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Acetylcholinesterase and Butyrylcholinesterase Are Expressed in the Spinal Meninges of Monkeys and Pigs

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Background: **Acetylcholinesterase inhibition at the spinal level has been shown to produce a potent antinociceptive effect. However, the site of cholinesterase inhibition is unknown. To determine whether the spinal meninges participate in acetylcholine metabolism, the spinal meninges of monkeys and pigs were assayed for cholinesterase activity.**

Methods: **Spinal cord, dura mater, and arachnoid mater specimens from anesthetized pigs and monkeys were mechanically homogenized and cholinesterase activity was determined quantitatively using a commercially available colorimetric assay. The ability of neostigmine to inhibit cholinesterase activity *in vitro* was also measured. Finally, the reverse transcriptase polymerase chain reaction (RT-PCR) was used to identify the cholinesterase metabolizing enzymes expressed by the spinal meninges.**

Results: **All spinal cord and meningeal specimens showed cholinesterase activity. In pigs, the dura mater showed less enzyme activity (36 ± 17.7 U/mg protein) than the arachnoid mater (73.4 ± 30.3 U/mg protein; $P < 0.05$), and the arachnoid mater showed less activity than the spinal cord (131.3 ± 55.2 U/mg protein; $P < 0.05$). In monkeys, the dura mater again showed less cholinesterase activity (45.8 ± 20.1 U/mg protein; $P < 0.05$), whereas cholinesterase activity in the arachnoid mater (90.3 ± 45.9 U/mg protein) and spinal cord specimens (101.9 ± 37.5 U/mg protein) were not significantly different. There were no significant species-related differences in cholinesterase activity. Neostigmine inhibited cholinesterase activity in a log-dose–dependent manner. The RT-PCR identified mRNA for acetylcholinesterase and butyrylcholinesterase in monkey pia-arachnoid mater.**

Conclusions: **These data show that the spinal meninges express acetylcholinesterase and butyrylcholinesterase; for monkeys, although not pigs, the level of cholinesterase activity is comparable with that found in the spinal cord. This finding suggests that the meninges may be an important site for acetylcholine metabolism and may play a role in the analgesic effect produced by intrathecally administered cholinesterase inhibitors. (Key words: Acetylcholine; arachnoid mater; cholinesterase; dura mater; enzymes; *Macaca nemestrina;* spinal cord.)**

THE spinal meninges traditionally have been considered simple physical barriers to the movement of drugs between the epidural space and the spinal cord. However, Kern *et al.*[1] recently showed that the spinal meninges also function as a metabolic barrier to drug penetration. In particular, we found that the spinal meninges contain the enzyme catechol-o-methyl transferase and can metabolize nearly all of the epinephrine that diffuses through them *in vitro*.[2] The physiologic function of spinal meningeal catechol-o-methyl transferase is unknown; however, teleologically it seems likely that the spinal meninges serve to remove "stray" neurotransmitters that escape the spinal cord and enter the cerebrospinal fluid (CSF). Thus the spinal meninges may contain many enzyme systems that can metabolize various neurotransmitters.

The purpose of this investigation was to determine whether the spinal meninges have significant acetylcholinesterase activity. We chose to study acetylcholinesterase because of recent work by Eisenach and Gebhart[3] and Yaksh *et al.*[4] showing that inhibition of acetylcholinesterase at the spinal level produces potent analgesia. To determine if the spinal meninges express cholinesterase activity, we used an *in vitro* model to quantify the cholinesterase activity in monkey and pig spinal meninges and compared the amount of activity in the meninges with that in the spinal cord. In addition, neostigmine inhibition studies and the reverse transcriptase polymerase chain reaction (RT-PCR) were used to determine which enzymes capable of metabolizing choline esters are present in the spinal meninges.

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Materials and Methods

Studies were approved by the University of Washington Animal Care and Use Committee, and guidelines of the American Association for Advancement of Laboratory Animal Care were followed throughout.

