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Effect of Low-Dose Sarin Exposure on the Neurochemistry of Different Brain Structures in Mice

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EFFECT OF LOW-DOSE SARIN EXPOSURE ON THE NEUROCHEMISTRY OF
DIFFERENT BRAIN STRUCTURES IN MICE.

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

Dhawal Oswal
B.Pharmacy, Pune University, 2007

2009
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY
SUPERVISION BY DHAWAL PRAVIN OSWAL ENTITLED “EFFECT OF LOW-DOSE SARIN
EXPOSURE ON THE NEUROCHEMISTRY OF DIFFERENT BRAIN STRUCTURES IN MICE” BE
ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
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Sarin (GB) is a toxic organophosphate (OP) nerve agent that was released in the Gulf War and was used in terrorist attacks in Japan. People who survive such attacks exhibit various long-term effects including alterations in neuropsychological performances. It has also been hypothesized that the Gulf War Illness could be a result of low level exposure to OP’s. In order to understand the effect of low dose exposure to GB on physiological and behavioral functions, we analyzed the levels of monoamines and their metabolites in different brain areas after exposure of mice to a sublethal dose of GB. Mice (male C57BL/6) were injected subcutaneously once a day for 2 days, with 0.05 LD50 or 0.4 LD50 of GB followed by behavioral testing in the open field environment, elevated plus maze and for fear potentiated startle. The mice did not show signs of cholinergic toxicity. They were sacrificed at 1, 4 and 8 weeks with collection of brains for neurochemical analysis. In both dose groups and time points, a significant decrease in the usage of dopamine (DA) was observed in the frontal cortex (FC) region of the brain which may account for a number of symptoms of the Gulf War veterans. There was an increase in the usage of DA in the amygdala at 4 weeks but not at 1, 8 weeks, indicating a reversible
effect. No significant change observed in the DA activity of the caudate nucleus which was consistent with no change in the motor activity in the open field studies. In addition to this, the levels of serotonin (5HT) were transiently elevated in all the brain regions studied. The FC is innervated mainly by the A10 group of dopaminergic cell bodies located in the ventral tegmental area (VTA) of the brain. The amygdala, in addition to A10, also receives projections from the A8 group of dopaminergic cell bodies in the retrorubral nucleus (RRN) – accounting for the difference from the FC. The caudate nucleus is innervated mainly by the A9 group of dopaminergic cell bodies located in the substantia nigra (SN) region of the brain. Data strongly suggests that even low dose of sarin has potent long-term, region specific effects on other neurotransmitter systems. Further experiments are necessary to evaluate the relationship between these modifications and the neuropsychological disorders reported after asymptomatic exposure to OPs.
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I. INTRODUCTION

Background

A United Nations report from 1969 defines chemical warfare agents (CWA) as “… chemical substances, whether gaseous, liquid or solid, which might be employed because of their direct toxic effects on man, animals and plants …” Among the CWA, the nerve agents have been around since World War I. The nerve agents are so called because they affect the transmission of nerve impulses in the nervous system.

All the nerve agents belong to a group of organophosphorus (OP) compounds. In 1934, Dr. Gerhard Schrader, a German Chemist at IG Farben, was given the task of producing pesticides. In doing so, he came across a highly toxic OP compound which was later called Tabun (GA). Following this, other nerve agents mainly Soman (GD) and Sarin (GB) were produced. These classic nerve agents are known as ‘G agents in American Nomenclature. By the mid-1950 a group of more stable nerve agents had been developed. They were 10-fold more toxic than GB. These agents came to be known as V-agents in the American nomenclature.

![Chemical structure of nerve agents](image)

**Figure 1** - Chemical structures of nerve agents (Augerson 2000)
Table 1 (Augerson 2000)

Nerve agent chemical structure

<table>
<thead>
<tr>
<th>Agent</th>
<th>X</th>
<th>R₁</th>
<th>R₂</th>
</tr>
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<tr>
<td>Tabun (GA)</td>
<td>CN</td>
<td>N(CH₃)₂</td>
<td>C₂H₅</td>
</tr>
<tr>
<td>Sarin (GB)</td>
<td>F</td>
<td>CH₃</td>
<td>CH(CH₃)₂</td>
</tr>
<tr>
<td>Soman (GD)</td>
<td>F</td>
<td>CH₃</td>
<td>CH(CH₃)C(CH)₃</td>
</tr>
<tr>
<td>Cyclosarin (GF)</td>
<td>F</td>
<td>CH₃</td>
<td>Cyclohexyl</td>
</tr>
<tr>
<td>VX</td>
<td>SCH₂CH₂N[CH(CH₃)₂]₂</td>
<td>CH₃</td>
<td>C₂H₅</td>
</tr>
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OP Insecticides have a similar structure. In the structure of insecticides (see figure 1) P (=O) has generally been replaced by P (=S) and a less reactive group than (-F), (-CN) or (-SCH₂CH₂N[CH(CH₃)₂]₂) is used.

