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Intrathecal delivery of fluorescent labeled butyrylcholinesterase to the brains of butyrylcholinesterase knock-out mice: Visualization and quantification of enzyme distribution in the brain

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Abstract

Exogenously delivered butyrylcholinesterase (BChE) has proven to be an efficient bioscavenger against highly toxic organophosphorus poisons and nerve agents. The scavenger properties of BChE when delivered via intramuscular, intravenous, subcutaneous, or intraperitoneal routes are limited to the body's peripheral sites because the 340 kDa enzyme does not cross the blood-brain barrier (BBB). Overcoming the BBB is an important step toward evaluating the neuroprotective properties of BChE within the central nervous system (CNS). This study examines the feasibility of delivering BChE to the brain and spinal cord by intrathecal (IT) injection. Mice completely devoid of BChE were injected intrathecally with either BChE (80 units) that was labeled with near-infrared fluorescent dye (BChE/IRDye) or a molar equivalent amount of carboxylate dye. The BChE/IRDye and carboxylate dye were tracked using an in vivo imaging system demonstrating the real-time distribution of BChE in the brain and the residence time in the brain and spinal cord through 25 h post-dosing. BChE/IRDye levels in the brain peaked at 6 h post-dosing. BChE enzyme activity was quantified in plasma and brain sections by BChE activity assays of plasma and of perfused tissues. Average BChE activity levels were 0.6 units/g in the brains of mice treated with BChE/IRDye at 4 h post-dosing. Intense fluorescent signal in the cortex, dentate gyrus and ventricles of the brain at 25 h post-dosing was visualized by confocal microscopy and the presence of BChE was confirmed with activity assays of frozen sections. This procedure proved to be an efficient, safe and rapid method to deliver BChE to the CNS of mice, providing a research tool for determining neural protection by BChE following OP exposure.

Keywords

Butyrylcholinesterase; Intrathecal injection; Fluorescent dye; Fluorescent imaging; Butyrylcholinesterase knock-out mouse

1. Introduction

Organophosphorus (OP) intoxication results in disruption of neurotransmitter signaling leading to physiological cholinergic crisis. In addition to profound peripheral effects, neuropathological damage and neurobehavioral deficits may occur subsequent to OP exposure (Shih et al., 2003). Therapeutic agents that are currently used to mitigate the effects of OP exposure include oximes, atropine and diazepam. These agents, however, have proven to be largely ineffective in the prevention of neuropathology (Dawson, 1994; van Helden et al., 1996; Lallement et al., 1998; Shih et al., 2007; Kapur, 2000). Exogenously delivered butyrylcholinesterase (BChE; accession #gi:116353) has proven to be an efficient bioscavenger against highly toxic OP poisons (Allon et al., 1998; Brandeis et al., 1993; Broomfield et al., 1991; Doctor and Saxena, 2005; Lenz et al., 2005; Raveh et al., 1993; Saxena et al., 2006). Encouraging data from pharmacokinetic and animal safety studies have culminated in human trials testing the safety of recombinant human BChE (Cerasoli et al., 2005; Huang et al., 2007; Saxena et al., 2005).

Effective bioscavenging in the brain and/or spinal cord could help prevent the neuropathology associated with OP toxicity; however, the BChE enzyme does not cross the blood–brain barrier when delivered via intramuscular (IM) injection (Duysen and Lockridge, 2008). Similarly, we have found that when BChE is administered by intravenous (IV), subcutaneous (SC), or intraperitoneal (IP) injection the enzyme does not cross the blood–brain barrier but remains in the plasma and tissues for extended periods (unpublished results from Lockridge laboratory). Kakkis and Dickson demonstrated in experimental studies with dogs that α -L-iduronidase was delivered by IT injection to the brain and meninges in the treatment of a lysosomal storage disorder, effectively bypassing the blood–brain barrier (Kakkis et al., 2004; Dickson et al., 2007). We hypothesize that a similar approach could be used to deliver exogenous BChE to the brain and spinal cord for the purpose of evaluating the enzyme's potential role as an OP bioscavenger within the CNS.

