

Pyridostigmine Crosses the Blood Brain Barrier to Induce Cholinergic and Non-Cholinergic Changes in Mouse Hypothalamus

Samuel A. Ropp, William C. Grunwald, Jr., Mariana Morris and David R. Cool*

Department of Pharmacology & Toxicology, Wright State University
Boonshoft School of Medicine, Dayton, OH 45435

*Corresponding Author:

David R. Cool, Ph.D.

Wright State University

Boonshoft School of Medicine

Department of Pharmacology & Toxicology

3640 Colonel Glenn Hwy

Dayton, OH 45435

T: 937-775-2457

F: 937-775-7221

david.cool@wright.edu

ABSTRACT

Pyridostigmine (PYR) is a quaternary amine whose ability to cross the blood brain barrier and affect acetylcholinesterase (AChE) activity in the brain has been rigorously debated. To determine whether PYR has regional brain effects, mice were sacrificed 15 min (acute) or 7 days (subacute) after treatment with PYR, PHY, or saline. AChE activity, protein and mRNA expression were analyzed in blood, hypothalamus and cortex. PHY inhibited blood, cortical and hypothalamic AChE activity in the acute and subacute groups. In contrast, PYR did not affect cortical AChE activity but inhibited hypothalamic activity after acute exposure. Subacute treatment with PYR increased hypothalamic AChE activity. Western blot analysis of the same tissues showed a significant increase in cortical and hypothalamic AChE protein following subacute PHY treatment. Subacute PYR treatment increased hypothalamic AChE protein levels (39%). Real-Time PCR analysis of AChE mRNA revealed significant increases in the cortex following subacute PYR (24%) and PHY (41%) and in the hypothalamus following subacute PYR (16%). Proteomic analysis of the cortex and hypothalamus by SELDI-TOF mass spectrometry suggested significant changes in the protein profiles from hypothalamus following both subacute PYR and PHY treatment. The results suggest that PYR crosses the blood brain barrier into the hypothalamus to affect AChE mRNA, protein and activity.

INTRODUCTION

The blood brain barrier (BBB) is a physical barrier that acts in both a neuroprotective and immunoprotective manner to prevent the movement of many substances from the circulation into the central nervous system [Pardridge, 2002]. Functionally, the BBB in most regions of the brain prevents entry of larger molecules >500 daltons while smaller non-ionic molecules pass freely. One exception to this rule is the molecule pyridostigmine (PYR) that has a mass less than 500 daltons, i.e., 261 daltons, but contains a quaternary amine that causes it to be ionized [Borland et al., 1985; Gawron et al., 1990; Izraeli et al., 1990; Arad et al., 1992; Cook et al., 1992; Wenger and Latzka, 1992; Wiley et al., 1992]. PYR is a reversible and relatively short-term inhibitor of acetylcholinesterase (AChE), the primary enzyme responsible for hydrolyzing acetylcholine [Hudson et al., 1985; Braga et al., 1989]. PYR has been used for many years to treat the autoimmune disease myasthenia gravis [Havard, 1973; Aquilonius et al., 1983; Aquilonius and Hartvig, 1986]. This use was supported by several studies in which prophylactic levels of PYR did not significantly affect brain AChE activity, impair performance or cognitive ability in humans [Borland et al., 1985; Gawron et al., 1990; Izraeli et al., 1990; Arad et al., 1992; Cook et al., 1992; Wenger and Latzka, 1992; Wiley et al., 1992]. The conclusion was that humans are not significantly affected because the quaternary amine prevents PYR from crossing the BBB. However, the caveat is that many of the studies on central effects of PYR were conducted on whole brain homogenates, which do not take into account the variable permeability of the BBB in different regions of the brain. In contrast, other studies have shown that peripherally administered PYR is capable of inducing neuronal apoptosis and exaggerated startle responses in rats, and can compromise the BBB resulting in seizures in mice [Chaney et al., 1991; Servatius et al., 1998; Li et al., 2000; Grauer et al., 2001]. Furthermore, peripheral administration of PYR to stressed mice has been shown to cause an inhibition of cortical AChE activity along with an increase in AChE mRNA levels, presumably through stress-induced disruption of the BBB [Friedman et al., 1996; Kaufer et al., 1998]. Contradictory effects on behavior have been observed as well. In behavioral studies from our group, PYR was reported to cause an exaggerated acoustic startle response in mice and decreased locomotor activity that was not correlated with exposure to stress [Dubovicky et al., 2007]. Other studies have shown no PYR-induced effects on open field behavior in mice and no effects on blood pressure or heart rate [Bernatova et al., 2003].

These findings are significant because, in the early 1990's, PYR was administered to an estimated 400,000 Persian Gulf War troops as a preemptive treatment against possible nerve agent exposure [Gavaghan, 1994; Karczmar, 1998; Shen, 1998], for time periods ranging from 1 to 7 days, with some troops extending treatment up to 21 days or more [Keeler et al., 1991; Jamal, 1998; Shen, 1998]. PYR's use as a prophylactic agent for chemical warfare has been further continued in the present "war on terrorism" in Iraq and Afghanistan, though the number of troops receiving it and dosages used are not yet fully documented. The symptoms appearing in Gulf War veterans, e.g., myalgia, fatigue, cognitive deficits, irritability mood swings and difficulty concentrating, are similar to symptoms associated with increased levels of acetylcholine [Doebbeling et al., 2000]. Due to this array of symptoms and the uncertainty of troop exposure to multiple factors, several studies have focused on the central nervous system to specifically address whether PYR may affect central cholinergic neurotransmission.

Interpretations of the results from these diverse studies are complicated due to the nature of the BBB and the types of assays being utilized. The presence of a traditionally tight BBB in most regions (99%) of the brain may prevent entry of small ionic molecules such as

PYR into these regions. However, in certain specialized areas called circumventricular organs (CVO), fenestrated capillaries prevail, which may be more permeable to small ionic molecules such as PYR. The hypothalamus contains this type of CVO. In support of this, PYR has been shown to affect the release of the anterior pituitary hormones, i.e., growth hormone, thyroid-stimulating hormone and adrenocorticotrophic hormone (ACTH) [Giustina et al., 1990; Giustina et al., 1991; Wehrenberg et al., 1992; Giustina et al., 1993; Murialdo et al., 1993; O'Keane et al., 1994; Llorente et al., 1996].

Inhibition of AChE by PYR and PHY causes an increase in acetylcholine that has the potential to regulate the neuroendocrine system in a cholinergic manner via muscarinic receptors [Huizen et al., 1994; Krsmanovic et al., 1998]. However, numerous other secondary effects have been described for cholinesterase inhibitors, such as organophosphates [Casida and Quistad, 2004], e.g., inhibition of serine hydrolases. This is significant because the enzymes responsible for processing pro-hormones in the neuroendocrine system are serine hydrolases that have a similar Asp-His-Ser catalytic triad as the cholinesterases [Bryan et al., 1986; Sussman et al., 1991; Nakayama, 1997]. Thus, inhibitors of AChE may have an effect on pro-hormone processing in the hypothalamus and pituitary through direct interaction with the catalytically active triad in pro-hormone convertases. The hypothalamic-pituitary axis, which represents the neuroendocrine system in this experiment, is ultimately responsible for secretion of peptide hormones that regulate endocrine and metabolic homeostasis throughout the body. Therefore, we wanted to determine if peptides or proteins other than AChE in these tissues were affected by either PYR or PHY treatment. To test this hypothesis, we used a relatively new technique, i.e., SELDI-TOF mass spectrometry, to generate a "proteomic" profile of the hypothalamic and cortex proteins and peptides after treatment with PYR or PHY. SELDI-TOF mass spectrometry (Surface Enhanced Laser Desorption/Ionization-Time of Flight Mass Spectrometry) utilizes ProteinChips® with different chemical characteristic properties, e.g., weak cation exchange, strong anion exchange, etc., that proteins bind to based on their particular affinity. The ProteinChips® can be washed with different buffers or water, causing removal of some peptides/proteins and retention of others. The proteomic profiles developed in this study provide a unique new method to evaluate the effects of specific drug treatment paradigms and provide further evidence for regional effects of PYR.