These nerve agents were not used largely in any of the wars. However, in the Gulf War a number of U.S. and UK personnel were exposed to them when a chemical depot storing such agents was destroyed. The other most widely publicized uses of nerve agents were the two terrorist incidents in Japan carried out by the operatives of the Aum Shinrikyo religious group.
**Chronic Health Effects of Acute OP Exposure**

The OP-CWAs exert their toxic effect mainly by disrupting the cholinergic system that includes acetylcholinesterase (AChE) and its natural substrate, the neurotransmitter acetylcholine (ACh) (Adou-Donia 1992). ACh is released in response to nerve stimulation and binds to post-synaptic ACh receptors, resulting in muscle contraction or gland secretions. Its action is rapidly terminated by hydrolysis with AChE via the serine hydroxyl in the catalytic triad of AChE (Koelle 1946). The three dimensional structure of AChE reveals an active center at the base of a narrow gorge (Sussman, Harel et al. 1991) which consists of the following sites (Figure 2):

1) The catalytic triad of Glutamate, Histidine and Serine;

2) An acyl pocket;

3) A choline subunit;

4) A peripheral site.

**Figure 2** - Active center of AChE (Adou-Donia 1992; Abou-Donia 2003)
OP esters including GB inhibit AChE by phosphorylating the serine hydroxyl group at the catalytic triad site. The phosphoryl group of GB reacts with the hydroxyl group of the serine residue in the active site of the enzyme as shown in the figure 3 below. Phosphorylated AChE undergoes ageing – a process that involves the loss of an alkyl group resulting in a negatively charged monoalkyl enzyme (Figure 4; Abou-Donia 2003) following which regeneration of phosphorylated AChE is not possible (due to adduct formation).

Figure 3 - Sarin phosphonyl enzyme: isopropyl methylphosphonyl AChE (Adou-Donia 1992; Abou-Donia 2003)
Figure 4 - Sarin aged phosphonyl enzyme: methylphosphonyl AChE (Adou-Donia 1992; Abou-Donia 2003).

Inhibition of AChE results in accumulation of ACh at both the muscarinic and nicotinic receptors in the central nervous system (CNS) and the peripheral nervous system (PNS) causing excessive cholinergic stimulation. This is seen as contraction of pupils, profuse salivation, involuntary urination and defecation, convulsions, paralysis, coma and eventually death due to respiratory failure (McDonough and Shih 1997; Taylor 2001; Shih, Duniho et al. 2003; Pope, Karanth et al. 2005).

OP-induced neurotoxicity is also well documented. Seizure induced by OP produce a characteristic pattern of neuronal damage that is specific for certain brain regions (Lemercier, Carpentier et al. 1983; Filliat, Baubichon et al. 1999). The degree and distribution of damage is correlated with seizure duration (Lallement, Carpentier et al. 1991; Lallement, Carpentier et al. 1991; McDonough, Dochterman et al. 1995).
Neuropathology is most commonly observed in the piriform cortex, amygdala, hippocampus, thalamus, neocortex and caudate nucleus.

**Current therapy for acute OP-poisoning**

The current therapy used for nerve agent toxicity mainly includes:

1) An anticholinergic - like atropine (ATR) as a symptomatic treatment. Atropine relieves the symptoms of the exposure but not the actual cause of the symptoms. It blocks the muscarinic ACh receptors and prevents excessive cholinergic stimulation. However, it does not act on the nicotinic receptors.

2) A cholinesterase (ChE) reactivator mainly an oxime such as pralidoxime (2PAM). OPs inhibit AChE activity through binding of a phosphoryl group (of the OP) to a serine hydroxyl group within the active site of the enzyme. Active AChE can be regenerated by the removal of the phosphoryl group by oxime drugs, of which 2PAM is currently used in the treatment of OP poisoning. However, this is not possible when the phosphorylated AChE undergoes aging.

3) In addition to these drugs the therapy may also include an anti-seizure medication such as diazepam. Brain damage in OP poisoning may be a result of seizure-induced inflammation (Zimmer, Ennis et al. 1997; Zimmer, Ennis et al. 1997; Svensson, Waara et al. 2001; Svensson, Waara et al. 2005; Chapman, Kadar et al. 2006) for which, an anti-seizure medication may be included.
Chronic Health Effects Low Level OP Exposure

The symptoms, neuropharmacological mechanisms and toxic consequences of high dose OP exposures have been well documented (McDonough and Shih 1997; Shih, Duniho et al. 2003; Pope, Karanth et al. 2005) and until the early 1990’s very little attention was given to the possible deleterious effects of low-dose exposure to OP’s. Low dose exposure of nerve gases has occurred in the Persian Gulf War of 1991 (Haley 1997) and in Matsumoto, Japan in 1994 (Nakajima, Ohta et al. 1999).