Tissue Preparation

Monkey (*Macaca nemestrina*) tissues were obtained from animals scheduled to be killed as part of the tissue distribution program of the University of Washington Regional Primate Research Center. Pig tissues were obtained from farm-bred pigs. All animals were anesthetized with thiopental and ketamine (monkeys, n = 11) or halothane in 100% oxygen (pigs, n = 9). Before removal of the meningeal specimens, all animals were rapidly exsanguinated to facilitate surgical exposure.

The spinal cords were exposed from T5 to L5 by laminectomy. The spinal cord was removed *en bloc*, and all three meningeal layers were carefully dissected from the spinal cord, preserving their normal anatomic relations. From this sheet of intact meningeal tissues, specimens of dura mater and arachnoid mater measuring approximately 1 cm^2 were removed. All meningeal specimens were rinsed with ice water to remove residual blood, mixed with 200 μl distilled water, and mechanically homogenized with a conical glass mortar and pestle. Spinal cord specimens approximately 1 cm long were similarly homogenized. The homogenates were centrifuged at 2,000 rpm for 5 min, and the resultant supernatant was assayed for cholinesterase activity. The time between tissue removal and measurement of cholinesterase activity was 45–60 min. During this time, care was taken to keep the specimens at approximately 4°C to prevent proteolysis.

Measurement of Cholinesterase Activity

Cholinesterase activity was measured quantitatively using the Sigma Cholinesterase (PTC) Reagent (Sigma Diagnostics, St. Louis, MO) as described in Sigma procedure no. 421. Briefly, the PTC reagent contains propionylthiocholine, which is metabolized by cholinesterase to propionate and thiocholine. Thiocholine subsequently reacts with 5,5′-dithiobis-2-nitrobenzoic acid present in the reagent to produce the yellow product 5-thio-2-nitrobenzoic acid. The rate at which 5-thio-2-nitrobenzoic acid is produced is measured at 405 nm using a spectrophotometer and is directly proportional to cholinesterase activity.

Specifically, in these experiments, the spectrophotometer (Beckman DU-50, Beckman Instruments, Irvine, CA) was calibrated with distilled water as a background reference. One milliliter of the Sigma PTC reagent was added to the spectrophotometer cuvette and 50 μl of the supernatant obtained from the tissue homogenate was added and immediately mixed by careful inversion so as not to produce bubbles in the cuvette light path. The change in absorbance at 405 nm between 15 and 45 s was used to determine the rate of change in absorbance (Δ A/min) from which cholinesterase activity was calculated as follows:

$$\text{Cholinesterase (U/L)} = \frac{\Delta \text{ A per min.} \times \text{TV} \times 1000}{13.6 \times \text{LP} \times \text{SV}}$$

Where:

Δ A per min = Change in absorbance per minute at 405 nm
TV = total volume (1.05 ml)
SV = sample volume (0.05 ml)
13.6 = millimolar absorptivity of 5-thio-nitrobenzoic acid
LP = light path of the cuvette (1 cm)
1,000 = conversion of units per milliliter to units per liter
U = 1 unit is defined as the amount of cholinesterase that will hydrolyze propionylthiocholine at the rate of 1 μmol/min at *p*H = 8.0 and T = 37°C

The Sigma PTC reagent and the cuvette were at room temperature, and the supernatant was chilled on ice. The temperature of the resultant mixture was measured immediately at the end of the assay (20.5 – 26.5°C) and was used to correct cholinesterase activity of the sample to 37°C according to a temperature conversion factor provided in Sigma procedure no. 421. Accutrol normal human serum (Sigma Diagnostics) was used to verify the accuracy of the assay on each day. The within-run coefficient of variation for this assay is 2%, and the between-run coefficient of variation is 3%.

The samples were also corrected for differences in protein content by assaying them for total protein using the Pierce BCA Protein Assay (Pierce, Rockford, IL). Briefly, Cu^{2+} in the BCA reagent is reduced to Cu^{+} by protein in the sample, and the reduced copper forms a stable complex with bicinchoninic acid present in the reagent. The bicinchoninic acid-Cu^{+} complex exhibits a strong absorbance at 562 nm, thereby allowing spectrophotometric measurement of total protein content.

