DOES PB CROSS THE BLOOD-BRAIN BARRIER DURING CONDITIONS OF STRESS?

Pyridostigmine bromide is a carbamate AChE inhibitor with a positively charged “quaternary ammonium” group, which is believed to prevent its penetration through the “blood-brain barrier,” a layer of cells that controls which substances may penetrate from the general circulation into the brain. Therefore, side effects of PB are typically those of heightened peripheral cholinergic activity (nicotinic and muscarinic). PB has been perceived as having an enhanced safety profile relative to such other carbamates as physostigmine, because of its relative disinclination to penetrate the blood-brain barrier. However, recent evidence suggests that PB can cross the blood-brain barrier under some circumstances, specifically in the context of stress or chemical combinations.

The ability of PB to cross the blood-brain barrier under circumstances of stress may render data on side effects of PB obtained during peacetime inadequate to predict effects that occur during war. Moreover, PB itself has been shown to enhance permeability of the blood-brain barrier, allowing penetration of normally excluded agents.

EVIDENCE

Published Research

In military reports dating at least to 1981, it was noted that the assumption of blood-brain barrier impermeability was not absolute (Kemp and Wetherell, 1981), that the blood-brain barrier was known not to be uniform even within individuals of the same species (Rapoport, 1976); and that evidence from animal studies suggests that PB can enter the midbrain, medulla, cerebellum, and cerebral cortex (Clement, 1977). Studies relating to PB penetration occurring in specific conditions of blood-brain barrier disruption (such as stress), and blood-
brain barrier disruption resulting from PB administration are few and, to our knowledge, recent. Results of the several published studies abstracted will be reviewed in turn. The first shows evidence that PB crosses the blood-brain barrier in a stress protocol in mice. The second suggests that there may be increased central and reduced peripheral side effects of PB in humans under war stress as compared to peacetime, suggesting similar stress-induced crossing of the blood-brain barrier. The third shows that PB itself enhances penetration of the blood-brain barrier by a virus. A fourth shows that heat-stress markedly increases blood-brain barrier permeability, though permeability to PB was not tested per se. The fifth abstract shows that concomitant exposure to chemicals amplifies the effect of stress on enhancing permeability of the blood brain barrier.

**Forced Swim Study in Mice**

The first study found that “stress” in the form of a forced swim increased PB penetration of the blood-brain barrier in mice. Researchers found that PB did not penetrate the blood-brain barrier in mice under normal conditions. However, in a four-minute forced swim (shown previously to simulate stress), an increase in permeability of the blood-brain barrier to PB was seen (Friedman, Kaufer, et al., 1996). This was demonstrated by a reduction in the dose of PB needed to achieve 50 percent inhibition of brain acetylcholinesterase (AChE). The dose needed for 50 percent inhibition was reduced from 1.5 mg/kg (~3.5 times the PB dose a 70 kg PGW vet would have received) in the absence of “stress” to 0.01 mg/kg (two-hundredths of the 70 kg PGW veteran dose) in the presence of stress—more than a hundredfold difference. A tenfold increase in penetration through the blood-brain barrier of compounds ordinarily relatively excluded by the blood-brain barrier was seen with these low levels of PB in stressed animals.\(^1\) More than a hundredfold increase in an indirect marker of brain neuronal activity\(^2\) was seen in stressed animals. A comparable effect occurred with 0.01 mg/kg PB (two-hundredths of the PGW dose) in stressed animals as occurred with 2 mg/kg PB (five times the PGW dose) in unstressed animals (in both instances leading to 95 percent inhibition of AChE in the cortex of the brain) (Friedman, Kaufer, et al., 1996).

It appears likely that these effects were the result of PB obtaining access to the brain, since PB was found to influence brain function similarly when applied directly to slices of brain tissue: application of PB (1 mM) to hippocampal slices

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\(^{1}\)These compounds consisted of dye bound to albumin or of AChE plasmid DNA, including the cytomegalovirus promoter.

\(^{2}\)This indirect marker of brain neuronal activity is brain c-fos mRNA determined by reverse transcription followed by polymerase chain reaction amplification.
reduced AChE activity within 30 minutes with similar efficacy to that observed in vivo at a dose of 2 mg/kg and induced a parallel hundredfold increase in brain levels of “c-fos oncogene” (the indirect marker of neuronal activity in the footnote 2). Moreover electrophysiological recording in slices of mouse brain showed increased stimulated neuronal activity in part of the mouse hippocampus (an area called “CA1”) following PB application (Friedman, Kaufer, et al., 1996).

