mice with a uniform genetic background. For toxicity studies we produced the BChE-/- mice by mating heterozygotes. The reason for this mating scheme was to have +/- and +/- controls that matched the test animals in genetic background, age, and living
conditions. BChE-/- mice are fertile, so we also maintain a BChE-/- line by breeding homozygotes.

The AChE-/- mice (Xie et al., 2000) are in strain 129Sv. The AChE knockout colony is maintained at the University of Nebraska Medical Center by breeding heterozygotes. Pregnant AChE+/- dams are fed 11% fat pellets as well as liquid Ensure (Duysen et al., 2002). AChE-/- mice are fed liquid Ensure in a dish after weaning on postnatal day 14. AChE+/- mice are now available from Jackson Labs (stock # 005987 129-Ache^{tm1Loc/J}).

Double knockout mice, AChE-/- BChE-/-, were made by breeding AChE+/+ mice to BChE-/- mice. AChE+/- rather than AChE-/- mice had to be used for generating the double knockouts because AChE-/- mice do not breed (Duysen et al., 2002). Double knockout mice were alive on embryonic day 19, when they were removed by C-section.

**Genotyping.** Mice were genotyped by PCR of DNA in hair (Schmitteckert et al., 1999). At least 12 hair follicles were pulled from the flank of day 14 or older mice with fine-pointed forceps and placed into a microfuge tube. DNA was extracted by adding 50 µl of 50 mM NaOH, heating for 15 min at 95°C in the thermocycler, and freezing for at least 15 min at -80°C. One µl of DNA solution was added to a 0.5 ml PCR tube containing 3 µl nuclease-free water, 5 µl of GoTaq Green Master Mix (a mixture of dNTP, buffer, dye, and DNA polymerase from Promega, Madison, WI, Cat#M7122), and 0.5 µl of each 0.2 µg/µl primer. The DNA was amplified for 35 cycles in a Perkin Elmer Cetus thermocycler, Waltham, MA.
The primers for the wild-type BChE gene were 5’ GTGGAATCCAAACAAACC (sense) and 5’ TTCTGTTTCTGGCTTCCTC (antisense). The 485 bp product encodes nucleotides 347-831 in accession # M99492, gi: 191579 encoding mouse BChE. The primers for the BChE-/- gene amplified the NEO gene to produce a 321 bp product. The primer sequences were 5’ AATGGGCAGGTAGCCGGATCAAGCG and 5’ ACAGACAATCGGCTGCTCTGATGC.

**Behavioral tests.** Grip strength was measured by placing the mouse on a screen and rotating the screen 180°. If the mouse could cling to the screen for 60 seconds or climbed to the top of the screen, it was scored as having normal grip strength. 3 BChE+/+, 6 BChE+/-, and 6 BChE-/- mice were tested for grip strength at age 24-30 days.

Righting reflex was measured as the time it took a mouse to return to an upright position after the mouse had been placed on its back. 4 BChE+/+, 2 BChE+/- and 6 BChE-/- mice were tested for righting reflex on postnatal day 4.

**Surface body temperature.** Body temperature was measured by placing a surface Microprobe MT-D, Type T thermocouple (Physitemp Instruments Inc., Clifton, NJ) in the armpit of the mouse. The probe was attached to a digital thermometer, Thermalert model TH-5.

**Tissue extraction.** Animals were euthanized with carbon dioxide and perfused transcardially with 50 ml saline to wash out the blood. Organs were homogenized
in 10 volumes of ice-cold 50 mM potassium phosphate pH 7.4, 0.5% Tween-20, in a Polytron homogenizer (Brinkmann Instruments, Canada) for 10 seconds. The homogenate was clarified by centrifugation in a microfuge. It was important to remove all turbid material because the particulate matter gave inconsistent activity readings in the Ellman assay. Each homogenate was centrifuged 3 times and the supernatant transferred to a new tube. The supernatant was ready for assay when centrifugation brought down no pellet. Free sulfhydryl groups in tissue extracts were depleted by 30 min preincubation with 0.5 mM dithiobisnitrobenzoic acid before addition of substrate.

**Enzyme activity assays.** Enzyme activity was measured in 0.1 M potassium phosphate pH 7.0 at 25°C in 2 ml reaction volume in a Gilford spectrophotometer interfaced via a MacLab data recorder (ADInstruments, Colorado Springs, CO) to a Macintosh computer. AChE activity was assayed with 1 mM acetylthiocholine in the presence of 0.01 mM ethopropazine to inhibit BChE. The ethopropazine concentration was increased to 0.1 mM when assaying liver and intestine extracts. BChE activity was assayed with 1 mM butyrylthiocholine at 412 nm (Ellman et al., 1961). The rate of hydrolysis of 50 µM benzoylcholine in 0.067 M sodium potassium phosphate buffer pH 7.4, 25°C, was recorded at 240 nm where ΔE=6700 M⁻¹ cm⁻¹. Samples assayed for carboxylesterase activity were preincubated for 30 min with 10 µM eserine to inhibit AChE and BChE, as well as with 10 mM EDTA to inhibit paraoxonase, before addition of p-nitrophenylacetate to a final concentration of 2.5 mM. The absorbance at 400 nm was recorded and µmoles product per minute were calculated from the
extinction coefficient $E = 9000 \text{ M}^{-1} \text{ cm}^{-1}$. A unit of AChE, BChE, and carboxylesterase activity was defined as micromoles substrate hydrolyzed per minute.