In the present study, butyrylcholinesterase knock-out (BChE^{−/−}) mice completely deficient in BChE, were injected intrathecally with near-infrared fluorescent dye labeled BChE. The BChE/dye was tracked using an in vivo imaging system demonstrating the real-time distribution of BChE and the residence time of the enzyme in the brain and spinal cord. BChE enzyme activity was quantified in plasma, brain sections, spinal cord and other organs by BChE activity assays of plasma, tissue homogenates and frozen sections.

By demonstrating that BChE can be effectively delivered to the brain and spinal cord by intrathecal injection and that the exogenously delivered BChE remains active in the brain through 25 h post-dosing, this study provides a first step toward the evaluation of BChE as a possible neuroprotective agent against OP poisoning.

2. Materials

2.1. Animal model

Animal work was conducted 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 Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center. Adult, male, butyrylcholinesterase nullizygotes (BChE^{−/−}) were bred at the University of Nebraska Medical Center from mice genetically engineered to disrupt the BCHE gene (Li et al., 2006). Male mice were used because they are generally larger than females allowing for easier visualization of the vertebral processes during intrathecal injections. BChE^{−/−} mice have no BChE activity in their tissues and plasma, and no distinguishable phenotype until they are

challenged with certain drugs (Li et al., 2008; Duysen et al., 2007). The genetic background of the BChE knock-out mice is strain 129Sv. The absence of BChE in these animals ensured that any detected enzyme had been delivered exogenously.

2.2. Test materials

IRDye 800CW protein labeling kit (928-38040) and the carboxylate form of IRDye 800CW (929-70020) were purchased from LI-COR, Lincoln, NE. Purified mouse diet with no chlorophyll containing ingredients was prepared by Harlan Teklad, Madison, WI (TD. 94048). Heparinized microvette blood collection tubes were purchased from Sarstedt, Nümbrecht, Germany (CB-300). Lyophilized horse BChE (C-1057) and all the reagents used in the BChE activity assays were purchased from Sigma, St. Louis, MO. The purified horse BChE from Sigma contains salt, so that 10.2 mg of the Sigma product contains 1 mg of BChE protein.

2.3. Imaging system

Imaging was performed using the Pearl™ Imager (LI-COR Biosciences). The Pearl Imager is a near-infrared fluorescent imaging system utilizing two lasers (685 and 785 nm) for excitation and a charge-coupled device detector for signal detection. The laser excitation provides deep tissue penetration, while near-infrared detection allows for high sensitivity due to the reduced tissue autofluorescence. Pearl Cam Software was used to standardize images and analyze the fluorescent signal in the brains of treated mice.

2.4. Confocal laser scanning microscope

Frozen brain sections from carboxylate control and BChE/IRDye injected animals were visualized on a Zeiss 510 Meta Confocal Laser Scanning microscope located at the University of Nebraska Medical Center's core facility. Equipped with 4 lasers and Nomarski optics this microscope provides superimposition of signals from the fluorophores overlaid with a differential interference contrast image of the tissue.

2.5. Labeling of BChE with fluorescent dye

Lyophilized Horse BChE (2 mg BChE in 20.4 mg lyophilized powder) containing 1440 units of BChE activity (1 unit hydrolyzes one micromole of butyrylthiocholine per min at pH 7.0, 25 °C, when the butyrylthiocholine concentration is 1 mM) was labeled with IRDye 800CW following the LI-COR product insert instructions. The dye bears an N-hydroxy-succinimide ester reactive group that makes a stable conjugate with available lysines on the BChE protein. The conjugate was dialyzed extensively against phosphate buffered saline to remove excess non-reacted dye. The labeled BChE bound 1 dye molecule per mole of protein and lost 6.7% of its initial activity. The labeled BChE was examined on a nondenaturing gel where it was found to consist primarily of tetramers of molecular weight 340,000.