The purpose of the present study was to analyze and compare the effects of PYR and PHY (PHY is a tertiary carbamate AChE inhibitor similar to PYR that can readily penetrate the BBB) in the hypothalamus and cerebral cortex in order to determine whether PYR has regional brain effects on AChE activity, AChE expression and general proteomic profiles, and compare them to the effects of PHY.

MATERIALS AND METHODS

ANIMALS

Male C57Bl mice (~25 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN) and housed individually in the WSU Laboratory Animal Resources Facility under a 12-hour light and dark cycle, and provided with food and water ad libitum. All animal experiments were conducted under an approved LACUC animal protocol in compliance with DOD, NIH and WSU guidelines.

DRUG TREATMENT

Pyridostigmine bromide (PYR) and physostigmine (eserine hemisulfate) (PHY) obtained from Sigma (St. Louis, MO) were dissolved in sterile saline and stored in 100 μ M aliquots at -80°C . Acute PYR and PHY (40 mg/kg and 11.5 mg/kg, respectively) or sterile saline (Sham) were delivered through subcutaneous injections. Acute experiments consisted of a single injection followed by decapitation after 15 minutes. For subacute treatment i.e., 7 days, Alzet mini-osmotic pumps (Cupertino, CA) containing PYR, PHY or saline, were surgically implanted subcutaneously on the mouse back. Mice were anesthetized with ketamine and xylazine (5:2), the intrascapular region of their back shaved and wiped with a betadine solution. A small cutaneous incision was made and the loaded mini-pump was inserted under the skin. After implanting the mini-pump, the incision was closed with sterile surgical sutures, and the mice were allowed to recover. The concentrations of PYR and PHY used for all subacute experiments were determined experimentally by those doses that produced approximately 40% inhibition of blood AChE, i.e., 10 mg/kg/day PYR, and 2.88 mg/kg/day PHY. To insure measurable acute responses to PYR and PHY, doses were increased 4 fold.

TISSUE COLLECTION

Upon the completion of treatment, the mice were decapitated, and the brains quickly removed. The hypothalamus and frontal cortex of each were dissected out and split into four equal portions to be used for AChE activity assays, western blotting, RealTime PCR and SELDI-TOF mass spectrometry. After removal of the brain, the anterior and neurointermediate pituitary glands were removed from the cranium via microscopic dissection. The samples were stored at -80°C until used.

PLASMA PYRIDOSTIGMINE ANALYSIS

Plasma levels of PYR were measured by Alturas Analytics, Inc. (Moscow, Idaho) using 10 mg/kg/day samples [Needham et al., 2003]. A PRP 1 (50 x 2.1 mm, 5 mm) HPLC column at ambient temperature was used for all sample analyses. Two Shimadzu 10 ADvp series pumps delivered a flow rate of 0.60 mL/min to the HPLC column. The mobile phase was 2% acetonitrile in water with 0.1% trifluoroacetic acid and 1 mM ammonium acetate. Under these HPLC conditions PYR and the PYR-D6 internal standard had a retention time of approximately 45 seconds. The HPLC effluent flowed directly into the electrospray interface of a Sciex API3000 triple quadrupole mass spectrometer with a turboionspray temperature of 400°C . The turboionspray gas flow was set to 7 L/min. The ESI interface was operated in the positive ion mode with an ESI voltage of 1500 V. The orifice and ring voltage of the instrument was set to 20 and 100 V, respectively. The instrument was operated in the multiple reaction-monitoring (MRM) mode for all sample analyses. The collision energy for both PYR and internal standard was 25 eV. Parent to product ion transitions m/z 181.2 to m/z 124 and m/z 187.2 to m/z 130 were monitored for PYR and the internal standard, respectively. The peak height of PYR was measured against the concentration to generate the standard curve results. A standard curve was prepared in mouse plasma with levels ($n=2$) of 0.5, 0.7, 1.0, 5.0, 50 and 100 ng/mL.

CHOLINESTERASE ACTIVITY ASSAY

Cholinesterase activity was analyzed by an adapted version of the Ellman colorimetric assay [Ellman et al., 1961] using a Packard Fusion plate reader. Briefly, samples were homogenized on ice in Na_2HPO_4 buffer (pH 8) containing 0.1% Tween 20 and centrifuged for 5 minutes at 13,000 rpm to remove cellular debris. The supernatant was removed, and 10 μ L was combined with 50 μ L of acetylthiocholine (ACTH), 50 μ L dithiobisnitrobenzoate (DTNB) and 90 μ L of sodium phosphate buffer (pH 8) in a 96 well plate and allowed to react. An

additional 2 μL of supernatant was used to determine protein concentrations using the Bradford method (Bradford, 1976). To determine blood cholinesterase, whole blood was diluted 1:100 with Na_2HPO_4 buffer (pH 8) prior to use in the reaction. For each diluted blood sample, half of the replicates were treated with tetraisopropylpyrophosphoramidate (iso-OMPA) to determine the relative activity of AChE and BuChE. Iso-OMPA selectively inhibits BuChE. The BuChE activity is derived by subtracting the AChE activity from the total activity. Since the hypothalamus and pre-frontal cortex have negligible amounts of BuChE activity, i.e., <2% of total cholinesterase activity (data not shown), the relative measurement of BuChE activity was only used when measuring cholinesterase activity in the blood. The reactions were monitored using a Packard Fusion plate reader and average slope (kinetic) values were generated for each well. By using the spectrophotometric data (slope values) and protein concentrations, the relative AChE activity was calculated for the given tissue.

SEMI-QUANTITATIVE WESTERN BLOT

Frozen tissue samples used for Western blot analysis were thawed and homogenized in 1X TE with 5 mM PMSF and centrifuged for 5 minutes at 13,000 RPM. The supernatant was recovered and the protein concentration was determined using the Bradford method. Prior to loading, the final sample volume was corrected to 10 μg of total protein in 15 μL using the appropriate amount of 1X TE with 5 mM PMSF. Sample proteins were separated on a 10% SDS-Page gel and then transferred to a PVDF membrane. The membrane was probed with antibodies to AChE and actin, an internal standard, and visualized by enhanced-chemifluorescence on a Fuji-FLA2000 fluorimager. Using Image Gauge software from Fuji, the relative amount of AChE, band size ~ 72 kDa, was determined after normalizing for protein loading inconsistencies with actin, band size approx. 43 kDa, on the same blot.

REAL-TIME PCR

Frozen tissue samples used for Real-Time PCR were thawed and homogenized in TRIzol® to extract total RNA via a chloroform/methanol extraction. Isolated RNA was dissolved in 1X TE and treated with DNase1 (DNA-free™, Ambion) for 20 minutes and DNase Turbo for 30 minutes. Approximately 50 ng of total RNA was used for a multiplex One-Step RT-PCR (Qiagen) reaction using the appropriate primers and TaqMan style probes (incorporated with quenched Texas Red and FAM) for AChE and MAX, respectively. No-RT reactions were used as a control for the detection of unwanted DNA amplification within the One-Step RT-PCR reactions. AChE primers and Taqman probes were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) with the following sequences: AChE-R 5'-ACCGAGAGTTGAGTGAA GACT-3': AChE-L 5'GCCGTCATACACATC CAAGGA-3': Taqman 5'-/5TexRdXN/ CCGCTCCGCTGTAGAAACCACCC/3BHQ-2/-3'.