**Nondenaturing gel electrophoresis.** 4-30% acrylamide gradient gels, 0.75 mm thick, were prepared in a Hoefer apparatus (SE600, Fort Lee, NJ). Electrophoresis was at 4˚C for 30 h at a constant voltage of 200 V. Gels were stained for BChE activity in the presence of 10 mM butyrylthiocholine iodide (Karnovsky and Roots, 1964). A higher butyrylthiocholine concentration than the normal 2 mM was used because the liver and intestine butyrylthiocholine hydrolases had a higher Km value than BChE. After 1 hour in the Karnovsky and Roots staining solution, the gel was counterstained for carboxylesterase activity by placing the gel into 100 ml of 50 mM TrisCl pH 7.5 with 0.05 g alpha-naphthylacetate (dissolved in ethanol) and 0.05 g of solid Fast Blue RR. Much of the Fast Blue RR remained solid, but this did not diminish the appearance of bands. After 10 min, the gel was rinsed with water, photographed, and stained with Coomassie blue. Other nondenaturing gels were stained in the presence of 2 mM acetylthiocholine iodide by the method of Karnovsky and Roots to reveal bands for both AChE and BChE.

**Treatment with succinylcholine.** Adult male BChE+/+, +/-, -/- and AChE-/- mice (n = 3 of each genotype) were treated intraperitoneally with 1.0 mg/kg succinylcholine chloride dissolved in saline. Since succinylcholine is unstable in
solution, a fresh succinylcholine solution was prepared for each experiment. The animals were 2-3 months old. The neurotoxicity of succinylcholine was evaluated with a functional observational battery of tests developed by McDaniel and Moser (McDaniel and Moser, 1993).

**Treatment with bambuterol.** Adult female BChE+/+ and BChE-/- mice (n =3 of each genotype) were treated with 75 mg/kg bambuterol IP. These doses are a huge excess over the 0.05 mg/kg IP that inhibits 94% of BChE activity in mouse plasma (Chatonnet et al., 2003). Animals were observed for 30 min post dosing. Surface body temperature was recorded at 5, 10, 15, 20, and 30 min post dosing. Plasma samples were collected into heparinized hematocrit tubes via the Saphenous vein before and 30 min after treatment with bambuterol. Plasma samples were tested for BChE, AChE, and carboxylesterase activity.

**Treatment with butyrylcholine, acetylcholine, propionylcholine, or fasciculin.** Adult male BChE+/+ and -/- mice (n =3 per group) were treated IP with 50 mg/kg butyrylcholine, acetylcholine, propionylcholine, or 0.5 mg/kg fasciculin 2.

**Treatment with (-)-Nicotine: nAChR function.** Adult female BChE +/- (n=4) and BChE -/- (n=4) mice were treated with 1.5 mg/kg nicotine subcutaneously (SC). The animals were tested 5 min post dosing for antinociceptive response to nicotine by submerging the animal’s tail in a 49º C water bath. The time for the animal to respond to the warm water with a flick of its tail was recorded. A cut-off
of 15 seconds was implemented to avoid damage to the tissue of the tail. One hour prior to treatment a baseline response time was recorded for each animal. Observations for toxicity and surface body temperatures were recorded prior to dosing and every 5 min through 30 min post dosing.

**Treatment with oxotremorine: M2 and M4 mAChR function.** Adult female BChE +/+ (n=5) and BChE -/- (n=5) mice were treated with 0.1 mg/kg oxotremorine SC. The animals were tested 30 min post dosing for antinociceptive and toxic response to oxotremorine by the same methods as described above in the nicotine testing section.

**Treatment with pilocarpine 150 mg/kg: M1 mAChR function.** Adult male BChE+/+ (n=5) and BChE-/- (n=5) mice were treated with 150 mg/kg pilocarpine IP. Body temperatures and observations were recorded every 5 min through 30 min, and every 15 min through 3 hours.

**Treatment with pilocarpine 50 mg/kg: cardiac effects.** The heart rate, ejection fraction, cardiac output, stroke volume, and respiration were measured in adult male BChE+/+ (n= 5) and BChE-/- (n= 5) mice following treatment with 50 mg/kg pilocarpine IP. A Vevo 770™ High-Resolution In Vivo Imaging System (VisualSonics, Toronto, ON, Canada) along with a RMVTM 707B “High Frame” Scan-head were used to image the heart of each mouse. The Scan-head has a center frequency of 30 MHz and a band frequency of 15-45 MHz. The mice were prepared for imaging by removing the hair on their ventral section with Nair®. The mice were anesthetized with 3% isoflurane and then maintained with 1.25% isoflurane. Each mouse was placed on a stage with ECG electrode
pads and an isoflurane nose cone. Care was taken to ensure that the anesthesia was delivered at the same rate and for the same length of time prior to recording the measurements in each animal. Mouse temperature and respirations were measured using a rectal probe and the heart rate was taken with the ECG electrodes. Warmed ultrasonic gel was applied to the ventral section of each animal. After the baseline heart rate and temperature were established each animal was injected IP with 50 mg/kg pilocarpine. A short-axis view of the left ventricle was imaged with the scan-head placed horizontally to the heart. The images were saved in M-Mode for analyses. All the data were analyzed by an Advanced Cardiovascular Software Package (VS-11560, VisualSonics, Toronto, ON, Canada). The analyses required a tracing of three full contractions in both systole and diastole. A second procedure involved marking the divisions between the intra-ventricular septum (IVS, the muscular wall between the heart ventricles), the left ventricle (LVID, the left lower chamber of the heart that receives blood from the left atrium and pumps it out under high pressure through the aorta to the body), and the left ventricle posterior wall (LVPW). From the measured parameters the cardiac function data were then automatically calculated by the software. The cardiac parameters were recorded at 5 min post dosing and every 15 min through 90 min post dosing.