For our studies, a standard absorbance curve was prepared using bovine serum albumin as the standard. Duplicate 10-μl samples from each specimen that had been assayed for cholinesterase activity and from the protein standards were placed in wells of a microplate. Freshly prepared bicinchoninic acid protein assay reagent (200 μl) was added to each well, and the microplate was incubated at 37°C for 30 min. The absorbance of each sample at 562 nm was measured using a microplate reader (model MR 650; Dynatech Laboratories, Alexandria, VA), and the values from the duplicate samples and the protein standards were averaged.

Identification of Cholinesterase Enzymes

Because acetylcholinesterase and butyrylcholinesterase (also known as plasmacholinesterase, pseudocholinesterase, atypical cholinesterase, cholinesterase, and EC 3.1.1.8) can hydrolyze choline esters, we performed additional studies to determine which enzymes were present in the spinal meninges.

Inhibition Studies. Both acetylcholinesterase and butyrylcholinesterase are inhibited by neostigmine, but acetylcholinesterase is much more sensitive to inhibition and is thus inhibited at a much lower neostigmine concentration than is butyrylcholinesterase.[5] To quantify the cholinesterase inhibition by neostigmine, homogenates of monkey pia-arachnoid mater were prepared as described before except that a mechanical homogenizer (Tissuemizer; Tekmar, Cincinnati, OH) was used to homogenize the tissue and the resultant homogenate was centrifuged at 3,000 rpm for 30 min. Cholinesterase activity was then determined as described before except that neostigmine in concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, and 1 mM was added to the spectrophotometer cuvette before addition of the tissue supernatant. A control was run with the same volume of supernatant but without neostigmine; this sample was used to determine the maximum possible enzymatic activity. Tissues from two monkeys were studied in this way.

Reverse Transcriptase–Polymerase Chain Reaction Studies. To further identify the enzymes responsible for the observed cholinesterase activity, the RT-PCR was used to determine whether mRNA for either acetylcholinesterase or butyrylcholinesterase was present in monkey pia-arachnoid mater. Briefly, pia-arachnoid tissue from *M. nemestrina* was removed as described before, immediately frozen on dry ice, and stored at −70°C for later use. Samples were thawed in 10 volumes chilled guanidine isothiocyanate (GulSCN) and homogenized with a polytron homogenizer (Tissuemizer) at 4°C. Total RNA was isolated from the homogenates by phenol/chloroform extraction followed by sodium acetate/isopropanol precipitation. RNA pellets were resuspended in GulSCN, extracted into phenol and chloroform until clean, precipitated with ethanol, and washed twice with 70% ethanol. RNA pellets were dried under vacuum, resuspended in DEPC-H_2O, and quantified spectrophotometrically. RT-PCR of the RNA samples was performed the Access RT-PCR System (Promega, Madison, WI) according to the instructions provided. RT-PCR was carried out in a Perkin Elmer DNA Thermo Cycler (Perkin Elmer, Norwalk, CT).

The oligonucleotide primers used to identify the butyrylcholinesterase gene were 5′-AGACTGGGTAGAT GATCAGAGACCTGAAAACTACCG-3pr and 5′-GAC-AGGCCAGCTTGTGCTATTGTTCTGAGTCTCAT-3′.[6] The primers for the acetylcholinesterase gene were 5′-CGGGTCTACGCCTACGTCTTTGAACACCGTGCTTC-3′ and 5′-CACAGGTCTGAGCAGCGATCCTGCTTGCTG-3′.[6] These primers span exons in acetylcholinesterase and butyrylcholinesterase genes,[7,8] allowing the detection of spurious genomic DNA amplification. Primers were purchased from GIBCO BRL (Grand Island, NY). Twenty-five picomoles of each oligonucleotide and 1 μg total RNA were used in each reaction. DNA reaction products were separated by electrophoresis on a 2% agarose gel in TAE buffer (40 mM Tris acetate, 1 mM EDTA), stained in a 5 μg/ml ethidium bromide in TAE buffer, visualized by ultraviolet radiation, and photographed using Polaroid 660 film (Cambridge, MA). Tissues from two monkeys were assayed for mRNA.