As an internal standard and for normalization, a predesigned primer and probe set was purchased for the nuclear protein Max (Rabbia et al. 2001, Cat No 4331182, Assay ID Mm00465485_m1, bought premixed as an "Assay on Demand" from Applied Biosystems, Foster City, CA). PCR products were generated, optimized and analyzed by fluorescent Real-Time PCR on a BioRad iCycler Real-Time PCR machine. The ΔCt for an individual sample, i.e., mouse #1 acute hypothalamus, was calculated by subtracting the Max Ct value from the AChE Ct value. The $\Delta\Delta\text{Ct}$ values for individual samples were then calculated by subtracting the average Sham ΔCt of the same sample group, i.e. acute hypothalamus, from the individual sample ΔCt . Using the ΔCt value, $2^{-\Delta\Delta\text{Ct}}$ was then calculated. This method of relative gene expression analysis has been described in detail by Livak and Schmittgen [Livak and Schmittgen, 2001]. The individual sample $2^{-\Delta\Delta\text{Ct}}$ values for each treatment group were

averaged and compared to the Sham controls to obtain the percent of control AChE mRNA. The formula for this is shown below.

$$2^{-\Delta\Delta CT} = 2^{-(Ct_{AChE} - Ct_{max}) - (Ct_{AChEave} - Ct_{maxave})}$$

PROTEOMIC ANALYSIS

Tissue for SELDI-TOF mass spectrometry was prepared as previously described [Cool and Hardiman, 2004; Hardiman et al., 2005]. Brain tissue was homogenized in 0.1 N HCl and centrifuged for 5 minutes at 13,000 rpm to remove tissue debris. The supernatant was then removed and protein concentrations were determined using the Bradford method. The protein concentrations were normalized to 1 µg/µL with 0.1 N HCl. The CiphergenWCX2 (weak cation exchange) ProteinChip® was used for the proteomic analysis and 1 µg of protein was applied to each spot. The samples spots were allowed to nearly dry (still damp), then rinsed individually with 50 µL of water to remove unwanted and unbound debris. The matrix (1 µL) or EAM (energy absorbing molecule) alpha-cyano-4-hydroxy cinnamic acid (CHCA) (in 50% acetonitrile and 0.1% TFA) was added to each spot to aid in the ionization of protein and peptides. The ProteinChips® were placed into the Ciphergen SELDI-TOF ProteinChip® Array for analysis. A spot and chip protocol were designed to take 10 transient reads at every five sites beginning at 20 and ending at 80 (each spot has 100 possible sites). The laser intensity was set to 160 for each sample with two warming shots at 165 intensity. The Ciphergen v3.01 software was used to analyze each spectra representing the proteome for that given sample spot.

STATISTICAL ANALYSIS

Statistical analysis of the SELDI-TOF MS proteomic data was completed with SPSS software using a multiple analysis of variance (MANOVA) with an ad hoc LSD post-test to determine the significance of the experimental findings ($p < 0.05$). All other statistical analyses were completed using one-way ANOVA with a significance level of $p < 0.05$, with Prism GraphPad Software, version 4.0.

RESULTS

Plasma Pyridostigmine Analysis

To determine the amount of PYR in blood, plasma from Sham and PYR-treated animals was analyzed using electrospray ionization mass spectrometry and compared against a standard curve of varying concentrations of PYR to determine concentration. The concentration of PYR observed in the blood was 50 ± 6 ng/mL (Figure 1).

AChE Activity Inhibition by Pyridostigmine and Physostigmine in Mice Treated with PYR, PHY or Saline.

The presence of specific AChE inhibitors, i.e., PYR or PHY, in any tissue is expected to inhibit AChE activity. Our first goal was to test the effect of PYR or PHY on AChE activity. Mice were treated acutely by subcutaneous injection (15 minutes) or subacutely by chronic subcutaneous infusion (7 days) with saline (Sham), PYR or PHY. Following treatment, blood, pre-frontal cortex and hypothalamus were removed and analyzed for AChE activity.

The blood AChE activity was inhibited to similar levels for both PYR and PHY acutely treated animals, i.e., 75 and 76% inhibition, respectively (Figure 2). Likewise, in subacutely treated animals, PYR and PHY caused 49% and 42% inhibition of blood AChE, respectively (Figure 2).

Having determined that AChE activity in blood was inhibited by both PYR and PHY, we studied their effects on hypothalamic and frontal cortex AChE activity. Cortical AChE was inhibited 76% and 26% by acute and subacute PHY treatment, respectively, (Figure 2). PYR had no effect on cortical AChE activity (Figure 2).

In comparison to blood and cortex, the hypothalamic response to PYR and PHY treatment was more complex. Both treatments, i.e., acute and subacute, significantly changed hypothalamic AChE activity. Acute treatment with PYR and PHY resulted in 51% and 79% inhibition of AChE activity, respectively. In contrast, following subacute treatment with PYR, AChE activity increased 35%, whereas subacute treatment with PHY caused a 31% decrease in AChE activity. Since PYR had effects at both acute and subacute treatments in the hypothalamus but not in the cortex, the results suggest that PYR enters the hypothalamus but not the cortex.

AChE Protein Levels are Modulated in Animals Treated with PYR and PHY

AChE protein should not be affected by acute treatment with PYR or PHY. To test this hypothesis, Western blot analysis was performed on the same cortex and hypothalamic tissues that were analyzed for AChE activity. Figure 3A shows a representative Western blot from the subacute cortex. To prevent membrane to membrane variance, Sham, PYR and PHY samples from each tissue were analyzed in alternative lanes (see Figure 3 legend for details).

Cortical AChE protein expression was not affected by either acute or subacute treatment with PYR (Figure 3B). Acute PHY treatment also had no effect. In contrast, subacute treatment with PHY resulted in a 27% increase in cortical AChE protein (Figure 3B).

Western blot analysis of the hypothalamus following the same treatment with PYR and PHY showed no significant changes in AChE protein levels (Figure 3B). However, following subacute treatment, a 39% increase in AChE protein was observed with PYR ($p < 0.05$).

AChE mRNA Level Changes Following Acute and Subacute Treatment

Since there was increased expression of AChE protein in hypothalamic tissue in response to subacute treatment with PYR and PHY, we wanted to determine if this could be correlated with an increase in AChE mRNA expression. To study AChE expression, RealTime PCR analysis of AChE mRNA was performed. RealTime PCR analysis of mRNA from acute PYR and PHY-treated cortical tissues showed no significant changes in AChE mRNA (Figure 4). In contrast, subacute treatment with PYR resulted in a 28% decrease in AChE mRNA. No changes were observed with PHY.

In the hypothalamus, acute treatment with PYR caused no increase in mRNA levels, while acute treatment with PHY caused an 80% increase ($p < 0.05$) in AChE mRNA (Figure 4). In contrast, subacute PYR -treatment caused a 40% increase ($p < 0.05$), whereas PHY caused no change in AChE mRNA levels.

Proteomic Changes in Response to PYR and PHY

Using the Ciphergen ProteinChip® reader, proteomic profiles for the four tissues, i.e., cerebral cortex, hypothalamus, anterior pituitary and neurointermediate lobe pituitary, were generated for Sham, PYR and PHY-treated mice from acute and subacute experiments. For each ion peak in the spectral profile, the area under the ion peak was integrated and an average obtained for each tissue from that group. These were then compared using a MANOVA analysis followed by LSD post-hoc test. MANOVA analysis indicated that only the subacute-treated hypothalamus showed significant changes ($p < 0.047$) (Table 1). The acute-treated hypothalamus showed a strong trend ($p = 0.076$), while there were no significant changes for either cortex treatment group, ($p > 0.5$).

Upon examination of the individual ion peaks in the hypothalamus profiles, numerous changes were observed following subacute treatment with PYR or PHY, e.g., 33 peaks were affected. Of these, twelve peaks showed similar changes following subacute treatment with PYR and PHY. Levels of the smallest ion peak, i.e., 1835 Daltons, were decreased with both PYR and PHY treatment, while levels of all the other peaks increased. The identity of these peaks is not currently known. However, the results clearly establish that PYR and PHY affected the proteomic profile in the hypothalamus but not in the cortex (Table 2).