**Statistics.** All the numerical data are reported and graphed ± standard deviation. The cardiac data were analyzed by independent samples t-test, assuming equal
variance using the Microsoft Excel statistical program. Significance was determined to be $p<0.05$. 
Results

Part of exon 2 deleted. The mouse has one gene for BChE and this gene is located in the middle of mouse chromosome 3 (GeneID 12038 Locus tag: MGI:894278). The 67 kb mouse BCHE gene contains 4 exons. Exons 2, 3, and 4 encode the 29 amino acid signal peptide and 574 amino acids of the mature BChE protein. The BChE knockout mouse was made by gene-targeted deletion of 485 bp from the 3' end of intron 1 and 406 bp from the 5' end of exon 2, deleting a total of 891 bp as indicated in Figure 1. The deletions removed the splice junction between intron 1 and exon 2, as well as the entire signal peptide including the translation start site, and the first 102 amino acids of the mature BChE protein. Asp 70, an important component of the peripheral anionic site, and Trp 82, the residue responsible for binding positively charged compounds in the active site, were deleted. These deletions ensured that no BChE protein could be synthesized. Southern blotting confirmed these deletions (Li et al., 2006).

Phenotype of BChE knockout mice. To date about 600 BChE-/- mice have been born. The BChE-/- mice have no apparent differences in appearance or behavior from littermates. They gain weight at the same rate, and attain the same adult weight as their littermates when they are fed a standard 5% fat diet. BChE-/- mice maintain a normal body temperature of 36.5-38°C by the age of weaning on postnatal day 21, at the same developmental stage as their BChE+/- and +/+ littermates (Figure 2).
The fertility of BChE-/- mice is normal. The average number of pups per litter born to BChE-/- mice is 6±0.5, the same as for BChE+/+ and +/- mice. The mice produce about 5 litters in year 1 of their life. They are bred starting at 7 weeks of age.

BChE-/- and +/- mice gained the righting reflex at the same age as wild-type mice on postnatal day 4. They have normal grip strength, normal body posture, and normal gait. Their skeletons are normal in structure. It is concluded that muscle strength is not adversely affected by absence of BChE.

We conclude that BChE-/- mice are healthy and indistinguishable from wild-type mice under normal conditions.

**Absence of BChE activity.** The BChE knockout mouse has no butyrylthiocholine hydrolase activity in plasma. This is visualized in Figure 3 where a nondenaturing gel stained for BChE activity with butyrylthiocholine shows a blank lane for serum from BChE-/- mouse (lane 2). By contrast, serum from wild-type mouse shows 3 BChE bands (lane 1). The slowest migrating band is tetrameric BChE; this band is the most intense and represents 95% of BChE activity in serum. Dimer and monomer BChE bands in wild-type serum stain weakly. When a gel is stained longer, weak trimer and a second type of BChE dimer band appear.

The BChE in serum is synthesized in the liver. Since BChE tetramers are stable in serum, but dimers and monomers are rapidly cleared, we had expected the liver BChE to consist predominantly of tetramers. However, lane 3 in Figure 3 shows a broad BChE band in the region of dimer and a faint tetramer band in
BChE+/+ liver. The broad band could be incompletely glycosylated BChE forms. No BChE was present in BChE-/- liver and intestine (lanes 4 and 5 in Figure 3).

Tissue extracts from BChE-/- mice had low but detectable activity with 1 mM butyrylthiocholine (Table 1). This activity was not due to spontaneous hydrolysis of substrate in buffer, but was enzyme catalyzed activity. The source of this activity was carboxylesterase.

All tissues tested in wild-type and heterozygous mice had BChE activity (Table 1). The highest BChE activity was in intestine, liver, and plasma, followed by heart, lung, diaphragm, brain, and quadriceps muscle. BChE+/- tissues had about 50% of the BChE activity present in BChE+/+ tissues.

**Carboxylesterase ES-10 hydrolyzes butyrylthiocholine.** Assay of BChE activity in BChE-/- tissue extracts gave the surprising result that BChE-/- tissues had low but detectable activity with 1 mM butyrylthiocholine (Table 1). The only tissue with zero BChE activity was plasma. Since butyrylthiocholine is hydrolyzed 30-50 fold more slowly (Hosea et al., 1995) by AChE (EC 3.1.1.7) than by BChE (EC 3.1.1.8) we first checked the possibility that the butyrylthiocholine hydrolase activity was due to AChE. Liver and intestine from double knockout embryonic day 19 mice, AChE-/- BChE-/-, were found to have about 0.1-0.2 units/g of activity with butyrylthiocholine, similar to the activity for BChE-/- liver and intestine. This result ruled out the possibility that the activity was due to AChE. Additional evidence that the butyrylthiocholine hydrolase enzyme was not AChE was the finding that acetylthiocholine was not hydrolyzed by AChE-/- BChE-/- tissues. In conclusion, the fact that AChE-/- BChE-/- tissues
hydrolyzed butyrylthiocholine, but not acetylthiocholine, means the butyrylthiocholine hydrolase is neither AChE nor BChE, but is a different esterase.

We hypothesized that the liver and intestine butyrylthiocholine hydrolase activity was due to carboxylesterase (EC 3.1.1.1) and performed additional experiments to characterize the enzyme.