Statistical Analysis

Within-species differences in cholinesterase activity among the tissues were assessed for statistical significance by one-way analysis of variance, and Fisher's protected least squares difference was used for *post hoc* testing. Differences between species in the amount of cholinesterase activity within the same tissue were assessed for statistical difference by repeated measures analysis of variance. A probability value <0.05 was considered significant. All values are reported as mean ± SD.

Results

Figure 1 is a representative plot of absorbance values for the dithiobis-2-nitrobenzoic acid product resulting

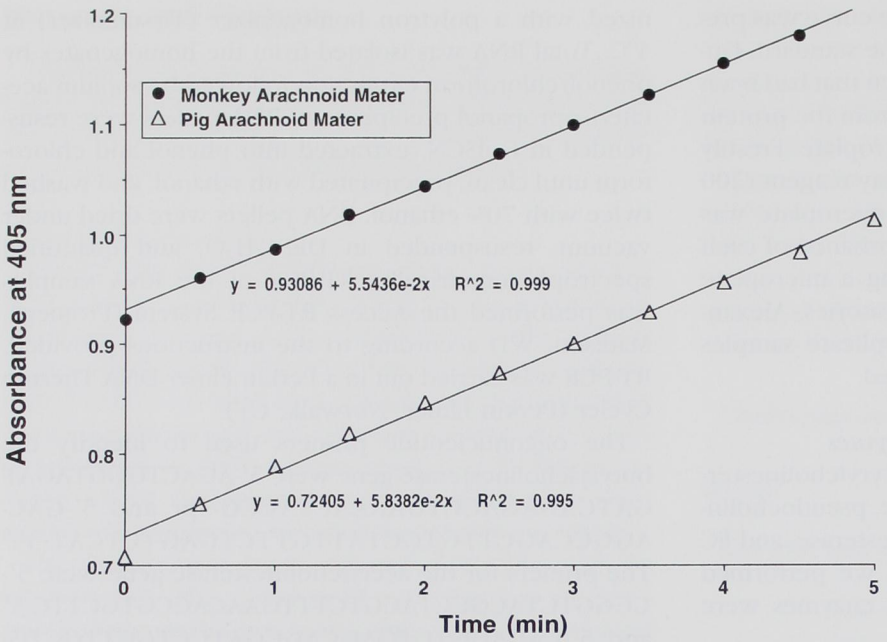

Fig. 1. Representative plots of change in absorbence over time for monkey and pig arachnoid tissue during cholinesterase activity assay. All studies had an r^2 value of 0.991 or greater.

from propionylthiocholine metabolism by meningeal cholinesterase. All experiments yielded comparable linear plots with chi-square values of 0.991 or more.

The total protein content of the dura, arachnoid, and spinal cord samples analyzed averaged 0.35 ± 0.09 μg, 0.86 ± 0.3 μg, and 0.94 ± 0.2 μg, respectively, for pig tissues and 0.10 ± 0.03 μg, 0.92 ± 0.3 μg, and 1.1 ± 0.4 μg, respectively, for the monkey tissues.

All meningeal samples and spinal cord specimens from both species showed cholinesterase activity (table 1), but there were significant differences in the amount of cholinesterase activity among the tissues in both species. In pigs, cholinesterase activity in the spinal cord exceeded that in the arachnoid mater by 79%, and the activity in the arachnoid mater was in turn 104% greater than that in the dura mater. In monkeys, cholinesterase activity in the arachnoid mater was 97% greater than the level of activity in the dura mater. However, there was no significant difference in cholinesterase activity between monkey arachnoid mater and spinal cord. There was no species-related difference in cholinesterase activity between monkeys and pigs.