DISCUSSION

The brain is a complex organ with numerous regions that express varying amounts of AChE enzyme. Since many AChE inhibitors cannot cross the BBB, a number of recent studies have focused on identifying the effects of AChE inhibitors on brain tissue [Abou-Donia et al., 1996; Abou-Donia et al., 1996; Lallement et al., 1998; Chaney et al., 2000; Grauer et al., 2000; Sinton et al., 2000; Kant et al., 2001; Tian et al., 2002]. Unfortunately, these studies have mainly focused on whole brain homogenates or brain regions other than those with circumventricular organs. Thus, the specific effect of AChE inhibitors in distinct regions where the BBB may be more permeable, i.e., in the hypothalamus, has never been carefully analyzed. Therefore, this study was designed to determine if a specific AChE inhibitor, PYR, has an effect on hypothalamic AChE, versus regions that have a traditionally tight BBB, i.e., cerebral cortex. The results obtained from PYR in this study were compared with those using another AChE inhibitor, PHY, which is permeable to cortex and hypothalamus and thus is expected to inhibit AChE in both regions. For the primary analysis, three different parameters of brain AChE were compared, i.e., AChE activity, AChE protein levels and AChE mRNA levels.

PYR should not inhibit AChE activity in the cerebral cortex due to the impenetrable presence of a tight BBB and the quaternary amine structure of PYR. We found that PYR had no effect on AChE activity in the cortex. In contrast, both acute and subacute treatment with PHY caused significant inhibition of AChE activity in the cortex. These results support our model (Figure 5) and the current literature concerning cortical AChE activity, i.e., PHY can penetrate the BBB whereas PYR cannot, and thus PYR did not produce cortical AChE inhibition [Arnal et al., 1990].

Conversely, since the hypothalamus has a more permeable BBB, we hypothesized that PYR should be able to cross into this tissue and affect AChE activity. The present study suggests that PYR gained access to the hypothalamic tissue at both acute and subacute time

points and thus, was capable of altering AChE activity. This is contrary to previously published studies with whole brain or non-CVO assays [Lallement et al., 1998; van Haaren et al., 1999; Grauer et al., 2000; Sinton et al., 2000; Kant et al., 2001]. Furthermore, since acute PYR and PHY produced nearly identical blood AChE inhibition we expected to find similar AChE inhibition in the hypothalamus. In acute experiments, treatment with PYR resulted in a 51% decrease in hypothalamic AChE activity while PHY produced a 79% decrease. The greater reduction in AChE activity produced by acute PHY suggests that higher concentrations of PHY were crossing into the hypothalamic tissue than PYR.

In contrast, the subacute-treatment data showed opposite effects following subacute PYR and PHY treatment. Treatment with PYR produced a 35% increase in AChE activity whereas PHY produced a 31% decrease. Since PYR and PHY both inhibit AChE activity, a measurable increase in AChE activity can only be explained by an up-regulation in AChE protein following prolonged treatment. We propose that the amount of PYR crossing into the hypothalamus would not be enough to overcome this AChE protein increase, and thus the result would be an increase in activity (Figure 5). When the AChE protein levels were examined after subacute-treatment with PYR, they were found to have significantly increased. This is consistent with our hypothesis and with previous studies in which AChE inhibitor-induced increases in cholinergic stimulation led to an increase in AChE protein levels [Nitsch et al., 1998]. While PHY did not produce a significant increase in AChE protein levels, there was a trend suggesting an increase. The opposite effects on AChE activity can be explained by more PHY entering the hypothalamic tissue than PYR. While the amount of PYR entering the hypothalamus in acute experiments is sufficient to inhibit AChE activity, the amount of PYR entering the tissue during subacute PYR treatment is apparently sufficient to initiate an increase in AChE protein and thus lead to an overall increase in AChE activity. In contrast, the amount of PHY that can enter the hypothalamus following subacute treatment appears to be in excess of what can be overcome by an up-regulation of AChE protein and thus an overall decrease in activity is observed.

Interestingly, when comparing the ratio of AChE activity to AChE protein in hypothalamus, the ratios were nearly identical for the Sham controls and PYR-treated, but were lower for the PHY-treated animals. The increase in hypothalamic AChE protein produced by subacute treatment with PYR appeared to cause an increase in the relative level of AChE activity compared to control animals. This is in contrast to PHY, where AChE activity remained depressed in both the cortex and hypothalamus. This further supports the explanation that the amount of PHY that can enter the hypothalamus is significantly higher than that of PYR. There was not a similar increase in PHY to offset the inhibition of AChE activity. Alternatively, the results could suggest that AChE levels (or AChE inhibition) are regulated differently by PYR and PHY. That is, if PHY were able to gain more access to the hypothalamus and have a greater impact on AChE activity, why wasn't it able to induce a higher production of AChE protein to compensate for the inhibition? The increase in AChE protein was not significant for PHY. This suggests that AChE might have reached a maximal expression limit in this tissue and was not capable of having a greater effect. It could also suggest that PHY and PYR are not equal in how they cause up-regulation of protein.

To develop a more complete understanding of the mechanisms of AChE expression and regulation by PYR and PHY, we investigated the mRNA levels in hypothalamus and cortex following treatment. There was a significant decrease in cortical AChE mRNA caused by PYR in the subacute group while PHY had no effect. In contrast, significant increases in

hypothalamic AChE mRNA were found with acute PHY treatment and subacute PYR treatment. The results clearly show that PYR affects AChE mRNA levels in the hypothalamus, consistent with our hypothesis. What is interesting is that PHY did not affect the AChE mRNA. We suggest that the excessive amount of PHY entering the hypothalamus would allow the cell to more quickly establish homeostasis of AChE mRNA returning it to a normal state. Accordingly, the lesser amount of PYR entering the hypothalamus would provide a continual chronic stimulation that could be enough to cause an increased expression of AChE mRNA. Since subacute PYR failed to significantly alter either AChE activity or protein levels in the cerebral cortex, the observed increase in subacute cortical AChE mRNA suggests an indirect link to the cortex from another tissue, e.g., the hypothalamus, or that a small amount of PYR is in fact entering the cerebral cortex.

Although our model did not include stress, the possibility exists that in some of the mice, stress levels may have been high enough to cause leakage across the BBB into the cortex. These results would then be consistent with other studies in which PYR was able to cross a stress-induced open BBB [Friedman et al., 1996; Kaufer et al., 1998]. These data further suggest that AChE mRNA may not be temporally regulated the same as AChE protein, and that the span of time between the acute and subacute treatments may not be ideal to demonstrate the region specific effects of PYR and PHY on AChE mRNA. To analyze the effects of PYR and PHY on AChE mRNA expression in greater detail, future experiments may use a more inclusive time course where the brain regions would be recovered at many time points varying from 5 minutes, to a few hours, to days.