Following the Persian Gulf War a large number of veterans exhibited a variety of symptoms, including weakness, fatigue, headache, memory loss, personality change, cognitive decline and increased susceptibility to infections (IOM 1995). A number of risk factors had been proposed to explain the development of these symptoms including combat-related post traumatic stress syndrome (PTSD) and exposure to OP pesticides, low doses of anticholinergic agents or nerve agents (Joyce C. Lashof 1996). Psychiatric interviews of a large number of veterans suggest that PTSD was relatively rare in Gulf War veterans (Ismail, Kent et al. 2002).

OP’s are hydrolyzed by paraoxonase, an enzyme that has two polymorphic forms; the Q form that hydrolyses nerve agents and the R form that hydrolyses OP pesticides (Davies, Richter et al. 1996). The US veterans from the Gulf War had low levels of the Q form of the enzyme (Haley, Billecke et al. 1999) whereas, the UK Gulf war veterans had low levels of both form of the enzymes (Mackness, Durrington et al. 2000) which suggest a
that exposure to OP pesticides or nerve agents could have caused the delayed onset symptoms mentioned above. In addition to this, occupational exposures of OP’s have also been reported to cause alterations in neuropsychological performances such as verbal and visual attention, visual memory, visuomotor speed, sequencing and problem solving (Rosenstock, Keifer et al. 1991; Stephens, Spurgeon et al. 1995; Bazylewicz-Walczak, Majczakowa et al. 1999; Farahat, Abdelrasoul et al. 2003).

Animal experiments studying asymptomatic exposure of OP’s have shown differential effects on physiological and behavioral functions (Russell, Booth et al. 1986; Scremin, Shih et al. 2003). Low levels of GB have been reported to cause an alteration of spatial orientation and spatial memory in rats (Kassa, Koupilova et al. 2001). Guinea pigs receiving 0.3, 0.4 or 0.5 × LD$_{50}$ repeated GB injections exhibited disrupted sleep patterns in the EEG (Shih, Hulet et al. 2006). Repeated exposure to low levels of GB have also been reported to cause persistent EEG changes in monkeys (Burchfiel and Duffy 1982).

The exact cause of these differential effects of low level OP exposure is not clear. AChE is the main target of OP pesticides as well as nerve agents however, there are also other serine hydrolases that could be the secondary targets of these agents (Casida and Quistad 2004). Studies carried out in AChE knockout mice have also shown that some of the effects of OP’s are independent of their cholinergic effects (Duysen, Li et al. 2001).

A number of mammalian synapses have presynaptic muscarinic autoreceptors. Low concentrations of OP’s can cause slow allosteric modulation of these receptors and thereby alter the presynaptic release of ACh as well as other neurotransmitters. Studies in brain slices have indicated that presynaptic muscarinic receptors are involved in
regulation of transmitter release in dopaminergic, GABAergic and glutaminergic synapses (Grillner, Bonci et al. 1999; Rocha, Santos et al. 1999).

**Monoamines – Dopamine and Serotonin**

**Role and Metabolism**

DA is a neurotransmitter from the monoamine family. Apart from being the precursor to norepinephrine (NE) and epinephrine, it has many important functions in the brain. Dopamine neurotransmission in different brain regions, particularly the frontal cortex (FC), amygdala and the caudate nucleus plays an important role in cognition, attention, motivation and reward, learning, creativity and motor activity. Dopaminergic cell bodies projecting to these regions of the brain are localized in ventral tegmental area (VTA), substantia nigra (SN) and the retrorubral field (RRF) (Fuxe, Hokfelt et al. 1974; Fallon, Koziell et al. 1978; Oades and Halliday 1987; Robert S. Feldman 1997; Inglis and Moghaddam 1999). 5-HT is also a monoamine neurotransmitter having important role in the regulation of sleep, mood, appetite and cognitive functions.

DA is metabolized by the actions of catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO) into homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC). The rate of usage of a neurotransmitter can be determined from the ratio of the metabolite to the parent compound e.g. HVA/DA or DOPAC/DA. HVA and DOPAC are the most significant DA metabolites. Of the two ratios for DA, HVA/DA ratio gives a very good estimate of DA usage because HVA [formed by the action of both catechol-O-methyltransferase (COMT) as well as monoamine oxidase (MAO)] is an extraneuronal metabolite of DA whereas DOPAC [formed by action of monoamine
oxidase (MAO)] is mostly a intraneuronal metabolite of DA (Figure 5; Robert S. Feldman 1997).