Neostigmine inhibited *in vitro* cholinesterase activity in monkey pia-arachnoid mater in a log-dose–dependent manner (fig. 2).

The RT-PCR amplified cDNA for acetylcholinesterase and butyrylcholinesterase in monkey pia-arachnoid mater, indicating that these tissues contain mRNA for

Table 1. Cholinesterase Activity in Meninges and Spinal Cord of Monkeys and Pigs

Species	Dura (units/g protein)	Arachnoid (units/g protein)	Spinal Cord (units/g protein)	Significance (*P*)	Significant Differences in Cholinesterase Activity between Tissues within Species
Monkey (n = 9)	45.8 ± 20.1	90.3 ± 45.9	101.9 ± 37.5	0.0113	Dura *versus* arachnoid Dura *versus* spinal cord
Pig (n = 9)	36.0 ± 17.7	73.4 ± 30.3	131.3 ± 55.2	0.0001	Dura *versus* arachnoid Dura *versus* spinal cord Arachnoid *versus* spinal cord

Values are mean ± SD. Statistical analysis was performed by one-way ANOVA; differences between tissues within species were determined by post hoc Fisher PLSD test. There were no statistically significant differences between species.

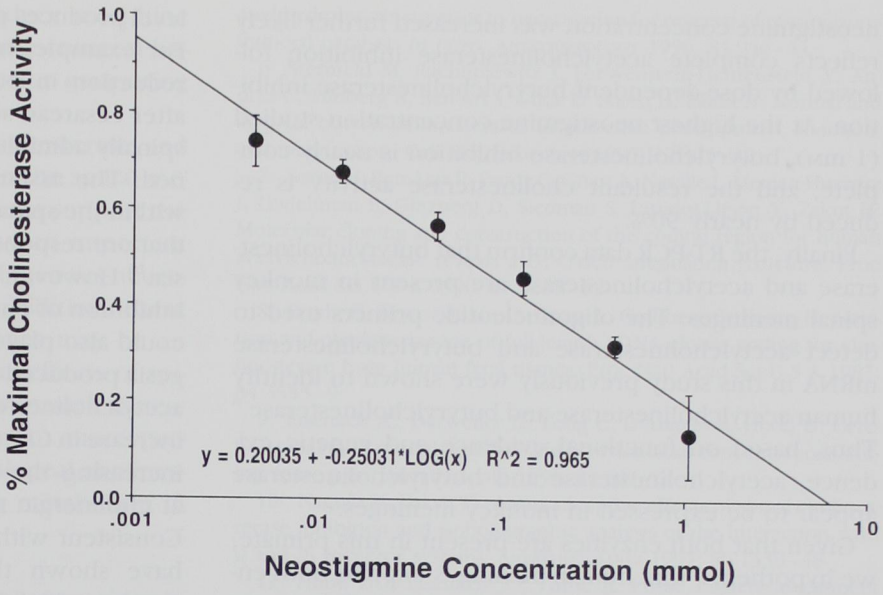

Fig. 2. Effect of increasing neostigmine dose on cholinesterase activity in monkey pia-arachnoid tissue.

both enzymes (fig. 3). The RT-PCR products produced using the acetylcholinesterase and butyrylcholinesterase primers were approximately 480 and 350 base pairs, respectively. This corresponds with the expected sizes (481 and 354, respectively) for the RT-PCR products produced with these primers.[6]

Discussion

These data provide multiple lines of evidence that the meninges of both monkeys and pigs express cholinesterase activity. Studies using the Sigma PTC reagent clearly demonstrate significant meningeal cholinesterase activity in monkeys and pigs. For comparison, the amount of activity in the meninges per gram of protein (36–131 U/g) exceeds that found in 1 ml human plasma (3–8 U/ml).§ However, because this assay does not distinguish between acetylcholinesterase and butyrylcholinesterase (*i.e.*, pseudocholinesterase or plasmacholinesterase), additional studies were conducted to positively identify the enzyme(s) responsible for the observed cholinesterase activity.