There are numerous secondary effects of cholinesterase inhibitors including inhibition of carboxylesterases and serine hydrolases, and as an antagonist on muscarinic and other receptors [Casida and Quistad, 2004]. The neuroendocrine system is responsible for producing peptide hormones from larger precursors or pro-hormones. Specialized enzymes are found in these tissues that can cleave the pro-hormone at paired-basic residues [Loh et al., 1984; Smeekens et al., 1992; Steiner et al., 1992]. As with AChE, these are serine proteases of the subtilisin family and have at their active site a similar catalytic triad of amino acids, Asp, His and Ser [Bryan et al., 1986; Sussman et al., 1991; Nakayama, 1997]. This catalytic triad has been found to be a target for organophosphate inhibition, i.e., diisofluorophosphate [Smeekens and Steiner, 1990; Meerabux et al., 1996]. Thus, the catalytic triad in the processing enzymes could be a potential target for AChE inhibitors such as PYR and PHY. If these processing enzymes were inhibited, we would expect to see a decrease in pro-hormone processing and a subsequent decrease in peptide hormone production. In previous studies on pro-hormone processing enzyme knockout mice we used SELDI-TOF mass spectrometry to analyze peptides and proteins from neuroendocrine tissue, i.e., the neurointermediate lobe pituitary [Hardiman et al., 2005]. Using the same mass spectrometric techniques in the present study, we showed that only the hypothalamus was affected by PYR or PHY. Of interest was that the smallest peptide at 1835 Daltons decreased, while the rest of the peptides or proteins increased. An explanation for this is that the pro-hormone processing enzyme activity in the hypothalamus was affected by PYR and PHY, allowing a buildup of the larger precursor peptides. The implication from the results is that PYR and PHY have both gained access to the hypothalamic region where the BBB is more open to affect the processing of proteins in the hypothalamus or the expression of processing enzymes.

Although behavioral studies were not conducted on the mice in this study, we could speculate from earlier studies by our group using the same concentrations of PYR, i.e., 10

mg/kg/day [Dubovicky et al., 2007] that specific effects should have been observed. Specifically, we would predict an acoustic shock response in the PYR-treated mice. Changes in locomotor activity and pre-pulsed inhibition were not observed in the earlier studies and would not have been expected here, except in the PHY-treated mice. Furthermore, studies from other groups have shown a correlation between stress and penetration of the BBB by PYR into other regions of the brain that could affect brain excitability and CNS functions [Friedman et al., 1996]. Although our study did not include a stress paradigm, in earlier studies from our group, the combination of shaker stress and PYR failed to produce significant changes in any of the behavioral variables [Dubovicky et al., 2007]. We conclude that some behavioral effects might be expected by treatment with PYR, but that more extensive testing would be required to specifically identify a cause-effect relationship.

CONCLUSIONS

The purpose of this study was to determine if the quaternary amine, pyridostigmine (PYR), could cross the BBB in specific regions of the brain in mice where the BBB is considered to be less restrictive to charged molecules. We have shown data that support this model and strongly suggest that PYR does cross the BBB into one of these specific regions, i.e., the hypothalamus (Figure 5). Subacute treatment with PYR caused changes in AChE activity, protein levels, mRNA and in non-AChE protein profiles. Although PYR's primary target is AChE, other serine hydrolases in the hypothalamus and other accessible tissues are also potential targets, whether through direct interaction with the active site on an enzyme or via changes in expression. Implications from these effects include the potential for PYR to perturb the endocrine/neuroendocrine system. In turn this implies there is a need to carefully monitor PYR use as a treatment prior to, or during, broad-use in the field.

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TABLES

Table 1. MANOVA Analysis of SELDI-TOF MS Proteomic Data.

Time	TISSUE	p- VALUE
Acute	Anterior Pituitary	0.627
	Posterior Pituitary	0.271
	Hypothalamus	0.076
	Cortex	0.545
SubAcute	Anterior Pituitary	0.545
	Posterior Pituitary	0.530
	Hypothalamus	0.047
	Cortex	0.544

A MANOVA analysis was used to examine proteomic data from the SELDI-MS analysis of each tissue. The resulting p-values indicate the probability of the treatment groups being different than the Sham treated. $P < 0.05$ is considered significant. Time indicates the length of time for the treatment with PYR or PHY, i.e., acute= 15 minutes, subacute= 7 days.

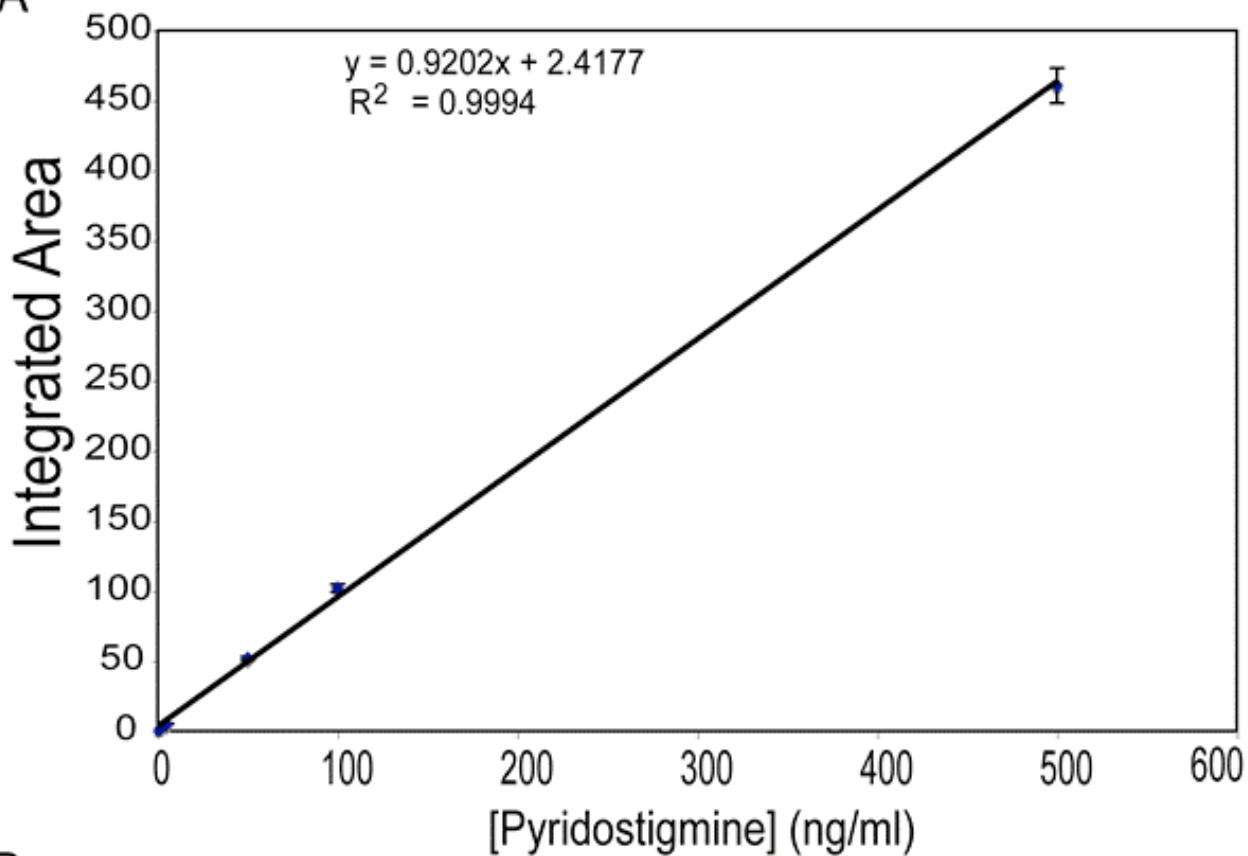
Table 2. Subacute effects of PYR and PHY on hypothalamus proteomic profiles.