The BChE-/- liver had a Km value for butyrylthiocholine of 6±2 mM, a value 100 fold higher than the Km value of mouse BChE (Hosea et al., 1995), but closer to the Km value of 40 mM for one of the carboxylesterases in rat liver (Mentlein et al., 1984). These results supported the conclusion that the enzyme was not BChE.

Benzoylcholine is a good substrate for BChE but not for carboxylesterase. It was found that BChE-/- liver and intestine did not hydrolyze 50 µM benzoylcholine.

Inhibitors of BChE activity were tested. Ethopropazine at 0.1 mM and bambuterol at 0.5 µM did not inhibit the activity in liver and intestines of BChE-/- mice. By contrast, BChE+/+ liver and intestine activities were inhibited 97% by these inhibitor concentrations. The classical cholinesterase inhibitor, eserine, which at 10 µM inhibits BChE activity completely, had only a slight effect, inhibiting activity about 20%.

A specific carboxylesterase inhibitor was tested. The commercially available diketone compound, benzil, was recently identified as a specific inhibitor of liver and intestine carboxylesterases from human and rabbit (Wadkins et al., 2005). We found that 4.5 µM benzil inhibited the liver and intestine
butyrylthiocholine hydrolase activity of BChE-/- mice about 90%. Benzil had minimal inhibitory effect on mouse AChE and BChE activity, in agreement with Wadkins et al., 2005, who found no inhibition of human AChE and BChE by concentrations of benzil as high as 0.1 mM. We concluded that the residual butyrylthiocholine hydrolase activity was due to a carboxylesterase in liver and intestine.

The carboxylesterase in mouse plasma is different from the carboxylesterases in mouse liver and intestine. Mouse plasma carboxylesterase did not have butyrylthiocholine hydrolase activity, and was not inhibited by 4.5 µM benzil. Proteomic analysis has identified the major carboxylesterase in mouse plasma as ES-1, accession # gi:22135640 in strain 129Sv/J mice (Bhat et al., 2005) and gi:6679689 in strain C57BL/6J x CBA F1.

The carboxylesterase inhibitors bis(4-nitrophenyl)phosphate and 2-(O-cresyl)-4H-1:3:2-benzodioxaphosphorin-2-oxide are considered to be specific inhibitors of rodent carboxylesterases (Mentlein et al., 1984; Maxwell et al., 1987). However, these compounds were not useful for our purpose because they inhibited both carboxylesterase and BChE.

To visualize the carboxylesterase responsible for residual butyrylthiocholine hydrolase activity in BChE-/- liver and intestine, we stained a nondenaturing gel for enzyme activity with butyrylthiocholine. Figure 3 shows that the butyrylthiocholine hydrolase activity in BChE-/- liver and intestine migrated at the same position as albumin in plasma. The carboxylesterase with butyrylthiocholine hydrolase activity is labeled ES-10. There was a single band of butyrylthiocholine hydrolase activity in BChE-/- liver. The same major band
was present in $\text{BChE}^{+/+}$ liver and in $\text{BChE}^{-/-}$ intestine. The $\text{BChE}^{-/-}$ intestine
had an additional 3 weak bands, which may be products of the same gene with
post-translational modifications such as aggregation, degradation by proteolysis,
or heterogeneity in glycosylation.

We conclude that a carboxylesterase in liver and intestine, but not in
mouse plasma, has the ability to hydrolyze butyrylthiocholine.

**Normal $\text{AChE}$ activity.** Plasma from $\text{BChE}^{-/-}$ mice has normal $\text{AChE}$ activity.
The bands of $\text{AChE}$ activity are visualized in Figure 4 on a nondenaturing gel
stained to reveal both $\text{AChE}$ and $\text{BChE}$ activity with acetylthiocholine.
Comparison of bands from $\text{AChE}^{-/-}$, $\text{BChE}^{-/-}$, and wild-type plasma allows
assignment of each band as either $\text{AChE}$ or $\text{BChE}$. There are 4 bands of $\text{AChE}$
activity and 5 bands of $\text{BChE}$ activity in wild-type mouse plasma (Figure 4, lane
2). The 5 bands in $\text{AChE}^{-/-}$ plasma (lane 3) are $\text{BChE}$; the fastest migrating
band is a monomer, the next two bands are dimers, followed by a weak trimer
band, and an intense tetramer $\text{BChE}$ band. The 4 bands in $\text{BChE}^{-/-}$ plasma
(lane 4) are $\text{AChE}$; the monomer band for $\text{AChE}$ is broad. The $\text{AChE}$ tetramer
migrates faster than the $\text{BChE}$ tetramer because $\text{AChE}$ is less glycosylated than
$\text{BChE}$ and therefore has a lower molecular weight. Mouse $\text{AChE}$ has 3 N-linked
glycans and 583 amino acids per subunit, whereas mouse $\text{BChE}$ has 7 N-linked
glycans and 574 amino acids per subunit (Rachinsky et al., 1990).

Tissues of $\text{BChE}$ deficient mice had normal $\text{AChE}$ activity, with brain
having the highest $\text{AChE}$ activity followed by plasma and muscle. All tissues
tested had AChE activity. There was a 20-fold range in AChE activity from 1.2 u/g in brain to 0.06 u/g in liver.

**Normal carboxylesterase activity.** Carboxylesterase activity was independent of BChE genotype. All mouse tissues had much higher carboxylesterase than AChE or BChE activity. The highest carboxylesterase activity was in liver and intestine where the activity was 110 u/g. Plasma carboxylesterase activity was about 20 u/ml. The lowest carboxylesterase activity was in quadriceps muscle, diaphragm, and brain where activity was 5-6 u/g.