The dose-dependent, neostigmine-mediated inhibition of cholinesterase activity in monkey pia-arachnoid mater suggests the presence of acetylcholinesterase and butyrylcholinesterase. *In vivo*, neostigmine inhibits human acetylcholinesterase at much lower concentrations (0.05 mM) than are required to inhibit human butyrylcholinesterase (5 mM).[5] Thus our finding that neostigmine concentrations of only 0.005 mM decreased cholinesterase activity by approximately 25% likely reflects partial inhibition of acetylcholinesterase alone. The greater inhibition of cholinesterase activity seen as the

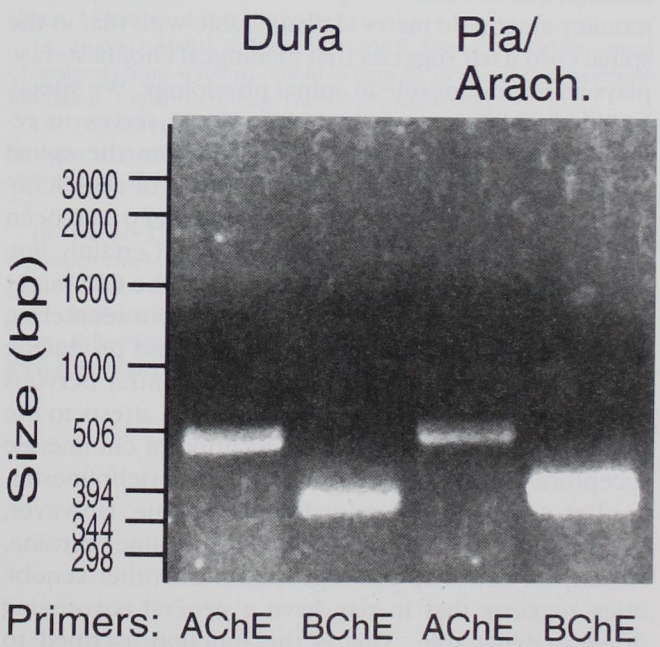

Fig. 3. Agarose gel stained with ethidium bromide showing RT-PCR products (DNA) from monkey dura and pia-arachnoid mater. In humans, the predicted RT-PCR products from these are 481 bp for acetylcholinesterase and 354 bp for butyrylcholinesterase. These sizes are comparable to the products found here. Because of the qualitative nature of RT-PCR, band intensity does not necessarily reflect the amount of mRNA present in the tissues. AChE-acetylcholinesterase; BChE-butyrylcholinesterase.

§ Sigma procedure no. 421. St. Louis, MO, Sigma Diagnostics.

neostigmine concentration was increased further likely reflects complete acetylcholinesterase inhibition followed by dose-dependent butyrylcholinesterase inhibition. At the highest neostigmine concentration studied (1 mM), butyrylcholinesterase inhibition is nearly complete, and the resultant cholinesterase activity is reduced by nearly 90%.

Finally, the RT-PCR data confirm that butyrylcholinesterase and acetylcholinesterase are present in monkey spinal meninges. The oligonucleotide primers used to detect acetylcholinesterase and butyrylcholinesterase mRNA in this study previously were shown to identify human acetylcholinesterase and butyrylcholinesterase.[6] Thus, based on functional evidence and genetic evidence, acetylcholinesterase and butyrylcholinesterase appear to be expressed in monkey meninges.

Given that both enzymes are present in this primate, we hypothesize that human meninges express both enzymes as well. Similarly, the presence of cholinesterase activity in the meninges of such disparate mammalian species as monkeys and pigs suggests that humans express such activity as well.

