

---

**EVIDENCE FOR NEUROTRANSMITTER DYSREGULATION  
IDENTIFIED OR PUBLISHED SINCE THE REVIEW PROCESS**

---

Information not included in the original discussion of neurotransmitter dysregulation is provided here. The theory of downregulation/dysregulation of ACh function, the principal theory of causation put forth by and supported in this report (other mechanisms are primarily adjunctive), was first articulated as part of this work for RAND in numerous briefings commencing in early 1997. Since then, and since much of this report was originally written, an increasing body of literature has emerged consistent with or supportive of a possible contribution of ACh dysregulation (and, as originally theorized, primarily downregulation), resulting from AChE inhibitors, and a possible contribution of such dysregulation to health conditions. Before the original draft was sent for review, acetylcholinergic dysregulation had not been articulated as a theory of illness in PGW veterans.

Both older, not-previously-discussed information and newer evidence continue to add support to the dysregulation theory, with differential effects on different receptors, enzymes (e.g., subtypes of AChE), muscles, and brain areas. The time-courses of some changes remain too small to account for truly long-term effects. Other time-courses are long-lived but have not been followed long enough to determine just how extended in time they are. Finally, it has been shown that exposure to PB may influence later response to PB (and perhaps other AChE inhibitors), although again the possible intervals between exposures that permit this effect have not been well-characterized.

Some particularly important work comes from the laboratory of Hermona Soreq and collaborators in Israel (briefed as part of a mission to the Middle East in October 1997). Prior work in animals by this group had shown that PB may penetrate the brain under stress—leading to increased ACh excitation—work that provided the conditions in which PB could exert an effect centrally. More-recent work by this group has shown that cholinergic excitation (such as that expected if PB penetrates the blood-brain barrier) leads to long-lasting changes in the brain resulting in delayed suppression of cholinergic transmission

(Friedman, Kaufer, et al., 1998) and that AChE inhibitors (of which PB is one) “induce multigenic transcriptional feedback response suppressing cholinergic neurotransmission” (Kaufer, Friedman, et al., 1999).

This work in animals, identifying mechanisms by which AChE inhibitors could exert long-lasting effects on ACh function, is complemented by epidemiological work that has suggested a relationship between PB adverse response (Haley and Kurt, 1997) and illness, or AChE exposure (PB and pesticide/insecticide exposure) and illness (Unwin, Blatchley, et al., 1999). Also critically important are clinical studies, such as those of Robert Haley and colleagues in Texas, that evaluate ill PGW veterans and controls on specific objective measures in an effort to identify subtle or marked deficits (for instance, neurological, neuropsychological, and cerebral blood flow or brain electrical activity alterations) in classes of ill PGW veterans, that may then be correlated with known aspects of cholinergic function to test and further refine (or conceivably refute) our understanding of how (and whether) disruption of the ACh system pertains to illness in some veterans. The cholinergic system has many subparts with distinct functions interacting in complicated ways, and only integration of work at many levels, including epidemiological, basic science, and clinical studies, will adequately further understanding of whether and how PB and ACh dysregulation may pertain to illness in PGW veterans.

## **POTENTIAL NONHOMOGENEITY OF EFFECTS**

Evidence continues to mount that the impact of AChE inhibitors or other augmenters of ACh action in altering regulatory systems may be nonuniform; alterations in regulation for one area of the brain, or one muscle, may differ from those in another area of the brain, or another muscle; the impact on one type of receptor, one type of nicotinic receptor, or one type of AChE molecule may differ from the impact on another type. These observations have important implications for investigation into the possible effects of PB; quantitative and qualitative differences in different parts of the system imply that negative or positive findings for one muscle, region, receptor, or molecule are consistent with positive, negative, or different findings at another muscle, region, receptor, or molecule.

### **Different Nicotinic Receptors**

Specifically, neuronal nicotinic receptors have been found to be functionally diverse. There exist subunits, or member of the gene family, whose functional role in neuronal nicotinic receptors remains unknown, and there may be member of the gene family not yet identified. Different genes encoding these subunits are expressed in discrete but overlapping sets of brain nuclei; almost all

known members are expressed in an area of the brain termed the medial habenula. Even adjacent cells may express different alpha subunit genes, suggesting major heterogeneity in receptor phenotype even in what are usually treated as a homogenous population of cells (e.g., Purkinje's cells in the cerebellum) (Patrick, Sequela, et al., 1993). Presence of different subtypes of receptors in different brain regions may participate in accounting for different responses of different brain regions to acute and prolonged ACh stimulation.