**Figure 5** – Metabolism of dopamine in the dopaminergic nerve terminal and synapse. DA = dopamine, COMT = catechol-O-methyltransferase, MAO = monoamine oxidase, 3-MT = 3-methoxytyramine, HVA = homovanillic acid, DOPAC = 3,4-dihydroxyphenylacetic acid.

OP’s have also been reported to alter monoamine levels, especially dopamine (DA) and serotonin (5-HT) in rat brains (Kant, Kenion et al. 1984; Coudraylucas, Leguen et al. 1987; Christin, Daulon et al. 2008). Cholinergic synapses are the major site of action of OP’s but they also have many other effects called non-specific or non-cholinergic effects, including the activation of other neurotransmitter systems in the central nervous system (CNS) (McDonough and Shih 1997). Low levels of OP’s may induce delayed onset
changes in the different neurotransmitter systems resulting in various neuropsychological symptoms associated with the Gulf War Illness.
II. HYPOTHESIS

Development of hypothesis

The neurological symptoms (Gulf War Illness) exhibited by the Persian Gulf War veterans could be due to exposure to subclinical levels of nerve gas. If exposure to low levels of OP causes the Gulf War Illness then the neuropsychiatric symptoms could be due to delayed onset changes in the different brain regions.

Hypothesis

Low doses of OP’s including the nerve agents cause delayed onset, long term neurochemical and neuropsychological effects which are non-cholinergic in nature.

Specific Aims

In order to study the possible neurochemical and neuropsychological effects following low level exposure of OP’s, we examined the effects of GB at sublethal doses on the levels of endogenous monoamines. The effects of such exposure on mainly serotonin (5-HT), dopamine (DA) and its metabolites, homovanillic acid (HVA) and 3, 4 – dihydroxyphenylacetic acid (DOPAC) were investigated in different brain areas particularly the frontal cortex (FC), amygdala and caudate nucleus.
**Aim 1** – To test the hypothesis that low doses of GB affect the neurochemistry in different brain structures in mice, particularly the FC, amygdala and caudate nucleus.

**Aim 2** – To test the hypothesis that low doses of GB affect the locomotor activity, anxiety and startle response of mice.
III. MATERIALS AND METHODS

Animals

Male C57BL/6 mice were obtained from Harlan Laboratories (Indianapolis, IN) weighing 20-25g, aged 3 months. Mice were maintained under a 12:12h light/dark cycle and housed in individual cages. A standard pellet diet and water were available ad libitum. All mice were allowed 7 days of acclimation to facilities and subjected to at least 3 days of handling before dosing. All procedures were approved by the Laboratory Animal Care and Use Committee of Wright State University, Dayton, OH.

Treatment

Sarin (USAMRICD, Aberdeen Proving Ground, MD) was diluted in 0.9% saline to concentrations of 8µg/kg (0.05 LD₅₀) and 64µg/kg (0.4 LD₅₀). Animals were administered subcutaneously (s.c.) in a volume of 0.5ml/100g body weight for 2 consecutive days. The animals were dosed for two days consecutively because rodents, as compared to humans, have a high amount of carboxylesterase (CaE). The excess CaE would be scavenged by GB rather than its natural substrate i.e. AChE; thus requiring more amounts of GB to produce its natural effects. Although two different low doses of GB were used, more emphasis was given to the 0.4 LD50 dose of GB as it is the highest dose of GB that does not produce any signs of cholinergic toxicity and has been widely
used for low dose OP studies. Controls received saline in the same volume and regime. Mice were euthanized at 1, 4 and 8 weeks.

**Locomotor activity**

Locomotor activity was evaluated in an automated open field system with infrared photo-beams (motor monitor, Version 6, 2000, Hamilton Kinder, Poway, CA). The open field arena had dimensions of 16 x 16 inches and was divided into central and peripheral zones. The mice were placed in the center of the open field arena and the following variables were recorded - locomotor activity, fine movement and rearing. Moreover, distance traveled, total time, rest time, number of entries and head pokes in individual zones were also recorded. All animals were handled for 3 days prior to testing in order to minimize handling-related stress. The mice were tested at 1 week, 4 weeks and 8 weeks after the last injection of GB. The animals were exposed to the open field arena for 10 min sessions in dim overhead light. After each session the number of fecal pellets was noted for assessment of emotional reactivity. The open field arena was then cleaned dry with 70% ethanol.