**Succinylcholine.** To determine whether the BChE knockout mouse is a model for BChE deficiency in humans, animals were treated with 1.0 mg/kg succinylcholine IP. This dose had very little effect on wild-type mice. The only signs of toxicity in wild-type mice were decreased activity and voiding of a large volume of urine. In contrast, all BChE-/- mice died of respiratory failure within 11 min. Heterozygous +/- mice did not die, but they had flattened posture, muscle fasciculation, fixed eyes, decreased response to being handled, ataxic gait, impaired mobility, reduction in body temperature, tachycardia, and they voided large volumes of urine. There was no lacrimation and salivation. The BChE+/- animals did not have breathing difficulties. BChE+/- mice completely recovered within 30 min, consistent with the short duration of action of succinylcholine in humans.

We conclude that BChE-/- and +/- animals are a suitable model for succinylcholine sensitivity in BChE deficient humans.
Succinylcholine treated AChE-/- mice had more pronounced signs of toxicity than succinylcholine treated BChE-/- mice and they died more quickly. In addition to the signs of toxicity described above for BChE+/- mice, the AChE-/- mice had severe muscle fasciculations, hunched posture, bulging eyes, bobbing head, and distressed breathing. Death was due to respiratory failure. All AChE-/- mice died within 6 min.

**Bambuterol.** BChE+/+ and -/- mice showed only mild signs of toxicity with 75 mg/kg IP and no signs of toxicity with lower doses. The mild signs were hunched posture, decreased activity, and piloerection. There was no decrease in body temperature. Activity assays of plasma samples showed that all of the BChE, 50% of AChE, and 20% of the carboxylesterase was inhibited in mouse plasma drawn 30 min after 75 mg/kg bambuterol IP. The behavioral response of BChE+/+ and -/- mice and the degree of enzyme inhibition were similar. In contrast, AChE-/ mice died from 0.05 mg/kg bambuterol injected SC, a dose that inhibited BChE in plasma by 94% but had no effect on BChE activity in brain (Chatonnet et al., 2003). AChE-/ mice died of respiratory failure (Chatonnet et al., 2003).

**Butyrylcholine, acetylcholine, propionylcholine, and fasciculin toxicity.** BChE-/ mice treated with 50 mg/kg butyrylcholine IP had severe cholinergic signs of toxicity including tonic-clonic convulsions, muscle fasciculations, body tremor, myoclonic jerks, piloerection, heaving respiration, lacrimation, and salivation. They died within 2-3 min. In contrast BChE+/+ mice had very mild
cholinergic signs of toxicity and survived. Control studies with 50 mg/kg IP of the sodium salt of butyric acid, and 50 mg/kg IP of choline chloride showed no toxic effects in BChE-/- mice. This result indicated that butyrylcholine, but not its hydrolysis products, were toxic.

Butyrylcholine was also toxic to mice that have no AChE. The AChE-/- mice had cholinergic signs of toxicity but they were different from those in BChE-/- mice. They had lacrimation, salivation, piloerection, and heaving respiration, but they had no convulsions, muscle fasciculations, body tremor, myoclonic jerks, and they did not die within 30 min. The milder toxicity in AChE-/- mice may be explained by hydrolysis of butyrylcholine to inactive products by BChE.

At 24 hours post dosing, the AChE-/- mice had a bloated abdomen. At 48 hours the distension had increased and animals were anorexic. Animals were euthanized and their internal organs examined. The stomach, cecum, large and small intestines were found to be severely distended with gas. No explanation for these signs of toxicity is available.

Propionylcholine and acetylcholine also caused severe cholinergic signs of toxicity in BChE-/- mice though they did not cause convulsions, and all mice recovered within 1 hour.

Fasciculin is a specific AChE inhibitor (Radic and Taylor, 2001). As expected from our studies with other AChE inhibitors (Duysen et al., 2007), the BChE-/- mice had severe cholinergic signs of toxicity after treatment with fasciculin and were moribund after 25 min. The wild-type mice also had severe cholinergic signs of toxicity but onset was slower and the mice became moribund after 18 h. In contrast, AChE-/- mice were resistant to the toxic effects of
fasciculin. The toxic effects of fasciculin are explained by accumulation of excess acetylcholine following inhibition of AChE. Since AChE-/- mice have no AChE, they are unaffected by specific AChE inhibitors including fasciculin, huperzine A, and donepezil (Boudinot et al., 2005; Duysen et al., 2007). On the other hand, AChE-/- mice are supersensitive to the toxic effects of acetylcholine. AChE-/- mice died within 30 sec of receiving 50 mg/kg acetylcholine IP, a dose that had only mild effects on wild-type mice.