The carboxylate form of IRDye 800CW had no N-hydroxysuccinimide ester. It was necessary to use dye missing the reactive ester group to prevent random labeling of proteins. Use of this control dye allowed comparison of distribution and clearance of the dye when it was free or bound to BChE.

2.6. Preparation of the BChE KO mouse for injection and imaging

One week prior to injection all test animals were placed on chlorophyll-free purified diet. This diet eliminated the background fluorescence in the stomach and intestines that results from the chlorophyll in standard pelleted diets. One day prior to injection the mice were shaved and a depilatory agent (Nair®) was used to remove hair from the ventral and dorsal

surfaces of the mice. Each animal was imaged prior to injection to establish a background level of fluorescence.

2.7. Intrathecal injection

All intrathecal injections were made using a method adapted from Hylden and Wilcox in which the lumbar vertebral column is accessed at the L5–L6 interspace (Hylden and Wilcox, 1980). Mice were anesthetized with isoflurane. Following treatment with an aseptic scrub, a small (1 cm) midline skin incision was made over the lumbosacral spine. The dorsal spinous process of the last lumbar vertebrae (L6) served as a landmark for these injections. The intervertebral space between L5 and L6 was identified by inserting the needle at a 90° angle just proximal to the spinous process of L6 (Fig. 1). Ten microliters of either BChE/IRDye (80 units) ($n = 8$) or carboxylate dye ($n = 8$) was injected using a custom 30-gauge needle fitted to a hubless Hamilton glass syringe (Hamilton Co., Reno, NV). Immediately following the injection procedure the animals were imaged to ensure proper injection placement.

2.8. In vivo imaging

Mice were anesthetized with 2.0% isoflurane prior to imaging with the Pearl™ Imager. When an acceptable level of anesthesia was achieved animals were placed on the heated stage of the imager and isoflurane was delivered continuously to the animals through a nose cone in the imaging drawer. Images were captured in less than 30 s at white light, 700 and 800 nm. Each animal was imaged pre-dosing, immediately post-dosing and at 5, 15, 30 and 60 min and 2, 4, 8, 11 and 25 h post-dosing. Immediately after each imaging session animals were removed from the stage and allowed to recover from the anesthesia in their home cage. Animals returned to full mobility 1–2 min after the isoflurane anesthesia was discontinued.

2.9. Determining in vivo levels of fluorescent signal in the brain

The fluorescent signal in the brains of BChE/IRDye and carboxylate treated mice was calculated in a semi-quantitative manner. Using the image analysis option on the Pearl Cam Software we calculated the fluorescent signal within an elliptical region of interest (ROI). Care was taken to analyze only images captured at the same resolution and of similar focus positions. The signal was determined by the equation $\text{signal} = [\text{total intensity for ROI} - (\text{background mean intensity} \times \text{area (pixels) for ROI})]$. Signals at each time point were averaged for animals within each treatment group.

2.10. Plasma collection

Plasma was collected from the saphenous vein into heparinized microvette tubes pre-dosing and at 4 and 25 h post-dosing. The volume of blood collected at each time point was approximately 75 μl . Plasma samples were analyzed for BChE activity.

2.11. Tissue collection for determination of BChE activity and fluorescent signal

At 4 h ($n = 3/\text{group}$) and 25 h ($n = 5/\text{group}$) post-dosing mice were euthanized by CO_2 asphyxiation. The animals were thoroughly perfused intracardially with 75 ml of ice cold phosphate buffered saline (PBS) to remove blood from organs. Following perfusion, organs were removed and imaged on the Pearl Imager. A sagittal cut was made along the midline of the brain to divide the brain in half. One half of each brain was placed in 10% buffered formalin for 24 h at 4 °C and then in PBS containing 20% sucrose for 24 h at 4 °C. Coronal sections from the fixed brains were either stained for BChE activity or imaged by confocal microscopy. The cerebellum, cerebrum and brainstem were dissected from the other half of the brain, flash frozen and stored at –80 °C until tested for BChE activity.