**Elevated Plus Maze**

Elevated plus maze (EPM) is a test of anxiety and was performed using an automated system by Kinder Scientific (Poway, CA). The EPM consists of two open arms (2 x 14 inches) and two closed arms (2 x 14 inches) with 6 inch high walls, connected by a 2 x 2 inch central square. The arms are elevated 30 inches above the floor. The mice were placed individually in the center of the maze facing the open arm and were allowed to explore the maze for 5 min. The number of entries into open and closed arms, as well as
the time spent in open and closed arms was recorded by infrared photocell beam breaks during the 5 min session. The mice were tested at 1 week, 4 weeks and 8 weeks after the last injection of GB. After each session the EPM was cleaned dry with 70% ethanol.

**Fear potentiated startle**

Fear potentiated startle (FPS) is used to access Pavlovian conditioned fear which is a form of associative learning. Conditioned fear in terms of FPS is the elevated startle response in the presence vs. absence of a stimulus that was paired with a foot shock. Mice were tested in the SM100 Startle Monitor System Version 6.12 (Kinder Scientific, 2001 Poway, CA). The test consisted of pre-training, training and post-training sessions. The pre-training was administered prior to Pavlovian fear conditioning. A 5 min acclimation period was followed by two white noise stimuli at two intensities, a 50 ms pulse at 75 and 80 dB. Startle amplitude was measured for 32 startle stimuli in the presence (CS + startle) and absence (startle alone) of a 30 sec, 12 kHz pure tone (70 dB) conditioned stimulus (CS) with an inter-trial interval of 1 min. The pre-training session is necessary to establish a baseline level of startle response to the tone before conditioning. In the training session the CS was paired with an electric shock of 0.4 mA intensity and 0.5 sec duration 29.5 sec after the tone. This session consisted of 5 min acclimation and then 20 tone + shock fear conditioning trials and is repeated for two days for a total of 40 trials. The inter-trial interval was 1-3.5 mins. The post-training session was the same as the pre-training session, except that it was conducted 24 hours after Pavlovian fear conditioning (training). The mean startle amplitudes were calculated for ‘startle alone’ as well as ‘CS + startle’ trials. The percent FPS was defined as an increase in the percent response to CS
from pre to post-conditioning tests and was computed by using the following formula –

\[
((\text{CS+startle} - \text{startle alone}) / \text{startle alone}) \times 100.
\]

**Cholinesterase activity**

The blood was collected (~50µl) 24 hours, 1 week, 4 weeks and 8 weeks after the last injection of GB either in the form of trunk blood (1, 4 and 8 weeks) or blood from facial vein bleed (24h) into heparinized tubes. It was stored on ice until assayed. Cholinesterase activity was determined by a modified version of the colorimetric assay of Ellman et al. (Ellman, Courtney et al. 1961) using a BIOTEK™ EL808 Microplate Analyzer. Fresh whole blood was diluted 1:100 with 0.1 M NaPO₄ pH 7.4 buffer. Total ChE activity was measured in the whole blood samples. Blood AChE activity was determined at 25°C by inhibiting butrylcholinesterase (BuChE) with iso-OMPA (tetraisopropyl-pyrophoramide). BuChE activity was then calculated by subtracting AChE activity from total ChE activity. Activity was reported as µmole/min/ml.

Brain tissue, namely the frontal cortex (FC), was assayed for ChE activity at the 1 week time point. FC was hand dissected using a razor blade. The tissue was placed on a foil and stored at -80°C until before the assay. Tissue was homogenized in 0.1M NaPO₄ ± 5% Tween-20, pH 7.4 buffer. The samples were centrifuged 24 × 4G at 4°C for 5 mins, and the supernatant was removed for assay use. Total ChE and AChE activities were determined as described above. A Bradford protein assay was performed on the supernatant to determine protein content and the ChE and AChE results were reported as nmole/min/µg protein.
Histology

Animals were sacrificed with the collection of brains. The brains were flash frozen in isopentane and stored at -80°C until sectioned. They were embedded in Tissue Tek® and sectioned at -20°C at a thickness of 10µm with a Thermo® cryotome. Sections were collected on Fischer Brand™ Plus slides. Slides were stored at -20°C until stained. Fluoro-Jade C staining was performed by placing 10µm sections in 0.001% Fluoro-Jade C solution (Histochem, Jefferson, AR, USA) in 0.1% acetic acid as described previously (Schmued, Stowers et al. 2005). The mounted sections were air dried and rehydrated in decreasing strengths of ethanol (100 to 70%) for 3 min then distilled deionized water for 1 min each. The slides were incubated in 0.06% potassium permanganate (15 minutes), washed in distilled water, and transferred to 0.001% Fluoro-Jade C staining solution (30 minutes). After staining, the sections were rinsed in distilled water, dried, immersed in xylene, and coverslipped with DPX (Sigma-Aldrich, St. Louis, MO, USA) to observe under microscope for histological damage.

