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Cholinesterase inhibitors and stress: Effects on brain muscarinic receptor density in mice

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ABSTRACT

Exposure to the reversible cholinesterase inhibitor, pyridostigmine bromide (PB), in conjunction with stress, has been suggested as a possible cause of Gulf War Syndrome. This work explores the hypothesis that PB exposure coupled with stress will alter cholinergic receptor density based on the rationale that prolonged exposure to PB and stress will lead to increased stimulation of cholinergic receptors due to the reduced capacity to degrade acetylcholine, leading to changes in receptor levels. Male C57Bl6 mice were exposed to PB (3 or 10 mg/kg/day) or physostigmine (2.88 mg/kg/day) for 7 days via ALZET mini-osmotic pumps implanted subcutaneously. The mice were stressed by shaking at random intervals (avg of 2 min/30 min) for 1 week, which was sufficient to increase blood cortisol levels. Brain tissue for autoradiographic analysis was collected on day 7 of treatment. While we examined many brain regions, analysis revealed that most of the significant changes ($p < 0.05$) were seen in cholinergic nuclei. Stress typically increased muscarinic receptor density, while PB and PHY generally decreased muscarinic receptor density.

1. Introduction

During the 1991 Gulf War U.S. and British soldiers were exposed to a variety of chemicals such as insecticides, vaccines and the prophylactic agent against nerve gas attack, pyridostigmine bromide (PB), as well as the emotional and physical stress of combat (IOM, 1997). Months to years later some veterans began to exhibit a group of neurological and neuromuscular symptoms (IOM, 1998) subsequently termed Gulf War Illness. One theory proposed to explain this phenomenon is that pyridostigmine bromide, and not the nerve agents it was meant to protect against, contributed to the development of Gulf War Illness (Shen, 1998).

Pyridostigmine bromide is a reversible carbamate inhibitor of cholinesterase enzymes (Aquilonius and Hartvig, 1986). The Veteran's Administration has reported that soldiers, who may have been exposed to cholinesterase inhibitors, are 2–3 times more likely to develop amyotrophic lateral sclerosis than are other veterans (Walsh, 2001). Symptoms of Gulf War Syndrome are varied but include memory loss, attention impairment, and difficulty in reasoning (Haley et al., 1997), symptoms also associated with a loss of receptors (Feldman et al., 1997; Nestler et al., 2001). The purpose of this study was to look at whether exposure to pyridostigmine bromide with or without concomitant exposure to sub-acute intermittent, unpredictable stress altered muscarinic receptor density in the mouse CNS.

Pyridostigmine bromide was chosen for use as a prophylactic cholinesterase inhibitor during the Gulf War due to its inability to cross the blood–brain barrier and clinical evidence showing that it had no neurological side effects (Somani et al., 1972; Aquilonius et al., 1980). Physostigmine is chemically similar to pyridostigmine bromide, but the absence of a quaternary amine group allows it to freely cross the blood–brain barrier (Pratt, 1990). While the ability of physostigmine to permeate the blood–brain barrier made it unsuitable for use by the soldiers during the Gulf War, this property was useful in this study as a positive control to compare with pyridostigmine.

Although many symptoms of Gulf War Syndrome are neurological, most studies attempting to demonstrate that pyridostigmine produces effects in the CNS have been negative. While one study has shown an increase in the permeability of the blood–brain barrier to pyridostigmine following a forced swim in mice (Friedman et al., 1996), others have been unable to replicate these results (Grauer et al., 2000; Lallement et al., 1998; Sinton et al., 2000; Shaikh et al., 2003; Shaikh and Pope, 2003). In fact, one study showed that the permeability of the blood–brain barrier to pyridostigmine was decreased in rats exposed to combinations of

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forced swim, restraint, and heat stresses (Sinton et al., 2000). All of these studies employed an acute stress paradigm in which the single stress/pyridostigmine exposure occurred immediately before the measurement of CNS AChE activity. To more closely model the experiences of the soldiers with respect to duration, frequency, and unpredictability of stress, we used an experimental paradigm for this study that induced intermittent moderate stress 24 h/day for 7 days with concomitant pyridostigmine exposure. We observed that this paradigm produced significant changes in muscarinic receptor density in multiple brain areas in animals exposed to stress alone and to the cholinesterase inhibitors, pyridostigmine and physostigmine.

2. Materials and methods

2.1. Materials

[^{3}H]-pirenzepine (70 Ci/mmol) was purchased from Perkin-Elmer (Boston, MA). [^{3}H]-N-methyl-scopolamine (83.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Atropine, pirenzepine, and (−)scopolamine were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Experimental animals

Male C57Bl6 mice weighing approximately 25 g (Harlan Sprague Dawley, Indianapolis, IN), were housed separately under a 12:12 light–dark cycle with light onset at 6:00 a.m. Water and laboratory rodent chow were provided ad libitum. Mice were treated with pyridostigmine bromide (3 or 10 mg/kg/day), physostigmine (2.88 mg/kg/day), or saline. Drug delivery for all time points was by subcutaneously implanted Alzet osmotic mini-pump. Anesthesia for the surgeries was a ketamine/xylazine (6:1) mixture injected intramuscularly. Mice were sacrificed at the end of treatment or 30 days later. Treatment groups each contained 10 animals.