2.12. Determination of BChE activity in plasma and brain homogenates

Plasma and brain BChE activity was measured by the Ellman method (Ellman et al., 1961) at 25 °C, in a spectrophotometer. Brain sections were extracted with and homogenized in 50 mM potassium phosphate pH 7.4 containing 0.5% Tween 20. Plasma samples (5 µl) and tissue supernatant (50 µl) were pre-incubated with 5,5'-dithio-bis (2-nitrobenzoic acid) in 2 ml of 0.1 M potassium phosphate buffer, pH 7.0, to react free sulfhydryl groups before addition of the substrate. BChE activity was measured with 1 mM butyrylthiocholine in 0.1 M potassium phosphate pH 7.0. Units of activity are defined as micromoles of substrate hydrolyzed per minute at pH 7.0, 25 °C.

2.13. BChE activity and fluorescence in frozen brain sections

Formalin fixed brains were cut at approximately bregma 0 mm to provide a landmark for sectioning. Each section was embedded in Tissue-Tek OCT compound and flash frozen in 2-methylbutane submerged in a dry ice/ethanol bath. The blocks were stored at -80 °C until they were sectioned by a microtome. Formalin fixed brains were cut coronally in 40 µm sections with every fifth and sixth section being placed on a separate slide labeled with the approximate bregma location. The sections were representative of regions from bregma 3.0 mm to bregma -6.0 mm. This procedure yielded an average of 42 sections/animal. Slides were air-dried and placed at -80 °C until they were either stained for BChE activity by the method of Karnovsky and Roots (Karnovsky and Roots, 1964) or examined by confocal microscopy.

Slides ($n = 13-15$ per mouse) were selected for BChE activity staining by choosing a range of sections from bregma 3.0 to bregma -6.0. The staining buffer was prepared immediately before use by mixing 10 ml of 0.2 M maleate (pH 6.0), 0.84 ml 0.1 M sodium citrate, 1.7 ml 0.030 M cupric sulfate, 1.7 ml 0.005 M potassium ferricyanide, and double distilled water to bring to a total volume of 16.7 ml. The staining solution was filtered through a 0.22 µm filter to remove particulates. The slides were brought to room temperature and then coupled to a Sequenza Coverplate (Thermo Shandon, Immunon, USA) Joining the slide and the cover-plate created a capillary gap for the staining reagents to flow through. The complex was placed into the cassette base. The slides were washed with 100 µl of staining buffer three times. Butyrylthiocholine (BTC) was added to the staining buffer to a final concentration of 2.0 mM. The slides were incubated at room temperature in 100 µl BTC-staining buffer. The staining solution was renewed every hour with fresh solution for a total of 4 h incubation. At the end of the incubation period the slides were washed with 100 µl of double distilled water 10 times and then fixed in 100 µl of 10% buffered formalin for 15 min. The slides were dehydrated in ethanol and xylene, and a cover-slip was added over Permount solution (Fisher Scientific). Sections were examined and photographed under brightfield microscopy using low and high magnification on a Nikon Eclipse 80i microscope.

Slides examined by confocal microscopy were brought to room temperature and 100 µl of Vectashield (H-1400) (Vector Laboratories, Burlingame, CA) was added to the tissue on each section and a cover-slip was placed over the tissue. Addition of this mounting media reduced the loss of fluorescent signal during microscopic examination and slowed photo-bleaching. Slides were examined by Confocal Laser Scanning microscopy at 680 nm. Regions from each section were photographed under constant acquisition conditions.

2.14. Statistical analysis

SPSS software was used to test the data for significance by *t*-test and ANOVA analysis. Averaged data are $\pm$ shown standard deviation or standard error.