Central effects of PB (that is, brain effects) were relatively more prominent than peripheral effects in conditions of stress, as compared to the unstressed condition. Control mice injected with 0.1 mg/kg PB (0.23 times the PGW dose) showed inhibition of serum butyrylcholinesterase (BChE) activity similar to that measured in humans in peacetime (18.8±3.5 percent human; 20.4±5.5 percent in mice), with no inhibition of brain AChE. The same dose in stressed mice (restraint stress) appeared to produce a lesser inhibition of BChE—with relatively suppressed peripheral nervous system (PNS) effects and relatively enhanced central nervous system (CNS) effects.

The studies were performed in mice, and on small samples (three to 11 mice per group; n = 8 with PB and forced swim). Also, “p values” and confidence intervals were not provided (a common “omission,” or difference in convention, in basic science literature). Nonetheless, the size of the effect precludes doubt regarding its importance in the animal model used. It remains to be determined what forms of “stress” produce such effects, to confirm that such an effect occurs in primates (and humans), and to determine what classes of agents normally excluded by the blood-brain barrier may gain access under conditions of stress.

**Comparison of Human and Mouse Data**

The second study compares human data during war and peace to data on stressed and unstressed mice. It found that human data in war (stress) and peace (nonstress) appear analogous to rodent data in forced swim (stress) versus none (nonstress); in both instances peripheral effects of PB appear to predominate in the unstressed case, while central effects become more pronounced in the presence of stress.

Friedman, Kaufer, et al. (1996) compared results from human studies on symptoms produced by PB in 35 healthy young volunteers during peace and on symptoms occurring during use of PB in 213 soldiers treated during the PGW. In peacetime volunteers, peripheral nervous system symptoms predominated. Symptoms included abdominal pain, diarrhea, frequent urination, increased salivation, rhinorrhea, and increased sweating, with an average of 18.8 percent complaining (range 5.5–38.9 percent). Only 8.3 percent (range 1–16.6 percent)
of participants reported symptoms related to CNS function (headache, insomnia, drowsiness, nervousness, difficulties in focusing attention, impaired calculation ability). In treated Gulf War personnel, CNS symptoms predominated: 23.6 percent (range 6.2–53.4 percent) reported CNS symptoms, while only 11.4 percent (range 6.1–20.4 percent) reported PNS symptoms. Results were felt to be consistent with the differential effects of stressed and unstressed conditions on mouse BChE and AChE, noted above.

However, the humans compared in peace and in war derive from different databases and are not necessarily comparable. Moreover, treated Gulf War veterans’ reactions, if they indeed result from PB (there was no placebo control group), might not reflect the effects of PB in war stress on others who did not present for treatment.

This study’s findings could have implications for possible long-term consequences of PB or of stress. The enhanced capacity of systematically administered plasmid DNA to reach the brain under stress may explain the vulnerability of stressed animals to viral infections (see Study 3, below). The time-course for this vulnerability remains to be determined. Transcriptional responses observed in this study were postulated to predict induction of secondary and tertiary processes with uncertain consequences and time-courses—effects that could conceivably have a role in long-term effects of PB on symptoms (Friedman, Kaufer, et al., 1996). At present, however, all long-term effects remain speculative. (See Chapter Fourteen, “Chronic Effects.”)

Effect of Cholinesterase Inhibition on the Blood-Brain Barrier

A third study on the effect of cholinesterase inhibition on the blood-brain barrier found that PB given to mice in subtoxic doses led to changes in the blood-brain barrier, allowing an infectious agent to gain entry to the brain and produce lethal CNS infection in a subset of animals not identifiably different from those not so affected.