As an example with possible clinical relevance, a study, published as an abstract, showed that "chronic" exposure of rats to the organophosphorus AChE inhibitor DFP, at a dose previously shown not to produce overt signs of toxicity (0.25 mg/kg/d for two weeks) led to sustained depression of hippocampal nicotinic receptors of a certain type, associated with sustained reduction in ability to learn a new task (Buccafusco, Prendergast, et al., 1997). Reduction of up to 50 percent was seen in the number of cortical, striatal, and hippocampal high-affinity (tritiated epibatidine) nicotinic receptors, measured by quantitative autoradiography. By three weeks after DFP withdrawal, hippocampal nicotinic receptor density did not recover significantly (but receptor densities in the other regions did). Significant impairment in learning a spatial navigation task (water maze) up to three weeks after withdrawal from DFP was seen; much less severe deficits occurred when training occurred prior to DFP administration (Buccafusco, Prendergast, et al., 1997). It was not stated whether testing beyond the three-week period was undertaken.

### **Different Forms of AChE**

In addition, multiple forms of AChE have been found. Six to 20 cholinesterase isoenzymes have been shown in the rat, with the pattern of isoenzymes influenced by the chemical given and by the assay employed (Nagayama, Akahori, et al., 1996). (Looking crudely at red blood cell and serum cholinesterase, the latter was reduced more in comparison to control animals with administration of fenthion, chlorpyrifos, bromophos, Diazinon, propaphos, DFP, Haloxon (Nagayama, Akahori, et al., 1996).) There exist sex differences in the pattern of cholinesterase isoenzymes in rats (Nagayama, Akahori, et al., 1996). Most effects by chemicals appeared to be greater in female rats, which start with more both true and pseudocholinesterase activity (Nagayama, Akahori, et al., 1996).

Another report, from a study in guinea pigs, notes that AChE is seen as a monomer (G1), dimer (G2), and tetramer (G4), with three asymmetric forms designated A4, A8, and A12; the A12 is the functionally important form at the neuromuscular junction (Lintern, Wetherell, et al., 1998). AChE inhibitors were noted to differentially inhibit different molecular forms of AChE in human brain and rat brain and diaphragm. In untreated guinea pigs, the highest AChE

activity was seen in the striatum and cerebellum, then the midbrain, medulla-pons, and cortex; the lowest activity was seen in the hippocampus. Activity in the diaphragm was in turn sevenfold lower than in the hippocampus. Differential impact of the AChE inhibitor soman (given at approximately one LD<sub>50</sub>, at 27 μM/kg) on brain regions and on AChE subtypes was seen with administration of soman in guinea pigs (Lintern, Wetherell, et al., 1998). One hour after soman administration, there was a dramatic reduction in AChE activity in all tissues (since soman is an AChE inhibitor). In the muscle, the three major molecular forms of AChE (A12, G4, and G1) were similarly inhibited and manifested similar rates of recovery, with normal function by seven days. However, in the brain, the G4 form of AChE was inhibited more than the G1 form; the hippocampus, cortex, and midbrain evinced the greatest reductions in activity; at seven days, activity had recovered in the cortex, medulla-pons, and striatum but inhibition remained in the hippocampus, midbrain, and cerebellum (Lintern, Wetherell, et al., 1998).

In chickens, there is a water-soluble form and several subtypes of membrane-bound AChE that differ in such characteristics as binding characteristics ( $K_m$ ), rate constant, turnover number, specificity constant, maximal velocity, and half life (the time to convert half the substrate to product) (al-Jafari and Kamal, 1994). Similarly, in humans there is a “minor form” of AChE, the water-soluble form and several subtypes of the “major form” of AChE that differ again in the kinetic properties noted above (Kamal and al-Jafari, 1996).

Existence of different species of AChE has gained importance by the finding of Israeli researchers that both stress and AChE inhibition by administration of PB (1 mM) or DFP (1 μM) led to “dramatic and persistent upregulation of AChE production in the central nervous system” (Soreq, 1999), which appears to result from calcium-dependent changes in gene expression (and alternate splicing), leading to increases in a minor, quickly migrating form of AChE (Kaufer, Friedman, et al., 1998). Studies in transgenic mice suggest that chronic increased AChE activity may delay onset of progressive deterioration in cognitive and neuromotor function (Andres, Beeri, et al., 1997; Beeri, Gnatt, et al., 1995), although whether the alterations in AChE seen in the stress/carbamate experiments would produce similar changes was not reported.