**Nicotine.** To determine possible functional changes of the nicotinic acetylcholine receptors (nAChR) in BChE-/- mice, the nAChR agonist nicotine was administered to BChE+/+ and BChE-/- mice at a dose (1.5 mg/kg) previously shown to have antinociceptive effects (Damaj et al., 1998). The response to the tail flick test was not significantly different between the genotypes. The pre-dose tail flick response times were 5.2±1.9 sec for BChE+/+ mice and 5.4±0.8 sec for BChE-/- mice. At 5 min post-dosing three animals of each genotype did not respond to the stimuli by the 15 sec cut-off, one animal in the BChE+/+ group flicked its tail at 6.1 sec and one animal in the BChE-/- group responded at 5.3 sec. No differences in toxicity between the genotypes were observed. Both the BChE+/+ and -/- mice experienced a 2ºC drop in body temperature by 10 min post dosing, with recovery to baseline temperature by 20 min post dosing. It was concluded that nicotinic acetylcholine receptor function appeared to be normal in BChE-/- mice.
Oxotremorine. The function of muscarinic acetylcholine receptor (mAChR) subtypes M2 and M4 was tested in BChE-/− mice using the mAChR agonist oxotremorine at a dose (0.1 mg/kg SC) shown to have significant antinociceptive effects in mice (Duttaroy et al., 2002). No significant differences in tail flick response were observed between the genotypes pre-dosing (BChE+/+ 6.2±2.0 sec; BChE-/− 5.6±3.0 sec) or at 30 min post dosing (BChE+/+ >15 sec/BChE-/− >15 sec) with oxotremorine. Observations of toxicity between the genotypes were similar with the exception of severe whole body tremors in 4 of 5 BChE+/+ mice compared to only 1 of the BChE-/− mice. Both genotypes showed a significant loss of body temperature through 60 min post dosing with the lowest temperature in both genotypes at 20 min post dosing (BChE 29.9±2.1ºC; BChE-/− 30.3±1.8 ºC). It was concluded that M2 and M4 muscarinic receptor function appeared to be somewhat altered in BChE-/− mice.

Pilocarpine toxicity. Functional changes of muscarinic acetylcholine receptors were tested with the nonselective muscarinic agonist pilocarpine. Following treatment with 150 mg/kg pilocarpine IP, BChE+/+ and BChE-/− mice had similar signs of toxicity including severe tremors, myoclonic jerks, opisthotonus posture, mucus covered feces, increased salivation, depressed respiration, ataxic gate, piloerection, rigid and extended limbs, impaired mobility and reduced activity. Both genotypes had an average drop in body temperature of 6 ºC by 90 min post dosing. A major difference in the response of BChE+/+ and -/- mice was that only the BChE-/− mice had convulsions. All of the BChE+/+ mice recovered their baseline body temperature and normal behavior pattern by 3 hours post dosing.
while the BChE-/- mice did not recover their baseline temperature and all BChE-/- animals died (n=4) or were moribund (n=1) by 24 hours post dosing. A reduction in M1 receptors causes resistance to pilocarpine-induced seizures (Hamilton et al., 1997) rather than the increased sensitivity observed in BChE-/- mice. We conclude that muscarinic receptor function appears to be altered in BChE-/- mice.

**Pilocarpine cardiac and respiratory effects.** Pilocarpine can have adverse effects on cardiac function (Wang et al., 1999). The possibility was tested that the lethal effect of pilocarpine was explained by cardiac or respiratory failure in BChE-/- mice. The heart rate, ejection fraction, cardiac output, stroke volume, and respiration rate were measured in BChE+/+ and -/- mice on a high-resolution in vivo imaging system. Animals not treated with pilocarpine had normal heart function regardless of genotype (Figure 5, panels A and B). It was concluded that BChE deficiency did not adversely affect heart function in mice, a result supported by studies of heart function in BChE deficient humans (Manoharan et al., 2007).

Anesthetized animals were treated with 50 mg/kg pilocarpine. A dose of 50 mg/kg rather than 150 mg/kg pilocarpine was used because the anesthetized mice could not tolerate the high dose used in awake animals. At 90 min post-dosing the animals had lower rates of respiration, fewer heart beats per min, and lower cardiac output compared to pre-dose animals (p<0.01, see Table 2). The stroke volume and ejection fraction had lower average values, but the standard deviations were too large for statistical significance. There were no significant
differences between the genotypes either pre-dosing or 90 min post-dosing in any of the measured parameters. The cardiac waveforms in Figure 5 illustrate the decrease in contraction strength and in heart rate that both genotypes experienced 60 min post-dosing with 50 mg/kg pilocarpine. It was concluded that BChE-/- mice had normal heart function, and that death of BChE-/- mice following treatment with pilocarpine was not explained by supersensitivity of the BChE-/- heart or the BChE-/- respiratory system.
Discussion

Carboxylesterase with butyrylthiocholine hydrolase activity

We had expected the BChE-/- mouse to have no detectable activity with butyrylthiocholine. However, all tissues with the exception of plasma, had residual activity. The characteristics of the butyrylthiocholine hydrolase were consistent with the interpretation that this activity was due to a carboxylesterase. Mice have 16 carboxylesterases encoded by two gene clusters on chromosome 8 (Ronai et al., 1993; Becker-Follmann et al., 1997). A graphic view of the carboxylesterase gene clusters, and the links to the gene and protein sequences, can be found at www.ncbi.nlm.nih.gov/mapview by selecting mus musculus Build 36 and searching for carboxylesterase, all matches. Rats have at least 6 carboxylesterases in liver and 1 major carboxylesterase in plasma (Mentlein et al., 1987). Of the 6 carboxylesterases purified from rat liver microsomes, one has been reported to hydrolyze butyrylthiocholine (Mentlein and Heymann, 1984; Mentlein et al., 1984). The rat carboxylesterase with butyrylthiocholine hydrolase activity is called the pl 6.0 enzyme or ES-10. It has a Km of 40 mM for butyrylthiocholine and does not hydrolyze acetylthiocholine or butyrylcholine. These characteristics agree with our findings of a high Km for butyrylthiocholine and lack of hydrolase activity with acetylthiocholine. Meintlein et al. (1984) cautiously suggested that his purified carboxylesterase preparation might be contaminated with cholinesterase. However, our finding that tissues from the double knockout mouse, AChE-/- BChE-/-, have butyrylthiocholine hydrolase activity rules out the possibility of contamination by cholinesterase. We conclude
that the low butyrylthiocholine hydrolase activity found in BChE-/- tissues is due to carboxylesterase ES-10.