A study conducted in Israel demonstrated that PB at doses of 0.4 mg/kg, about equal to one PGW PB dose (actually, 0.93 of a dose) and approximately a tenth of the LD$_{50}$ (the LD$_{50}$ is the dose lethal in 50 percent of animals), led to increased blood-brain barrier permeability in mice, as gauged by ability of a peripherally introduced nonneuroinvasive neurovirulent Sindbis virus strain (SVN) to cause CNS infection (Grauer, Ben Nathan, et al., 1996). (A strain that is “nonneuroinvasive is one that does not ordinarily invade the CNS. A “neurovirulent” strain may cause disease in the CNS once there.) Though a single oral dose in PGW veterans was 0.4 mg/kg, perhaps 7 percent of the oral dose is available as blood PB, so that the injected dose given to mice is in fact substantially larger than that given to humans, on a per-weight basis (see
0.1 LD<sub>50</sub> of soman and physostigmine led to similar effects. CNS infection occurred six to eight days following inoculation in about 40 percent of mice in which 0.1 LD<sub>50</sub> of PB had been introduced at the time of peak viremia (the peak of virus detectable in the blood, which occurred at about one day following inoculation) and in about approximately 20–40 percent of mice exposed to soman and physostigmine. High viral titers (levels of virus) were recorded in brain tissue of sick mice, with up to 5.210<sup>6</sup> PFU (plaque-forming units, a measure of the amount of virus) in sick mice as compared to no virus in mice that appeared healthy. Survival was 100 percent in mice exposed to PB or SVN alone but only about approximately 60 percent in those exposed to both. The differences were not due to changes in viral traits, e.g., neuroinvasiveness or neurovirulence, since no virus was observed when the virus isolated from brains of soman-treated mice was injected intraperitoneally to nontreated mice. Since there are cholinesterases located in the blood-brain barrier, the effect described was speculated to result from direct inhibition of the enzymes located in the capillary wall.

One could speculate whether a similar effect could underlie part of the observed increase in toxicity reported when PB is mixed with other agents, such as insecticides (see Chapter Nine, “Interactions Between PB and Other Exposures”). No virus was detected in the 60–70 percent of mice that appeared healthy following the same soman treatment. (See Chapter Eight, “Individual Differences in Reactions to PB.”)

**Effect of Heat on the Blood-Brain Barrier**

Heat stress in rodents (exposure to 38° C for four hours, not out of range of possible experience in the PGW) has been shown to markedly increase blood-brain barrier permeability to tracers, including Evans Blue albumin and labeled sodium (Sharma, Nyberg, et al., 1992); PB was not a focus of that study. Another study, published as an abstract, found no increase in PB penetration with heat alone in guinea pigs (25° C, 39° C, or 43° C for two hours). This was interpreted as demonstrating that penetration of PB into the brain under stress depends on the experimental conditions used. Differences in animal species, duration of stress, and nature of stressor may be pertinent factors. Both heat stress and restraint stress had been shown to markedly increase blood-brain barrier permeability in rats and mice, respectively, although heat stress was maintained for four hours for the rats.

**Effect of Chemicals and Heat on the Blood-Brain Barrier**

Dr. Abou-Donia (Duke University) is currently conducting animal studies on effects of physical/emotional stress (restraint stress, involving placement of an
animal in a Plexiglas restraint tube each morning after chemical treatment, if
any), heat stress, and chemicals on the blood-brain barrier in animals. Combi-
nations of PB (5 mg/kg/d orally, or 3.8 times the PGW dose) with high doses of
other chemicals agents (DEET 500 mg/kg/d subcutaneous; permethrin, 500
mg/kg/d in oil, daily for 30 days) influence the blood-brain barrier such that PB
can cross and produce central cholinesterase inhibition (inhibition that is
region-specific), and charged (radioactively labeled) chemicals that ordinarily
do not cross the blood-brain barrier may do so. Findings suggest that addition
of restraint-stress increased the effect of chemicals alone on the blood-brain
barrier (Abou-Donia, Abdel-Rahman, et al., 1997). The smallest effect occurs
with stress followed by chemicals, the largest when stress and chemical expo-
sures occur concomitantly (Abou-Donia, 1998). One investigator found in pre-
liminary work that heat stress (exposure to 38°C for four hours prior to sacrifice)
may produce effects on the blood-brain barrier similar to those produced by
restraint stress in mice (Abou Donia, 1998).

An abstract by another group found that shorter heat exposures (two hours) in
guinea pigs, even at higher temperatures, did not lead to detectable entry of
intravenously injected radiolabeled PB into the CNS, as evaluated auto-
radiographically. However, exposure to 43°C did itself result in partial
inhibition of brain AChE activity (Lallement, Foquin, et al., 1998). At present, no
direct evidence supports or refutes the possibility that heat might increase
blood-brain barrier penetrability in humans.

**Blood-Brain Barrier Breaches**

Blood-brain barrier permeability is increased in other settings that continue to
be defined. For example, headache may actually produce neurogenic inflam-
mation (through antidromic stimulation to the trigeminal-vascular junction
leading to release of pro-inflammatory peptides); indeed, it is thought to be for
this reason that the antimigraine drug sumatriptan is ineffective for mild
headache, in which the drug cannot penetrate the CNS, but is effective for
moderate or severe headache, in which neurogenic inflammation permits CNS
access for sumatriptan (Rothrock, 1999).