Subacute Hypothalamus							Significance (p < 0.05)		
Peak (M/Z)	Average Integrated Area						Sham vs PYR	Sham vs PHY	PYR vs PHY
	Sham	S.E.	PYR	S.E.	PHY	S.E.			
1835.2	32.3	5.7	12.3	3.2	16.3	5.2	Yes	Yes	-
4471.5	25.6	8.0	58.8	12.6	64.8	11.4	Yes	Yes	-
5444.3	70.1	11.5	173.2	35.2	356.9	27.2	Yes	Yes	Yes
5706.6	56.9	21.8	211.8	54.3	410.5	36.5	Yes	Yes	Yes
6224.8	78.9	27.1	192.4	45.4	313.5	20.5	Yes	Yes	Yes
6573.5	29.1	4.8	54.6	11.6	81.4	7.1	Yes	Yes	-
6901.6	103.0	41.8	419.5	100.9	457.9	53.2	Yes	Yes	-
7013.8	165.5	71.0	737.8	180.7	1014.6	109.3	Yes	Yes	-
8398.4	329.5	63.8	693.6	115.4	1155.3	86.4	Yes	Yes	Yes
9915.1	64.3	22.8	234.8	50.4	353.0	29.3	Yes	Yes	Yes
11325.9	118.9	48.2	466.9	104.6	517.2	66.4	Yes	Yes	-
13810.2	95.1	38.1	324.6	73.3	405.0	43.1	Yes	Yes	-
14035.9	251.5	100.6	869.9	180.8	1146.4	148.0	Yes	Yes	-
14988.4	274.3	95.4	1017.0	235.4	1575.0	253.5	Yes	Yes	-
16791.7	358.1	88.5	1132.5	173.3	1528.9	168.0	Yes	Yes	-

Subacute (7 day) effects of PYR and PHY on hypothalamus proteomic profiles as determined by SELDI-TOF MS analysis of samples on a Ciphergen ProteinChip® Array. Average integrated areas for 45 ion peak M/Z values for Sham, PYR and PHY were compared using an ANOVA array in order to determine significance (Yes = significant difference, p < 0.05). S.E. indicates standard error of the mean.

FIGURES

A



B

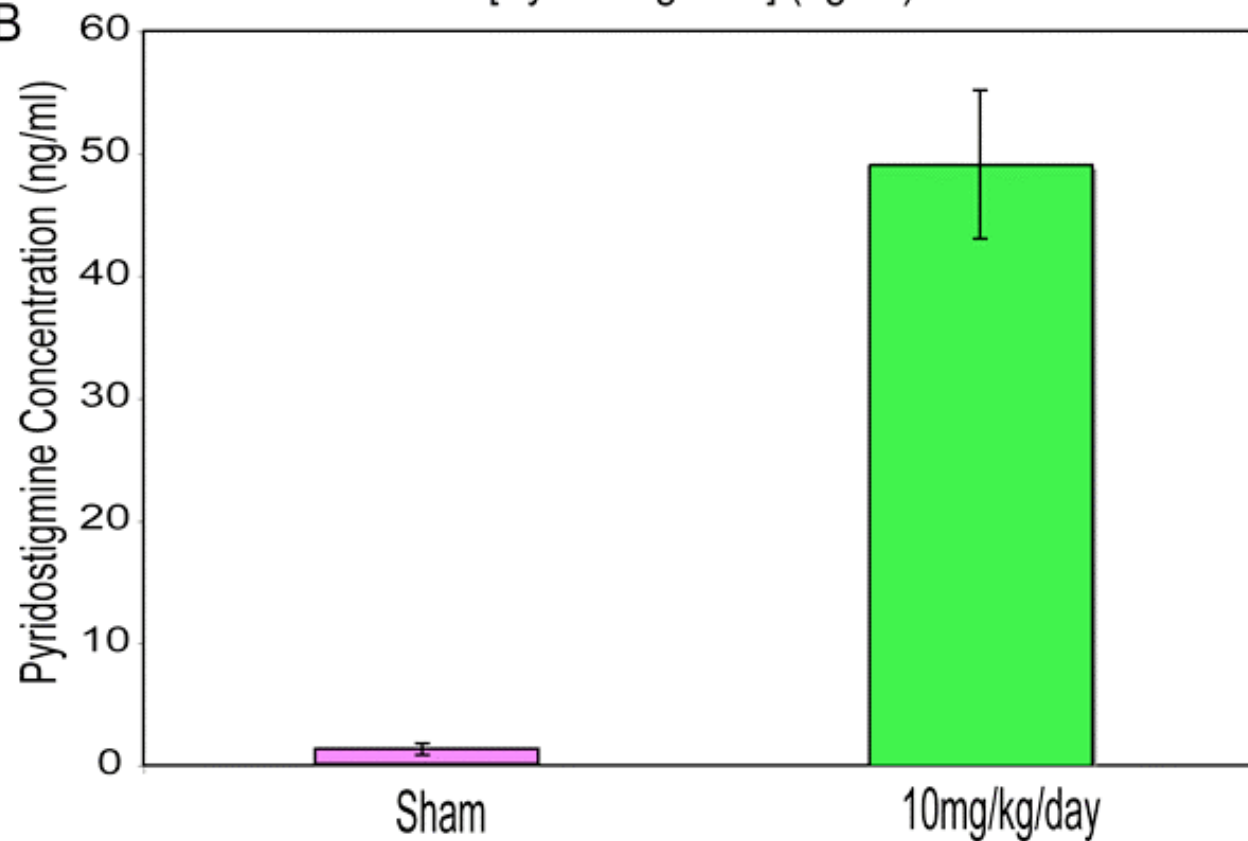


Figure 1. Measurement of Blood PYR Levels. PYR levels in blood were determined using a Sciex API3000 triple quadrupole mass spectrometer [Needham et al., 2003].
(A) A standard curve was established using increasing concentrations of PYR (ng/mL) in serum. These results were then compared to serum from mice implanted with Alzet pumps containing 10 mgs/kg PYR.
(B) The results represent the mean PYR concentration (ng/mL) \pm SEM from three separate mice.