Neurochemical Analysis

Animals were sacrificed by decapitation 1, 4 and 8 weeks after the last GB injection. The brains were removed and the FC was hand dissected. The remainder of the brain was placed in a brain matrix (Ted Pella, Inc; Figure - 6) and sliced into 1mm or 2mm slabs rostral to the interaural line, which were then stored at -80°C until further dissection (Figure - 7). For this study we choose to study three different brain regions, FC, amygdala and caudate nucleus. We dissected the amygdala and the caudate nucleus using landmarks in The Mouse Brain Stereotaxic Coordinates (2008) as a guide (Figure – 8 and
9). The FC, amygdala and the caudate nucleus were homogenized in 300μl of 0.2N perchloric acid (HClO₄). The homogenates were centrifuged at 24 × 4G at 4°C for 20 minutes and the supernatant was separated into 2 aliquots. These aliquots were stored at -80°C until further analysis by reverse phase high performance liquid chromatography (HPLC).

**Figure 6** - Brain Blocker - used to dissect the brain into 1mm slabs.
Figure 7 - Sagittal section of the mouse brain. The vertical lines rostral to the intraural line indicate the slots where razor blades were placed for dissection.
Figure 8 - Coronal section of the mouse brain between 2-3mm rostral to the intraural line (Keith B. J. Franklin 2008). The marked triangle shows the region dissected for the amygdala.
Figure 9 - Coronal section of mouse brain between 4-5mm rostral from the intraural line (Keith B. J. Franklin 2008). The circled region shows the region dissected for the caudate nucleus.
HPLC is a type of liquid chromatography in which the mobile phase is forced through a relatively short, narrow bore column by a high-pressure pump. Specialized detectors are attached to the system to identify the separated components of the sample as they are eluted from the column. One of the commonly used detectors is an amperometric detector. In an amperometric cell the column effluent passes electrodes to which a voltage is applied. As the molecules in the column effluent pass the electrodes they either get oxidized or reduced. In either case, the movement of electrons causes a current to flow that is proportional to the concentration of the molecules in the column effluent (Robert S. Feldman 1997)

The aliquots were injected directly into the chromatography system (20µl). Chromatographic separation was conducted using an ESA 80 × 4.6mm column packed with 3µm C18 resin and a LC-4B amperometric detector (Bioanalytical Systems Inc. BASi) optimized to 0.75V. The mobile phase consisted of a stock of 3.45g sodium phosphate, 14.7g sodium citrate, 0.1g ethylenediaminetetraacetic acid (EDTA), 1.1g diethyamine hydrochloride and 0.24g 1-octanesulfonic acid per liter. The pH was adjusted to 3.1 using phosphoric acid. During analysis, 250 ml of stock was used plus an additional 120 mg of 1-octanesulfonic acid (varied with age of the column), 12 ml acetonitrile and 5.5 ml dimethylacetamide. The flow rate was kept fixed at 0.6 ml/min.

A standard containing known amount of the monoamines - norepinephrine (NE), dopamine (DA), homovanillic acid (HVA), 3, 4 – dihydroxyphenylacetic acid (DOPAC) and serotonin (5-HT) was injected in the chromatography system twice daily, before and at the end of the analyzing the samples. The concentrations of monoamines in the samples were determined by comparing the peak amplitude from the samples to that
obtained from known standard concentrations. Previous work done in this lab has shown that there is no significant loss of monoamines in the process of homogenization. For this reason the use of an internal standard was avoided. The concentrations of NE, DA, HVA, DOPAC and 5-HT were determined and expressed as ng/mg of wet tissue. The usage of the neurotransmitter was determined by measuring the ratio of the metabolite to the parent compound e.g. HVA/DA or DOPAC/DA (Robert S. Feldman 1997).

**Statistical Analysis**

Data was analyzed by using STATISTICA (Statsoft, v6) data analysis software. One way ANOVA was used to evaluate overall significance. A Fisher Least Significant difference (LSD) post hoc test was used to identify individual group differences. The results are presented as mean ± SEM. The confidence limit of p < 0.05 was considered statistically significant.
IV. RESULTS

Clinical Signs and Body Weights

During the whole experiment no animal died and no signs of cholinergic intoxication were observed. The mice in the 0.4 LD50 dose group showed a significant weight loss on the first day, after the last GB injection, but the weights rapidly recovered by the end of the third day (Figure 10). The mice in the 0.05 LD50 dose group did not show any significant weight loss as compared to the controls (data not shown).

Figure 10 - Change in body weights (F (1, 35) = 10.00, p = 0.003) * p < 0.05 – significant difference from the controls
Locomotor Activity

The motor activity was determined in an open field arena. The dose of GB that was used to study the locomotor activity was 0.4 LD50 because it is a national standard established for low dose exposure studies. The parameters recorded for locomotor activity included basic movements, rearing and the time spent in center vs. periphery. There was no significant difference in the locomotor activity between the GB treated mice and the controls (Figure 11).