**Receptor function.** Nicotinic receptor function appeared to be normal in the BChE-/- mouse as measured by response to nicotine. However, muscarinic receptor function appeared to be altered, based on behavioral observations in response to oxotremorine and pilocarpine. BChE-/- mice were resistant to oxotremorine, but hypersensitive to pilocarpine. Downregulation of all muscarinic receptors, as seen in the AChE-/- mouse (Bernard et al., 2003; Chatonnet et al., 2003; Li et al., 2003; Volpicelli-Daley et al., 2003; Adler et al., 2004), would have resulted in resistance to both oxotremorine and pilocarpine. The opposite response to two different muscarinic agonists may be explained by tissue specific alterations.

BChE-/- mice died after treatment with pilocarpine. The lethal effect was not explained by supersensitivity of the heart or respiratory system, but could be due to pilocarpine-induced seizures. The BChE-/- mice, but not wild-type mice, had tonic-clonic convulsions after treatment with pilocarpine. Pilocarpine induces status epilepticus in rodents by stimulating muscarinic receptors (Maslanski et al., 1994). Our results suggest that muscarinic receptors in the brain of BChE-/- mice are hypersensitive to pilocarpine.

**Butyrylcholine binds to cholinergic receptors.** BChE-/- mice were more sensitive than BChE+/+ mice to the toxic effects of butyrylcholine, propionylcholine, and acetylcholine, suggesting that BChE detoxifies these
esters. The finding that butyrylcholine causes cholinergic signs of toxicity is consistent with the interpretation that butyrylcholine binds to cholinergic receptors. Additional evidence for the presence of receptors capable of binding butyrylcholine is the finding that butyrylcholine stimulates cardiac neurons (Darvesh et al., 1998). The signs of toxicity presented by both BChE-/- and AChE-/- mice, namely lacrimation and salivation, are probably explained by overstimulation of peripheral muscarinic receptors by butyrylcholine.

Butyrylcholine seems to act not only on peripheral muscarinic receptors but also on muscarinic receptors in the brain. Butyrylcholine, like pilocarpine, induced convulsions in BChE-/- mice. Since pilocarpine is known to be a muscarinic agonist, it suggests that butyrylcholine is also a muscarinic agonist.

**Model for BChE deficiency in humans.** People with silent BChE are healthy, fertile, and live to old age (Manoharan et al., 2007). Similarly, BChE knockout mice have a normal phenotype. The suitability of the BChE-/- mouse as a model for BChE deficiency in humans was demonstrated by the adverse response of BChE-/- mice to the muscle relaxant, succinylcholine. The BChE-/- mice stopped breathing and died when they received a dose that was not harmful to BChE+/+ mice. People with silent BChE also stop breathing in response to succinylcholine, though people are kept alive by a ventilator (Lockridge, 1990).

Bambuterol, a slow release drug for asthma, had no significant toxic effects on BChE-/- mice at 75 mg/kg ip, a dose three orders of magnitude higher than that required to inhibit 94% of the BChE activity in a wild-type mouse. This suggests that bambuterol is a highly specific inhibitor of BChE. On this basis,
bambuterol is predicted to be safe in BChE deficient humans, though bambuterol is not expected to have beneficial effects because the active anti-asthma drug, terbutaline, is not likely to be produced.

Pilocarpine, a drug used topically to reduce the intraocular pressure of glaucoma and used orally to increase salivary flow in people who have dry-mouth, induced seizures and death in BChE-/- mice. This raises the possibility that humans deficient in BChE may be unusually sensitive to pilocarpine.

Huperzine A and donepezil are AChE specific inhibitors used for slowing the progression of Alzheimer's disease. BChE-/- mice died when treated with huperzine A and donepezil at doses that were not toxic to BChE+/+ mice (Duysen et al., 2007). This result suggests that BChE has a function in neurotransmission and that this function is made evident when AChE activity is inhibited. Furthermore, this result suggests that BChE deficient people will not tolerate huperzine A and donepezil.

Conclusions

The BChE knockout mouse is a model for BChE deficiency in humans, suitable for testing susceptibility to drugs that specifically react with BChE or AChE.

Carboxylesterase ES-10 in mouse liver and intestines has butyrylthiocholine hydrolase activity.
References


Footnotes

This work was supported by Edgewood Biological Chemical Center Contract W911SR-04-C-0019 and Eppley Cancer Center grant P30CA36727.
Legends for figures

Figure 1. Structure of the mouse BCHE gene. A) The 67 kb mouse BCHE gene contains 281 bp in exon 1, 1528 bp in exon 2, 167 bp in exon 3, and 122 bp in exon 4. Exon 2 encodes 84% of the mature protein as well as the translation ATG start site. The BChE-/- mouse was made by gene-targeted deletion of 891 bp from the BCHE gene (485 bp from intron 1, and 406 bp from exon 2). B) The missing BCHE regions were replaced by a 1.9 kb NEO gene cassette, oriented opposite to the BCHE gene.

Figure 2. Surface body temperature of BChE deficient mice as a function of age. The surface body temperature of 3-6 male mice of each genotype was measured starting on postnatal day 5 through day 34.