2.3. Shaker stress

Half of each treatment group for days 7 and 37 were subjected to shaker stress. The shaker power unit No. 5901 (Eberbach, Ann Arbor, MI) was used to induce shaker stress. It was set for a linear horizontal excursion of 2.86 cm at a speed of 150 cycles per minute. The total time of shaking was 90 min per day over 7 days distributed by computer into 45, 2 min periods separated by randomized still periods with a mean duration of 30 min (13–44 min). No handling of the mice was required as they were kept in their home cages which were on a cage rack mounted onto the shaker. The cage rack had an automated watering system and food pellets were placed on the bedding to avoid having food cups what would rattle during shaking (Bernatova et al., 2002).

2.4. Tissue preparation

Saline, pyridostigmine, and physostigmine treated animals were sacrificed by decapitation at time points described above. Brains were removed, quickly frozen in isopentane, and stored at −80 °C until sectioned. 10 μm coronal sections were prepared on a precision microtome contained in a cryostat, thaw mounted on to UltraStick™ slides (GoldSeal Products, Portsmouth, NH), air dried for ∼12 h and stored at −20 °C until analyzed. Coordinates for sections relative to bregma are as follows (in mm): diagonal band nuclei, 0.86; caudate putamen, limbic (cingulate) cortex, lateral septal nuclei, olfactory tubercle, 0.62; nucleus basalis, −0.46; and hippocampus, hypothalamus, thalamic nuclei, amygdala, and retrosplenial cortex −1.82 (Paxinos and Franklin, 2001).

2.5. Cholinesterase measurement

Trunk blood was collected immediately following decapitation into heparinized tubes and analyzed for cholinesterase activity using the Ellman method (Ellman et al., 1961). To minimize reactivation of the enzyme by dissociation of the carbamates, samples were kept on ice and diluted immediately before initiating the activity measurement.

2.6. Differential binding assay

To characterize changes in muscarinic receptor density by subtype, the differential binding assay developed by Flynn and Mash was used (Flynn and Mash, 1993). M_1 receptors were labeled by incubating slices with 3 nM ^{3}H-pirenzepine in 20 mM Tris buffer (pH 7.4) with 1 mM $MnCl_2$ for 45 min at 25 °C followed by 3 brief dips (approximately 3–5 s) in buffer at 4 °C. M_2 receptors were labeled by preincubating slices with 0.3 μM pirenzepine in 50 mM PO_4 buffer (pH 7.4) with 1 mM $MgCl_2$ for 1 h at 25 °C followed by 3 brief dips in buffer at 4 °C. The tissue slices were then incubated in 0.5 nM [^{3}H]-NMS in 50 mM PO_4 buffer (pH 7.4) with 1 mM $MgCl_2$ for 2 min at 25 °C followed by 3 brief dips in buffer at 4 °C. M_3 receptors were labeled by preincubating tissue slices in 0.5 nM NMS in 50 mM PO_4 buffer (pH 7.4) with 1 mM $MgCl_2$ for 5 min followed by 3 brief dips in buffer at 4 °C. The slices were then incubated with 0.5 nM [^{3}H]-NMS in 50 mM PO_4 buffer (pH 7.4) with 1 mM $MgCl_2$ for 60 min at 25 °C followed by 3 brief dips in buffer at 4 °C. Dissociation was accomplished by incubating slices with 1 μM atropine in 50 mM PO_4 buffer (pH 7.4) with 1 mM $MgCl_2$ for 60 min at 25 °C followed by 3 brief dips in buffer at 4 °C. Nonspecific binding for each subtype was determined by the addition of 1 μM atropine to the incubation buffer.

2.7. Fuji imaging plate and image gauge software

Dried tissue slices and tritiated microscales (Amersham Corp., Arlington Heights, IL) were apposed to a FujiFilm Tritium Imaging Plate (FujiFilm, Stamford, CT) for 10 days. Fluorescent Image Analyzer FLA-2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan) plate reader and MacBas ImageReader (Version 1.3E) software (Fuji Photo Film Co. Ltd., Tokyo, Japan) were used to capture the autoradiograms. MacBas Image Gauge (Version 2.5) software (Fuji Photo Film Co. Ltd., Tokyo, Japan) was used to quantitate the intensity of binding. Regions of interest were identified and quantitated by placing 1–5 squares of standard size (0.02 mm^2) on the brain region of interest in each hemisphere. Background was subtracted and the optical densities were converted to units of fmoles of radioligand bound per milligram dry tissue by comparison to the microscale standards. The following formula was used for the conversion:

$$\text{fmol/mg} = \frac{(\text{optical density} \times \text{calibration slope})}{\text{specific activity} \times 1000}.$$

2.8. Statistical analysis

Receptor density was analyzed using the GLM procedure of SAS (SAS, 1995). Data were analyzed as a 3 × 2 factorial design with the main effects of stress, drug treatment and stress × drug treatment interactions. Means were considered to be statistically different if the p value was < 0.05.

3. Results

Region and treatment specific changes in muscarinic receptor density were observed after 7 days exposure to stress, PB, or PHY. The observed changes in receptor density were also subtype