The physiologic role for meningeal cholinesterase is unclear, but the fact that cholinesterase activity in the monkey arachnoid mater is comparable with that in the spinal cord itself suggests that meningeal cholinesterase plays an important role in spinal physiology. We speculate that meningeal acetylcholinesterase serves to remove acetylcholine that has escaped from the spinal cord and entered the CSF. The presence of acetylcholine in the spinal CSF of humans and sheep has been clearly demonstrated by Eisenach *et al.*[9] Certainly limiting the amount of free acetylcholine in the CSF might be important to prevent acetylcholine from reentering the spinal cord or brain at remote sites and producing unwanted neuronal activity. The dire central nervous system consequences of cholinergic crisis attests to the undesirability of uncontrolled activation of cholinergic receptors. The physiologic role for butyrylcholinesterase has not been determined for any tissue. However, its ability to hydrolyze several drugs (cocaine, heroine, succinylcholine, and chloroprocaine) and other xenobiotics suggests that it may have a general scavenging or detoxifying role. This is the function ascribed to butyrylcholinesterase by Sternfeld *et al.*,[6] who identified this enzyme in the human placenta as well.

Acetylcholine has been identified as an important modulator of pain transmission in the spinal cord,[10] and Eisenach and Gebhart[3] and Hood *et al.*[11] recently showed that inhibition of cholinesterase at the spinal level produces potent analgesia in animals and humans. For example, neostigmine allows a dose-independent reduction in postoperative morphine use in patients after cesarean section.[12] However, the site of action of spinally administered neostigmine has not been identified. The assumption has been that neostigmine acts within the spinal cord at the muscarinic nerve terminals that are responsible for acetylcholine-mediated analgesia.[10] However, the findings of this study indicate that inhibition of cholinesterase within the spinal meninges could also play a role in the cholinergic-mediated analgesia produced by neostigmine. Inhibition of meningeal acetylcholinesterase would be expected to result in an increase in CSF concentrations of acetylcholine, thereby increasing the bioavailability of this neurotransmitter at cholinergic nerve terminals within the spinal cord. Consistent with this hypothesis, multiple investigators have shown that acetylcholinesterase inhibition increases spinal CSF concentrations of acetylcholine.[13–15]

Even if a physiologic role for cholinesterase enzymes within the spinal meninges is not yet clear, our data suggest that the traditional view of the spinal meninges as simple anatomic barriers may need revision. The current study extends earlier work by Kern *et al.*[1] that showed that the spinal meninges also express significant catechol-o-methyl transferase activity. Together these studies indicate that the spinal meninges are more than a mere anatomic barrier between the epidural space and the CSF. The meninges may also prove to be an important metabolic barrier to protect the underlying central nervous system from endogenous neurotransmitters and exogenous drugs. The potential clinical importance of this function for the spinal meninges is that they may well play a role in the elimination of drugs that we administer clinically or that we may administer in the future. If this is so, we may be able to favorably alter the spinal bioavailability of endogenous neurotransmitters and exogenous drugs by manipulating spinal meningeal metabolism. In fact, we have already found that we can markedly increase the bioavailability of epinephrine by inhibiting meningeal catechol-o-methyltransferase.[2]

Another potential way to use meningeal cholinesterases might be through the use of choline-ester containing "pro-drugs" that are cleaved by cholinesterases to generate active metabolites. A possible example of this is the use of intrathecal heroin, which is a potent intrathecal analgesic in humans.[16,17] However, heroin is an inactive opioid that must be metabolized to morphine to produce analgesia. The enzyme that cleaves

the acetate groups from heroin to produce morphine in plasma is butyrylcholinesterase. Thus meningeal butyrylcholinesterase may be responsible for generating morphine from intrathecal heroin.

In conclusion, significant butyrylcholinesterase and acetylcholinesterase activity is present in the spinal meninges of monkeys and pigs. These findings support the idea that the spinal meninges may play an important role in limiting the bioavailability of endogenous neurotransmitters and exogenous drugs. In addition, these findings suggest that the spinal meninges may be an important site of action for the analgesic effects of spinally administered cholinesterase inhibitors such as neostigmine. Further studies are necessary to more precisely define the physiologic role of meningeal cholinesterase in particular and meningeal drug metabolism in general.