Figure 3. Nondenaturing gel to show absence of BChE activity in BChE-/- serum and to identify the carboxylesterase with butyrylthiocholine hydrolase activity. The gel was first stained for activity with butyrylthiocholine for 1 hour (lanes 1-5). Lane 1 shows BChE tetramers, dimers, and monomers in BChE+/+ serum. Lane 2 is blank because the BChE-/- serum has no BChE activity. Lanes 3 and 4 are from BChE+/+ and -/- liver. Lane 5 is from BChE-/- intestine. The butyrylthiocholine hydrolase band present in BChE -/- liver and intestine is carboxylesterase ES-10. The gel was counterstained for activity with alpha-naphthylacetate and Fast Blue RR for 10 min to show bands with carboxylesterase activity (lanes 6-10). The major carboxylesterase in mouse serum, ES-1 (lanes 6 and 7), migrates just above albumin. The ES-10 band in
liver and intestine (lanes 8-10) migrates to the same position as albumin in serum. Lastly, the gel was stained for protein with Coomassie blue (lanes 11-15). 3 µl of serum and 30 µl of tissue homogenate (extracted with 10 volumes of buffer) were loaded per lane.

Figure 4. Nondenaturing gel stained for AChE and BChE activity with acetylthiocholine. Human plasma (lane 1) has 4 BChE bands and one AChE band, indicated as B and A. Wild-type mouse plasma (lane 2) has 5 BChE bands and 4 AChE bands. AChE/- mouse plasma (lane 3) has 5 bands for BChE. BChE/- mouse plasma has 4 bands for AChE.

Figure 5. Cardiac waveforms (white horizontal lines) in BChE+/+ mouse pre-dose (A), 60 minutes post-dose with 50 mg/kg pilocarpine (C), and BChE-/- mouse pre-dose (B) post-dose (D). Figures C and D demonstrate a decrease in contraction strength (flattened contractions) consistent with a decrease in heart rate and cardiac output.
Table 1. Butyrylthiocholine hydrolase activity in tissues of BChE deficient and wild-type male mice.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>BChE-/- activity, u/g</th>
<th>BChE +/- activity, u/g</th>
<th>BChE+/+ activity, u/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>0.2±0.06</td>
<td>4.5±1.1</td>
<td>8.5±2.3</td>
</tr>
<tr>
<td>Liver</td>
<td>0.09±0.02</td>
<td>1.2±0.3</td>
<td>2.7±0.3</td>
</tr>
<tr>
<td>Plasma</td>
<td>0±0</td>
<td>0.82±0.1</td>
<td>1.46±0.2</td>
</tr>
<tr>
<td>Heart</td>
<td>0.02±0.01</td>
<td>0.43±0.1</td>
<td>0.75±0.1</td>
</tr>
<tr>
<td>Lung</td>
<td>0.01±0.01</td>
<td>0.21±0.2</td>
<td>0.38±0.1</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>0.03±0.03</td>
<td>0.2±0.1</td>
<td>0.35±0.1</td>
</tr>
<tr>
<td>Brain</td>
<td>0.02±0.01</td>
<td>0.09±0.03</td>
<td>0.2±0.05</td>
</tr>
<tr>
<td>Muscle-quadriceps</td>
<td>0.02±0.02</td>
<td>0.08±0.01</td>
<td>0.14±0.01</td>
</tr>
</tbody>
</table>

Activity was assayed with 1 mM butyrylthiocholine. Units of activity are micromoles per minute per gram wet weight or per ml of plasma. The background activity for buffer containing all reagents except mouse homogenate was 0.001 u/g. Tissues from 4 to 6 animals were tested. Male mice were 3 to 4 months old.
Table 2. Cardiac function measured in BChE+/+ and BChE/-/- mice pre-dosing and 90 min post-dosing with 50 mg/kg pilocarpine IP. Data are shown ± standard deviation.

<table>
<thead>
<tr>
<th></th>
<th>BChE+/+</th>
<th>BChE/-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-dose/ Post-dose (% change)</td>
<td>Pre-dose/ Post-dose (% change)</td>
</tr>
<tr>
<td>Respiration (breaths/min)</td>
<td>124±26/</td>
<td>124±41/</td>
</tr>
<tr>
<td></td>
<td>58±13a (53%)</td>
<td>52±19a (58%)</td>
</tr>
<tr>
<td>Heart Rate (beats/min)</td>
<td>399±116/</td>
<td>422±92/</td>
</tr>
<tr>
<td></td>
<td>288±94a (28%)</td>
<td>282±81a (33%)</td>
</tr>
<tr>
<td>Cardiac Output (ml/min)</td>
<td>18±4/</td>
<td>22±4/</td>
</tr>
<tr>
<td></td>
<td>11±4a (38%)</td>
<td>11±4a (50%)</td>
</tr>
<tr>
<td>Stroke Volume (µl)</td>
<td>47±9/</td>
<td>51±6/</td>
</tr>
<tr>
<td></td>
<td>33±17 (30%)</td>
<td>39±17 (24%)</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>60±4/</td>
<td>62±7/</td>
</tr>
<tr>
<td></td>
<td>51±18 (15%)</td>
<td>53±11 (15%)</td>
</tr>
</tbody>
</table>

aPredose values are significantly different from 90 min post-dosing values (p<0.01).
Fig. 4

Human  wt mouse  AChE-/-  BChE-/-

1  2  3  4

A tetramer
A dimer
A monomer