![Locomotor activity graph]

Figure 11 - Locomotor activity at 1, 4 and 8 weeks. There was no significant difference in the locomotor activity between the controls and GB treated mice.
Elevated Plus Maze

EPM test was carried out to get a measure of anxiety. No significant difference was observed with respect to the time spent in open arms or closed arms between the 0.4 LD50 GB treated mice and the controls (Figure 12).

![Time spent in Open Arms](image)

**Figure 12** – Elevated plus maze – time spent in open arms at 1, 4 and 8 weeks. There was no significant difference in the locomotor activity between the controls and GB treated mice.

Fear potentiated startle

Fear potentiated startle (FPS) is used to access Pavlovian conditioned fear which is a form of associative learning. FPS was carried out in two groups of mice injected with saline and 0.4 LD50 dose of GB. FPS which was defined as an increase in the percent response to CS from pre to post-conditioning tests was calculated. The results for FPS
were inconclusive because the learning behavior was not observed in the controls making the data for the GB treated mice of little use (Figure 13).

**Figure 13** – The percent FPS as calculated for controls and GB treated mice. The results were inconclusive because the learning behavior was not observed in the controls.

**Blood Cholinesterase Activity**

Blood cholinesterase activity was determined at different time points for mice injected with 0.4 LD50 dose of GB. A one way ANOVA revealed that there was a significant decrease in blood ChE activity on day 1 after the last GB injection. A Fisher’s LSD post hoc test revealed a significant decrease in AChE activity at 24 hours post GB exposure. The levels of all enzymes returned to control values within 3 days post GB exposure (Figure 14).
Figure 14 - Blood cholinesterase activity observed for saline treated (Controls) and GB treated mice at Day 1, 2, 3 and at 1 week post GB exposure. Data are presented as mean ± SEM. [F_{ChE} (3, 40) = 9.70, * p = 0.014; F_{AChE} (3, 39) = 9.68, ** p < 0.001; F_{BuChE} (3, 40), p = 0.25]

**Brain cholinesterase activity**

The FC ChE activity was measured 1 week after the last injection of 0.4LD50 of GB. No significant changes in enzyme levels were observed (Figure 15)
Figure 15 - Brain cholinesterase activity measured in FC at 1 week. No significant changes in enzyme levels were observed.

**Histology**

Fluoro-jade staining was performed to identify the areas in brain affected by GB induced brain damage. Fluoro-Jade is an anionic fluorochrome capable of selectively staining degenerating neurons in brain slices. The histochemical application of Fluoro-Jade results in a simple, sensitive and reliable staining of degenerating neurons and their processes resulting from exposure to a variety of neurotoxic insult (Schmued, Albertson et al. 1997). No Fluoro-Jade C positive cell were found in the brain sections (Figure 16)
Figure 16 – No positive cells for Fluoro-Jade C staining in the dentate gyrus of hippocampus of mice treated with two daily doses of 0.4 LD50 GB.

Neurochemical Analysis

The studies for neurochemical analysis were done with two doses of GB namely 0.4 LD50 and 0.05 LD50. However more emphasis was given on the 0.4 LD50 dose of GB because this dose had been well characterized in this lab and all further studies were carried out with this dose including the behavioral tests. The concentrations of NE, DA, HVA, DOPAC and 5-HT were determined and expressed as ng/mg of wet tissue. The
usage of the neurotransmitter was determined by measuring the ratio of the metabolite to the parent compound e.g. HVA/DA or DOPAC/DA (Robert S. Feldman 1997).

There was a decrease in the DA usage in the FC consistently at all dose groups and time points. We observed a significant decrease in the HVA/DA ratio in the FC at all time points in the mice dosed with 0.4 LD50 of GB (Figure 17A). A similar decrease in DOPAC/DA ratio was also seen at the same dose group in the FC; Figure 17B].

Figure 17A - HVA/DA ratios observed in the FC at 1, 4 and 8 weeks in mice treated with a GB dose of 0.4 LD50 [F<sub>1 week</sub> (3, 44) = 9.65, p < 0.001; F<sub>4 weeks</sub> (3, 44) = 9.65, p < 0.001; F<sub>8 weeks</sub> (3, 44) = 9.65, p = 0.0018] * p < 0.001; ** p < 0.05 – significant difference from controls.
Figure 17B - DOPAC/DA ratios observed in the FC at 1, 4 and 8 weeks in mice treated with a GB dose of 0.4 LD50 [F₁ week (3, 37) = 20.08, p = 0.108; F₄ weeks (3, 37) = 20.08, p < 0.001; F₈ weeks (3, 37) = 20.08, p < 0.001] * p < 0.001 – significant difference from controls.