**OTHER RESEARCH**

In one peer-reviewed study, intracranial placement of heat-killed BCG (bacillus
Calmette-Guerin), which normally would not cross into the brain, followed by
subsequent peripheral exposure to BCG (in the presence, however, of a power-
ful adjuvant), precipitated a demyelinating response in rats (Matyszak and
Perry, 1995). This is a case in which proximal events allowing substances entry
to the CNS led to temporally distant responses.
Another peer-reviewed study demonstrated that aluminum levels in mouse brain increase following administration of aluminum adjuvanted vaccines (Redhead, Quinlan, et al., 1992). Moreover, the paper cites increasing evidence that aluminum ions can contribute to increased permeability of the blood-brain barrier, acting synergistically with iron ions. Therefore, it is conceivable that aluminum-adjuvanted vaccines, such as the anthrax and botulinum toxoid vaccines administered to some military personnel during the PGW, could have increased blood-brain barrier permeability independently from—and perhaps in addition to—effects produced by PB, chemicals, heat, or stress. However, such effects remain to be demonstrated in humans, and it is unknown whether effects from these sources would be additive, synergistic, or neither.

**DISCUSSION**

Our understanding of the possible immediate and delayed consequences of the introduction to the brain of foreign substances normally excluded from the CNS is limited at best. Data are consistent with the possibility that some neuroactive substances, including PB, may gain access to the CNS through impaired blood-brain barrier function in conditions of heat (around 100° F), chemical combinations, and/or physical/emotional stress. The scope of definitions of “stress” that may produce leakiness in the blood-brain barrier continues to be characterized. It is not clear how many well and ill PGW veterans may have been exposed to comparable or “relevant” forms of stress. The doses of chemicals used in these experiments are extreme, and it is difficult to compare their stress protocols to experiences of PGW veterans.

It is also possible that breaches in the blood-brain barrier could lead to enhanced susceptibility to neurovirulent infectious agents or neurotoxic effects from concurrent chemical exposures. Therefore data on side effects from PB obtained without “stress,” or sustained heat, or perhaps chemical combinations, may not adequately reflect effects that may occur in the presence of these factors. Consequently, it is possible that data on acute (and chronic) effects of PB obtained using PB alone, in peacetime conditions, may not adequately reflect effects that occur in an environment in which such factors as stress, heat, or chemical exposures are also present.

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3Refer to A Review of the Scientific Literature As It Pertains to Gulf War Illnesses, Vol. 3: Immunizations (Golomb, forthcoming), in the chapter relating to effects of aluminum in vaccines.
CONCLUSIONS

At present, evidence from animal studies suggests that disruption of the blood-brain barrier in situations of heat, chemical exposure, and physical/emotional stress, some of which may have been present in the PGW, may lead to central effects from drugs, chemicals, and infectious agents normally denied access to the CNS. The practical effect of this disruption in such conditions as those seen in the Gulf War is uncertain. Moreover, whether chronic or delayed consequences of these exposures may occur is a matter for further study.

SCIENTIFIC RECOMMENDATIONS

Further research should help provide the following information:

- The nature of the “stresses” and chemical combinations that disrupt the blood-brain barrier.
- The nature and duration of blood-brain barrier disruption following stress.
- The classes of normally excluded agents that gain access to the CNS with such blood-brain barrier disruption.
- The effects of stress using drugs and chemicals in doses and through administration routes more similar to those used by military personnel.
- A determination of whether such effects occur or continue with subacute or subchronic exposures similar to those expected in combat.
- What becomes of agents that cross the blood-brain barrier due to a leaky barrier after the barrier function has been restored; are such agents “trapped” in the CNS, and if so, what are the implications, if any?

SUMMARY ANALYSIS

Exposure: It is unknown whether PGW veterans would have been “exposed” to breaches in the blood brain barrier. Animal data indicate that some forms of stress, high-dose chemical combinations, heat, AChE inhibitors, and aluminum all may contribute to enhanced blood-brain barrier permeability. Different veterans would have experienced different combinations and different “doses” of these factors. Whether these would be adequate to significantly enhance blood-brain barrier permeability in some instances is not known.

Compatible symptoms: Increased symptoms compatible with central AChE inhibition were reported following PB administration during the PGW compared to peacetime. This is consistent with the existence of breaches in the blood-brain barrier.
Link between exposure and symptoms: There is no information to determine whether factors thought to promote breaches in the blood brain barrier were more likely to be present in individuals reporting central symptoms following exposure to PB.