3. Results

3.1. In vivo detection of BChE/IRDye

The success of the intrathecal injection technique (Fig. 1) was verified by observing the movement of the injected dye along the spine and into the brains of treated mice over a 25 h period (Fig. 2). All animals ($n = 16$) were injected successfully. The appearance of fluorescence in the muscle surrounding the injection site suggested that there was some leakage either during the injection process or following injection from the site itself.

The fluorescent signal in the brains of BChE/IRDye and carboxylate dye treated mice was measured over a 25 h period (Fig. 3). The signal peaked in both groups at 360 min post-dosing. At 25 h post-dosing the signal in the brains of carboxylate dye treated mice returned to background levels while the signal in the brains of mice injected with BChE/IRDye remained at a level equivalent to that observed 15 min post-dosing. The rapid clearance of the carboxylate dye is consistent with published reports (Kovar et al., 2007).

At 4 h post-dosing ($n = 3/\text{group}$) and at 25 h post-dosing ($n = 5/\text{group}$) with BChE/IRDye or carboxylate dye mice were euthanized and perfused. Organs and tissues were dissected out and imaged by placing directly on the stage of the Pearl Imager. At 4 h post-dosing intense fluorescent signal was observed in the brain, spinal column, liver and kidneys of animals treated with BChE/IRDye and carboxylate dye. Mice treated with BChE/IRDye had measurable levels of fluorescent signal remaining in the tissues and organs 25 h post-dosing (Fig. 4e–h) in contrast to tissues and organs from carboxylate dye treated mice that exhibited no detectable signal (Fig. 4a–d). The brain in BChE/IRDye treated mice (Fig. 4e) exhibited the highest fluorescent signal in the cistern region (prepontine, magna and supracerebellar). The spinal column from BChE/IRDye treated mice (Fig. 4f) showed the most intense signal at the site of injection, in the muscle surrounding the injection site and within the spinal cord. Fluorescent signal in the liver and kidneys (Fig. 4g and h) of the BChE/IRDye treated mice demonstrates the elimination of the labeled BChE through processing in the liver and excretion from the kidneys.

3.2. BChE activity in the plasma and brain following IT injection

Plasma activity levels of animals treated IT with BChE/IRDye 800CW or carboxylate dye were determined pre-dosing ($n = 8/\text{group}$), 4 ($n = 8/\text{group}$) and 25 ($n = 5/\text{group}$) hours post-dosing. No BChE activity was detected in the carboxylate dye treated group at any time point. The BChE/IRDye 800CW treated mice had no activity pre-dosing, 42 ± 11 units/ml at 4 h post-dosing and 23 ± 4 units/ml at 25 h post-dosing. BChE activity in the cerebellum, cerebrum and brain stem of the BChE/IRDye 800CW treated animals was measured at 4 ($n = 3/\text{treatment}$) and 25 ($n = 5/\text{treatment}$) hours post-dosing (Fig. 5). Activity was 0.4–0.7 units/g. Analysis by one way ANOVA showed no significant differences between the levels of BChE in the separate brain sections at each time point. Mice treated with the carboxylate dye had no measurable BChE activity in the brain sections. BChE activity was further evidenced by staining frozen brain sections from animals euthanized 25 h post-dosing with either BChE/IRDye or carboxylate dye (Fig. 6a–d). The most intense BChE staining was observed around the ventricles, dentate gyrus and cortex in animals treated with BChE/IRDye. Sections from carboxylate dye treated mice that were used as negative controls showed very little background staining (Fig. 6d).