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References

1. Kern C, Mautz DS, Bernards CM: Epinephrine is metabolized by the spinal meninges of monkeys and pigs. ANESTHESIOLOGY 1995; 83:1078–81

2. Kern C, Bernards CM: Ascorbic acid inhibits spinal meningeal catechol-o-methyl transferase *in vitro* resulting in a marked increase in epinephrine bioavailability. ANESTHESIOLOGY 1997; 86:405–9

3. Eisenach JC, Gebhart GF: Intrathecal amitriptyline. Antinociceptive interactions with intravenous morphine and intrathecal clonidine, neostigmine, and carbamylcholine in rats. ANESTHESIOLOGY 1995; 83:1036–45

4. Yaksh TL, Grafe MR, Malkmus S, Rathbun ML, Eisenach JC: Studies on the safety of chronically administered intrathecal neostigmine methylsulfate in rats and dogs. ANESTHESIOLOGY 1995; 82:412–27

5. Yang H, Goudsouzian N, Martyn J: Pseudocholinesterase-mediated hydrolysis is superior to neostigmine for reversal of mivacurium-induced paralysis *in vitro*. ANESTHESIOLOGY 1996; 84:936–44

6. Sternfeld M, Rachmilewitz J, Loewenstein-Lichtenstein Y, Andres C, Timberg R, Ben-Ari S, Glick D, Soreq H, Zakut H: Normal and atypical butyrylcholinesterases in placental development, function, and malfunction. Cell Mol Neurobiol 1997; 17:315–32

7. Soreq H, Ben-Aziz R, Prody C, Gnatt A, Neville L, Lieman-Hurwitz J, Levlehman E, Ginzberg D, Siedman S, Lapidot-Lifson Y, Zakut H: Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G, C-rich attenuating structure. Proc Natl Acad Sci U S A 1990; 87:9688–92

8. Prody C, Zevin-Sonkin D, Gnatt A, Goldberg O, Soreq H: Isolation and characterization of full-length cDNA clones coding for cholinesterase from human fetal tissues. Proc Natl Acad Sci U S A 1987; 84:3555–9

9. Eisenach JC, Detweiler D, Tong C, D'Angelo R, Hood D: Cerebrospinal fluid norepinephrine and acetylcholine concentrations during acute pain. Anesth Analg 1996; 82:621–6

10. Naguib M, Yaksh TL: Antinociceptive effects of spinal cholinesterase inhibition and isobolographic analysis of the interaction with μ and ∂_2 receptor systems. ANESTHESIOLOGY 1994; 80:1338–48

11. Hood DD, Eisenach JC, Tuttle R: Phase I safety assessment of intrathecal neostigmine methylsulfate in humans. ANESTHESIOLOGY 1995; 82:331–43

12. Krukowski JA, Hood DD, Eisenach JC, Mallak KA, Parker RL: Intrathecal neostigmine for post-cesarean section analgesia: Dose response. Anesth Analg 1997; 84:1269–75

13. Detweiler D, Eisenach J, Tong C, Jackson C: A cholinergic interaction in alpha 2 adrenoreceptor-mediated antinociception in sheep. J Pharmacol Exp Ther 1993; 265:536–42

14. Hood D, Mallak K, James R, Tuttle R, Eisenach J: Enhancement of analgesia from systemic opioid in humans by spinal cholinesterase inhibition. J Pharmacol Exp Ther 1997; 282:86–92

15. Togashi H, Matsumoto M, Yoshioka M, Hirokami M, Tochihara M, Saito H: Acetylcholine measurement of cerebrospinal fluid by in vivo microdialysis in freely moving rats. Jpn J Pharmacol 1994; 66:67–74

16. Fogarty D, Milligan K: Postoperative analgesia following total hip replacement: A comparison of intrathecal morphine and diamorphine. J R Soc Med 1995; 88:70–2

17. Milligan K, Fogarty D: The characteristics of analgesic requirements following subarachnoid diamorphine in patients undergoing total hip replacement. Reg Anesth 1993; 18:114–7