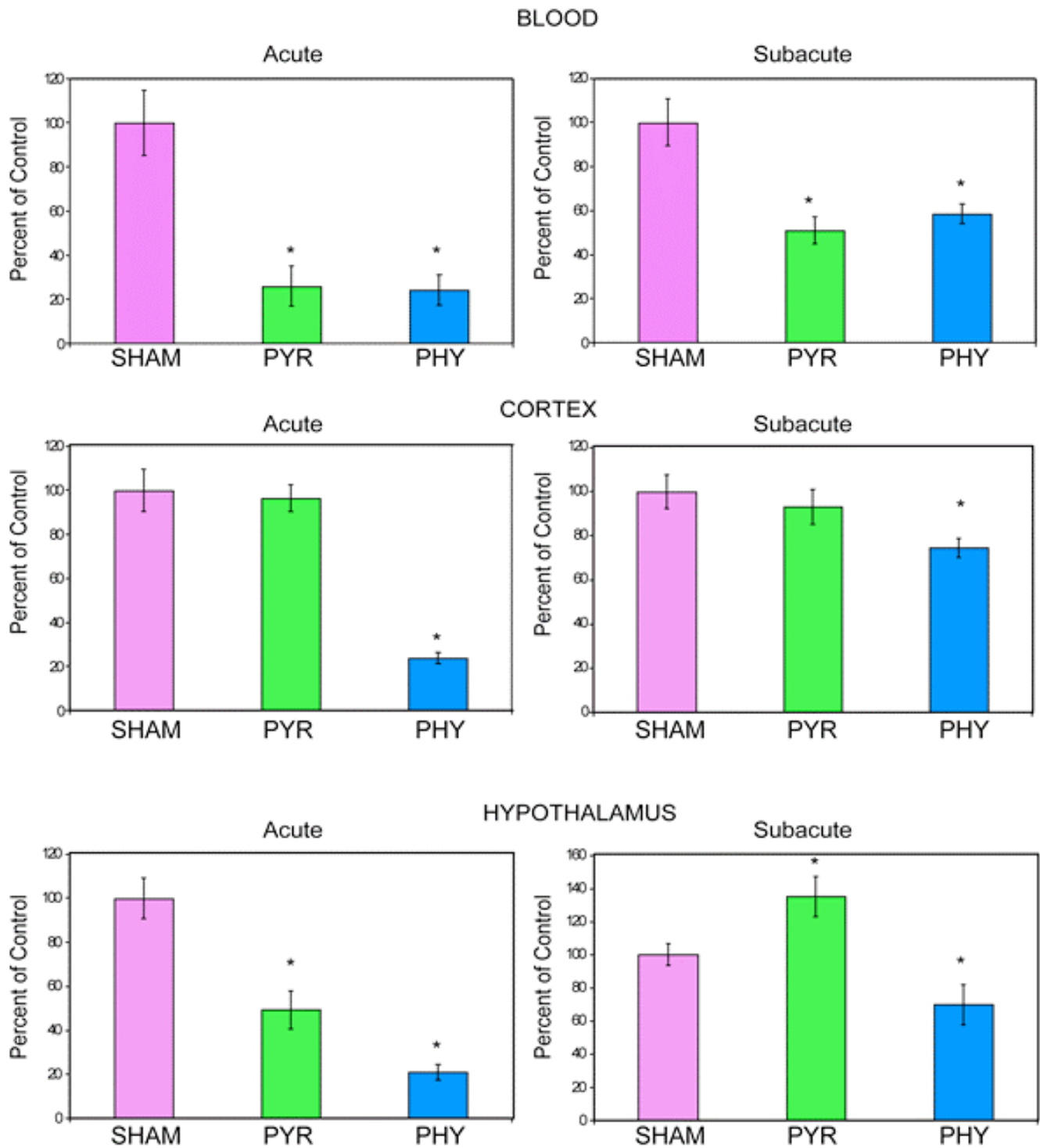


Figure 2. Effect of PYR and PHY on AChE activity in blood, cortex and hypothalamus. AChE activity was measured using the modified Ellman assay. The data are presented as the average percent of control \pm SEM from 10 different animals per group assayed in triplicate, i.e., N=10 for each group. Significance was determined at $p < 0.05$.

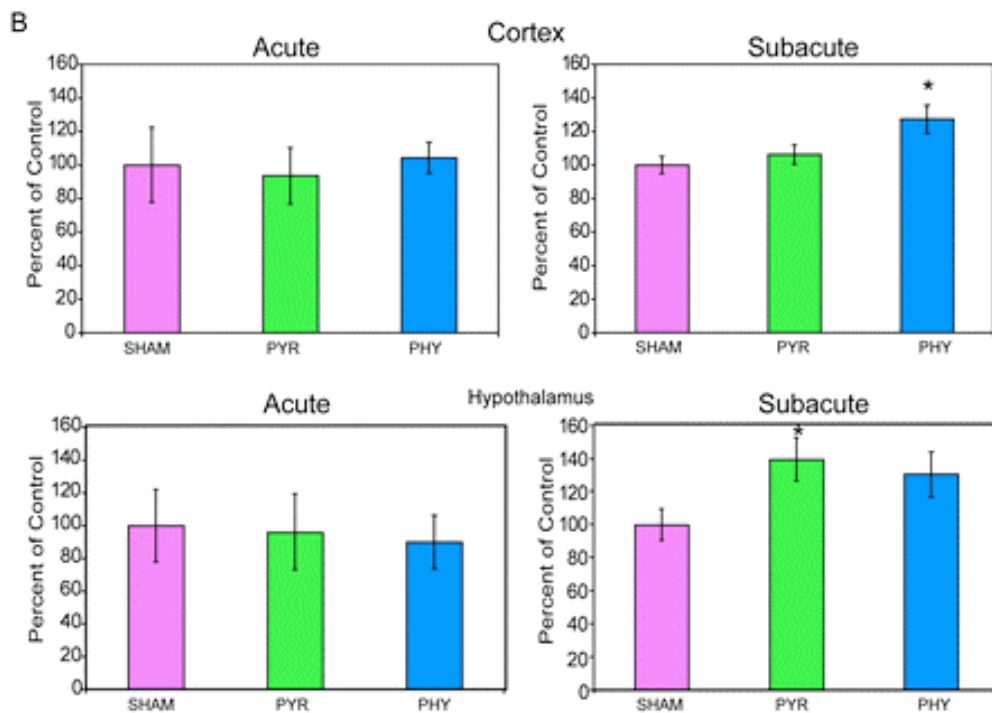
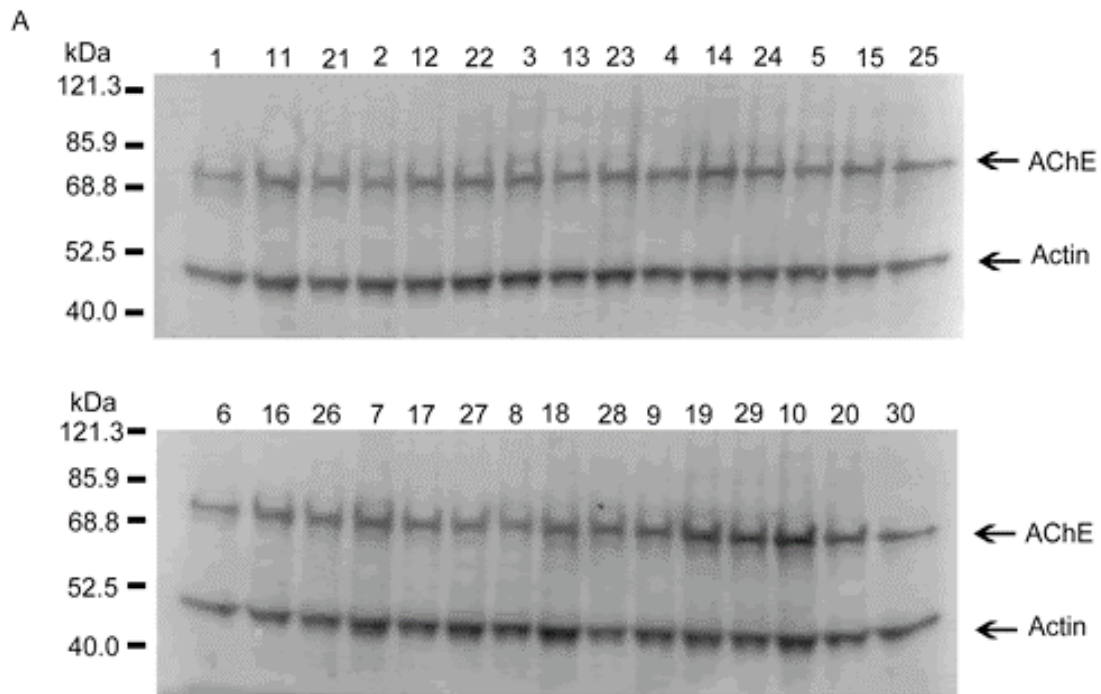


Figure 3. Effect of PYR and PHY on AChE protein levels in cortex and hypothalamus.

AChE protein was analyzed by western blotting using an antibody to AChE and an antibody to actin that was used to normalize the protein loading. Numbers 1-10 represent Sham; 11-20 represent PYR-treatment; and 21-30 represent PHY-treatment. The samples were grouped in an alternating pattern to prevent inter and intra-blot differences. FUJI ImageGauge software was used to analyze the density associated with each band. The data are presented as the average percent of control \pm SEM from 10 different animals per group, i.e., N=10 for each group. Significance was determined at $p < 0.05$.