3.3. Detection of BChE/IRDye in specific regions of the brain by confocal laser microscopy

Frozen sections from the brains of mice treated with either BChE/IRDye or carboxylate dye and euthanized 25 h post-dosing were analyzed by confocal laser microscopy at 680 nm (Fig. 6e–h). Sections from bregma 3.0 to bregma –6.0 ($n = 13$ sections/animal) were

scanned. The highest fluorescent signal was observed in the tissue surrounding the ventricles (Fig. 6e), within the cortex (Fig. 6f), and the dentate gyrus (Fig. 6g). Lower levels were detected in the striatum, and other hippocampal regions. Very low background signal was detected in sections from carboxylate treated mice as seen in figure (Fig. 6h).

4. Discussion

BChE labeled with fluorescent dye provided a convenient tracking procedure and a semi-quantitative method to measure residence time. Using IRDye 800CW that emits in the near-infrared (805 nm) reduced absorbance and scatter from animal tissues resulting in higher sensitivity and specificity (Kovar et al., 2007). The fluorescent BChE combined with a sensitive imaging system provided immediate feedback on the success of the intrathecal injections. This technique proved to be: rapid, the procedure was completed within a minute after the animal reached an acceptable level of anesthesia; efficient, all the animals were successfully injected; and safe, no damage to the spinal cord resulted from the injection. Using the BChE knock-out mouse assured that all measured BChE activity was a result of exogenously delivered enzyme, as no background levels had to be considered.

Previous work in our laboratory demonstrated that when BChE/IRDye was administered by IM injection the enzyme remained active in tissues through 16 days post-dosing. No BChE activity was detected at any time point in the brain following IM injection (Duysen and Lockridge, 2008). Similarly when BChE was injected IV, SC, or IP the enzyme did not cross the blood-brain barrier but remained in the plasma and tissues for extended periods (unpublished results from Lockridge laboratory). Movement of the BChE enzyme into brain tissue was the aim of this research. A commentary on breaching the blood-brain barrier by Lebowitz (2005) noted that direct injection of proteins into the cerebrospinal fluid allows proteins to contact many surfaces of the brain tissue. For the protein to penetrate the underlying parenchyma there must be diffusion through the ependymal cell layer. Large concentration gradients exist between the cerebrospinal fluid and the tissue beyond the ependymal layer. Lebowitz theorized that transport across the ependymal cell layer would require the delivery of high levels of protein through the cerebrospinal fluid. Following published reports that IT injection of a high concentration of iduronidase into the spinal fluid facilitated movement of the enzyme across the ependymal lining of the brain and into the cells beneath (Kakkis et al., 2004; Dickson et al., 2007) we hypothesized that this same method of bypassing the blood-brain barrier may apply to the transport of BChE delivered to the brain. Injecting 80 units of BChE by IT injection represented a large increase above normal BChE brain levels in wild type mice (0.44 total units) and normal plasma levels (0.84 total units) (Li et al., 2008). IT injection of this high concentration BChE produced no signs of toxicity. Saline perfusion of the mice ensured that none of the brain BChE activity or fluorescence could be attributed to enzyme present in the vasculature. The IT injection of BChE/IRDye achieved an average 0.6 units/g at 4 h post-dosing and 0.5 units/g 25 h post-dosing in all sections of the brain. This was the delivered equivalent of 0.24 and 0.2 total units of BChE in an average 0.4 g mouse brain compared to the endogenous level of 0.44 total units in the brain of a wild type mouse. High levels of BChE activity were detected in the plasma through 25 h post-dosing, indicating leakage from the injection site into the musculature surround the spine and/or transfer into the bloodstream from the CNS. The BChE/IRDye complex is processed and eliminated by the liver and kidneys as evidenced by the high level of fluorescence in these organs through 25 h post-dosing. Kovar et al. (2007) demonstrated that more than 90% of the fluorescence from the unconjugated dye is eliminated from the mouse by 24 h post-dosing and all of the dye is gone by 48 h. In contrast mice injected IM with the conjugate BChE/IRDye 800CW had detectable fluorescent signal for at least 17 days post-dosing (Duysen and Lockridge, 2008). Visualization of the labeled protein by confocal microscopy and activity assays of frozen

sections verified high levels of the enzyme in the in cortex, striatum and dentate gyrus regions as well as around the ventricles of the brain. The wide distribution of the enzyme in brain sections demonstrates that transport was achieved across the ependymal lining and that the enzyme remained through 25 h post-dosing.