### **Differences Across Brain Regions**

A study in rats showed that stimulation of the nicotinic classes of ACh receptors, with long-term nicotine treatment (high dose: 50 mg/kg in drinking water for nine to 41 weeks), led to no significant changes in activity of the enzyme cholineacetyltransferase (which catalyzes production of ACh) or in muscarinic binding sites (receptor sites) (Nordberg, Wahlstrom, et al., 1985). However, 24

hours after withdrawal from 41 weeks of nicotine treatment, there was a significant 46 percent increase in the number of (radiolabeled) nicotine-binding sites in the hippocampus, with a concurrent 44 percent decrease in nicotine-binding sites in the cortex, and no change in the midbrain, consistent with mixed upregulation and downregulation (Nordberg, Wahlstrom, et al., 1985). Of note, these changes had disappeared on the fourteenth day of abstinence and had not occurred when only nine weeks of nicotine treatment had occurred. Although in these conditions (with nicotine as the ACh agonist, in rats) the effects examined were not long-lasting, these effects illustrate that the impact of ACh augmentation may be regionally specific and present in one region but absent in another.

Another study (abstract) in mice, reviewed above, also supports differential impact of AChE inhibitors on different brain regions; specifically, there were differential changes in density of radiolabeled epibatidine type of high-affinity nicotinic receptors following two weeks of low-dose DFP administration; reduction in receptor number was seen in cortex, striatum, and hippocampus but was sustained only in the hippocampus (measured at three weeks post-DFP); this was accompanied by reduced performance in a water-maze learning task, both the learning and spatial elements of which would be expected to involve the hippocampus (Buccafusco, Prendergast, et al., 1997).

AChE activity in mice brain following exposure to stress (in the form of forced-swim) showed differential changes according to brain region. At 80 hours after stress, cortical AChE activity remained markedly increased, nearly double that of controls; while cerebellar AChE, which had initially dropped, was restored to control values, and hippocampal AChE, which had initially increased to approximately 75 percent over controls, was reduced to approximately 50 percent of control values. Thus, the direction and time-course of changes in AChE was markedly different from one brain region to another, potentially consistent with differential dysregulation (Kaufer, 1998). (Changes were only followed to 80 hours poststress.) Similar changes in AChE activity were reportedly seen following carbamate exposure (1 mM PB) but graphs showing the time-course of response were provided only for the instance of stress.

Another study in rats found that physostigmine (a close relative of PB that more readily crosses the blood-brain barrier) leads to opposite-direction changes in different parts of the hippocampus: using 2-deoxyglucose as an index of regional brain activity, an increase was seen in the outer zone of molecular layer of the dentate gyrus and in stratum lacunosum-moleculare of an area of the hippocampus, termed area CA3, suggesting an increase in perforant path input during theta rhythm; a decrease was seen in the hilar dentate region, felt to reflect hilar inhibition by local circuits during theta rhythm generation (Sanchez-Arroyos, Gazetlu, et al., 1993).

### **Differences Across Muscle Types**

Studies performed in the neuromuscular junction of mice have shown that PB induces different changes in different forms of AChE, with different time-courses in different muscles (Lintern, Smith, et al., 1997). "Repeated treatment with PB in mice can have a profound delayed effect on the activity of AChE in skeletal muscle. The changes varied in magnitude, direction and time-course depending on the type of muscle examined." The differences were felt to suggest a sensitization of the metabolic pathways for synthesis of the precursor G1 form, and/or assembly of precursor forms into the functional A12 form of AChE. Oscillations in activity seen in some cases suggested possible disruption of the fine homeostatic control of the enzyme levels (Lintern, Smith, et al., 1997). A mechanism for these changes was proposed, based on prior evidence that PB can induce synthesis of beta-endorphin in motoneurons, and beta-endorphin can increase synthesis of AChE in myotubes. One source for differential responses across different muscles is the fact that ACh release is intermittent in nerve signals to "fast" muscle fibers, while the nerves to slow fibers are more continuously active. Following PB administration, the G4 and A12 forms of AChE were reduced in the diaphragm but increased in the extensor digitorum longus and soleus muscles (limb muscles); at a week later, all forms were increased in all three muscles. At two weeks, the activity had returned to normal in the diaphragm but not in the muscle of either tested limb.

### **DYSREGULATION AND CHRONIC EFFECTS: PB EXPOSURE MAY ALTER SENSITIVITY TO LATER EXPOSURES**

Another potentially important finding was that PB treatment altered sensitivity of muscles to later exposures (Lintern, Smith, et al., 1997). A single dose of PB was given to mice that had been pretreated with PB for three weeks, then left untreated for two weeks; and to control mice. Controls showed no effect on enzyme activity in the diaphragm at up to five days, but decreases in enzyme activity in the extensor digitorum longus and increases in enzyme activity in the soleus were seen. In contrast, in the pretreated group all three examined forms of AChE were increased in the diaphragm, especially the A12 form, and the soleus and extensor digitorum longus showed prolonged decreases in all forms, although in the soleus the A12 remained above normal. Thus, the nature, direction, and time-course of changes differ according to the specific enzyme subtype and the muscle, and they depend on prior exposure.