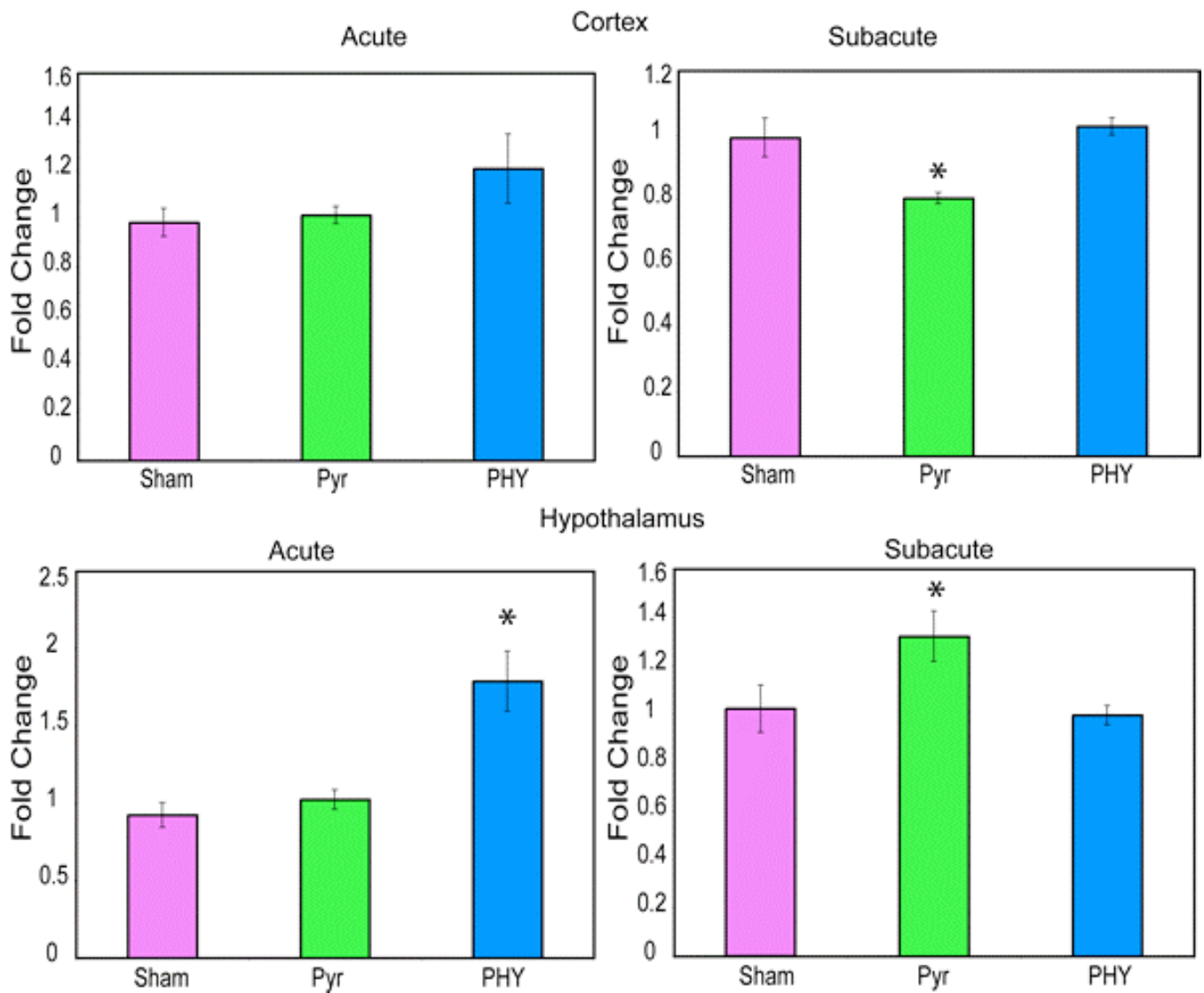


Figure 4. Effect of PYR and PHY on AChE mRNA levels in cortex and hypothalamus. mRNA was extracted and analyzed by semi-quantitative RealTime RT-PCR using a probe for AChE and the housekeeping gene MAX in each well. The data resulting from this was analyzed using the $2^{-\Delta\Delta CT}$ method as described by Livak [Livak, 2001]. The data represent the average fold change \pm SEM from 10 independent samples assayed in duplicate. Significance was determined at $p < 0.05$.

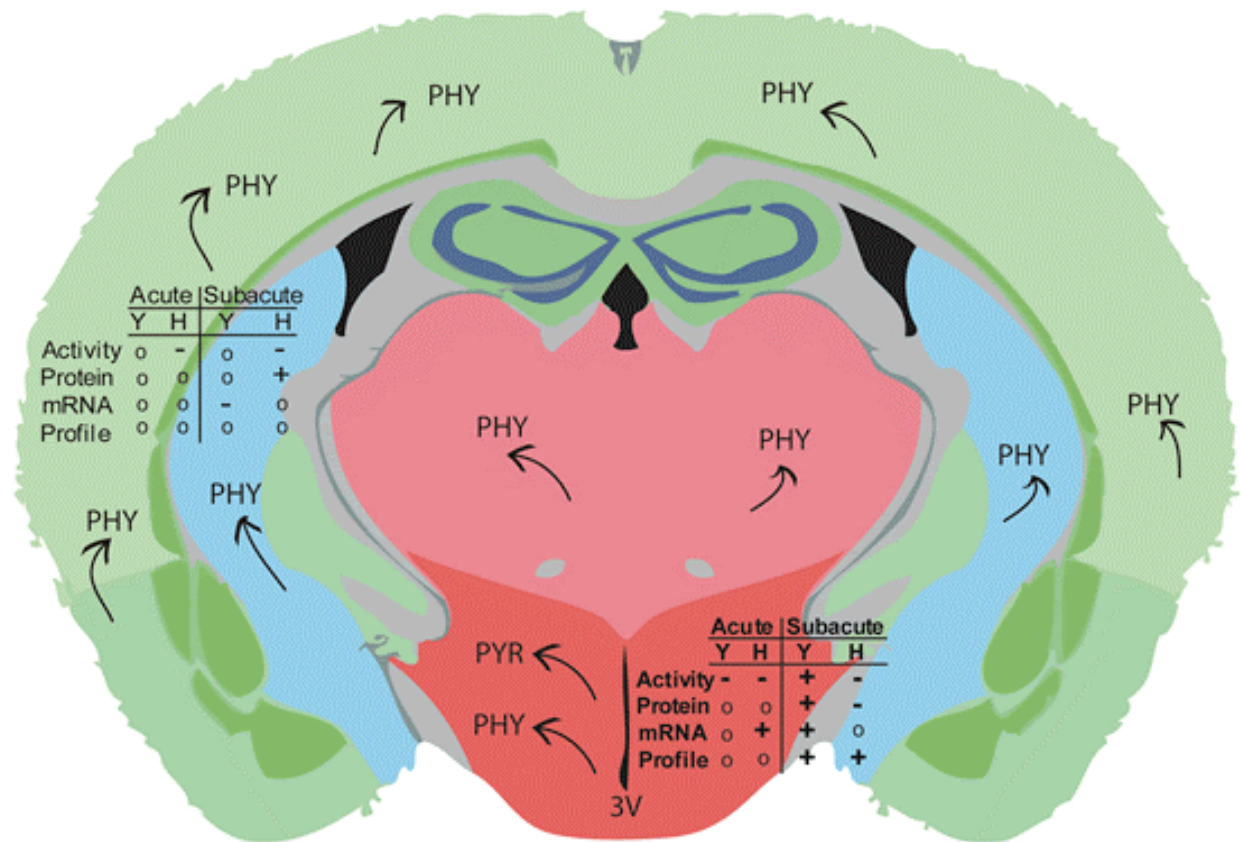


Figure 5. Model drawing showing a coronal section of the mouse brain (coronal level 68: Bregma -1.355 mm) with general regions colorized [adapted from the Allen Reference Atlas; Brain Info (2007), Neuroscience Division, National Primate Center, University of Washington, <http://www.brain-mapl.org>]. Major regions: light green indicates cerebral cortex; red indicates the hypothalamic region; pink indicates the thalamus; light blue indicates the caudate/amygdala regions; and the black vertical line indicates the 3rd ventricle in the hypothalamus (3V). Curved arrows indicate entry of PHY or PYP in the tissue. The superimposed table gives the qualitative results for changes caused by Acute and Subacute treatments, Y= PYP and H=PHY. The changes are indicated by (-) for a decrease, (+) for an increase or (o) for no change in AChE activity, protein, and mRNA expression levels. Changes in protein profiles, as determined by mass spectrometry, are also indicated by the same symbols.

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