Central nervous system effects immediately following OP intoxication may include altered behavior and mental status, loss of consciousness, seizures and apnea (Cannard, 2006). Long-term neurological effects include chronic sequelae that are demonstrated by reductions in intellectual function, academic skills, flexibility in thinking and simple motor skills (Savage et al., 1988). BChE has proven to be effective as a bioscavenger for OP in the body's peripheral system, reducing toxicity and subsequent physiological damage. Information on the efficiency of BChE to mitigate central nervous system effects following OP exposure has not been demonstrated due to the inability of the enzyme to cross the blood–brain barrier. This study demonstrates the successful intrathecal delivery of BChE in the cerebrospinal fluid, across the epithelial membrane lining of the ventricles into the brain. This method of delivery will facilitate testing the neuroprotective effects of exogenously delivered BChE in the brain following OP intoxication.

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Fig. 1.

Illustration of the needle placement (arrow) for IT injection of BChE/IRdye or carboxylate dye. The needle was placed at a 90° angle proximal to the spinous process of L6 in the intervertebral space between L5 and L6. Computed tomography (CT) image of the mouse skeleton provided by Dr. R. Lee Mosley and Dr. Nan Gong, University of Nebraska Medical Center.

Fig. 2. Fluorescent signal (800 nm) in the spinal cord and brain following intrathecal injection with BChE/IRDye in a BChE^{-/-} mouse pre-dosing (a), 4 (b) and 25 (c) hours post-dosing. (Dorsal view).

Fig. 3.

In vivo determination of fluorescent signal (800 nm) in the brains of mice 0–240 min ($n = 8$), >240 min ($n = 5$) treated with either BChE/IRDye (closed squares) or carboxylate dye (open circles). Using the image analysis option on the Pearl Cam Software the fluorescent signal within an elliptical region of interest (ROI) was calculated for the whole brain of each animal. The insert demonstrates the measured ROI. The size of the ellipse region remained constant and care was taken to analyze only images captured at the same resolution and of similar focus positions. Error bars are $\pm$ standard error. Data were analyzed by independent t -test, (a) is significantly different than (b) $p = 0.04$; (c) is significantly different than (d) $p < 0.001$.

Fig. 4. Fluorescent signal 25 h post-dosing in the brain (a and e), spinal cord (b and f), liver (c and g) and kidneys (d and h) from mice treated with either carboxylate dye (a–d) or BChE/IRDye (e–h). All images were linked in the Pearl™ Imager software program and standardized for intensity.

Fig. 5. BChE activity in the cerebellum (solid bars), cerebrum (striped bars) and brain stem (dotted bars) of BChE^{-/-} mice treated intrathecally with 80 units of BChE/IRDye 800CW measured at 4 ($n = 3/\text{group}$) and 5 ($n = 5/\text{group}$) hours post-dosing. BChE^{-/-} mice treated intrathecally with the carboxylate dye had no measurable activity. ($\pm$ standard deviation).

Fig. 6.

Brain sections from BChE^{-/-} mice treated with BChE/IRdye (25 h post-dosing) visualized by brightfield microscopy (200×) after staining for BChE activity (a–d) and by confocal scanning laser microscopy (e–h). High levels of BChE activity and fluorescent signal (680 nm) were seen in tissue surrounding the 3rd ventricle (3V) approximate bregma –2.5 (a) surrounding the dorsal 3rd ventricle (D3V), approximate bregma –2.5 (e), in the of the cortex (b and f), and throughout the dentate gyrus (DG) (c and g). Brain sections from BChE^{-/-} mice treated with carboxylate dye showed no BChE activity (d) or fluorescent signal (h) 25 h post-dosing.