Mice injected with GB dose as low as 0.05 LD50, there was a significant decrease in the HVA/DA ratio at all time points; (Figure 18A). There was also a significant decrease in the DOPAC/DA ratio in the same dose group (Figure 18B).
**Figure 18A** - HVA/DA ratios observed in the FC at 4 and 8 weeks in mice treated with a GB dose of 0.05 LD50 \( [F_{4\ weeks\ (2, \ 16)} = 19.17, \ p < 0.001; \ F_{8\ weeks\ (2, \ 16)} = 19.17, \ p < 0.001] \) * \( p < 0.001 \) – significant difference from controls.
**Figure 18B** - DOPAC/DA ratios observed in the FC at 4 and 8 weeks in mice treated with a GB dose of 0.05 LD50 \[F_{\text{4 weeks}} (2, 16) = 23.0, p < 0.001; F_{\text{8 weeks}} (2, 16) = 23.0, p < 0.001\] * p < 0.001 – significant difference from controls.

A decrease in the ratio of HVA/DA and DOPAC/DA was observed because of significantly elevated DA levels in the FC of mice treated with 0.4 LD50 GB (Figure 19A and B) as well as in mice treated with a GB dose of 0.05 LD50 (Figure 20A and B).

**Figure 19A** - Elevated DA levels observed in the FC of mice injected with 0.4 LD50 dose of GB \[F_{\text{1wk}} (3, 51) = 4.95, p < 0.05; F_{\text{4wk}} (3, 51) = 4.95, p < 0.05; F_{\text{8wk}} (3, 51) = 4.95, p < 0.001\] * p < 0.05 – significant difference compared to controls. No significant change in concentration of HVA.
Figure 19B - Elevated DA levels observed in the FC of mice injected with 0.4 LD50 dose of GB [F_{1wk} (3, 51) = 4.95, p < 0.05; F_{4wk} (3, 51) = 4.95, p < 0.05; F_{8wk} (3, 51) = 4.95, p < 0.001] * p < 0.05 – significant difference compared to controls. No significant change in concentration of DOPAC.
**Figure 20A** - Elevated DA levels observed in the FC of mice injected with 0.4 LD50 dose of GB [$F_{4\text{wk}} (2, 17) = 18.34, p < 0.001; F_{8\text{wk}} (2, 17) = 18.34, p < 0.001$] * $p < 0.001$ – significant difference compared to controls. No significant change in concentration of HVA.

![Comparison of DOPAC and DA levels in FC at GB dose of 0.05 LD50](image)

**Figure 20B** - Elevated DA levels observed in the FC of mice injected with 0.05 LD50 dose of GB [$F_{4\text{wk}} (2, 17) = 18.34, p < 0.001; F_{8\text{wk}} (2, 17) = 18.34, p < 0.001$] * $p < 0.001$ – significant difference compared to controls. No significant change in concentration of DOPAC.

In the amygdala there was an increase in DA usage at 4 weeks but not at 1, 8 weeks. A significant increase in the HVA/DA ratio was observed in both the dose groups at the 4
week time point (Figure 21 and 22). Similarly there was also an increase in DOPAC/DA ratio in both the dose groups at the 4 weeks (Figure 21 and 22). Both these ratios returned to control values at 8 weeks.

![DA usage in Amygdala for GB dose of 0.4 LD50](image)

**Figure 21** - HVA/DA and DOPAC/DA ratios observed in the amygdala at 1, 4 and 8 weeks in mice treated with a GB dose of 0.4 LD50 \( F_{\text{HVA/DA}} (3, 36) = 3.57, p = 0.0026; \) \( F_{\text{DOPAC/DA}} (3, 40) = 2.81, p < 0.05 \) * p < 0.05 – significant difference from controls.
Figure 22 - HVA/DA ratios and DOPAC/DA ratios observed in the amygdala at 4 and 8 weeks in mice treated with a GB dose of 0.05 LD50 \( [F_{\text{HVA/DA}} (2, 16) = 5.06, p < 0.05;\]
\( F_{\text{DOPAC/DA}} (2, 17) = 14.04, p < 0.001] \) * \( p < 0.05 \) ** \( p < 0.001 \) – significant difference from controls.

In both the dose groups an increase in HVA/DA ratio was seen at 4 weeks because of significantly elevated HVA levels (Figure 23A and 23B).
**Figure 23A** - Elevated HVA levels observed in the amygdala of mice injected with 0.4 LD50 dose of GB \( [F_{0.4,LD50} (3, 43) = 5.44, p < 0.001] \) *p < 0.001 – significant difference compared to controls. No significant change in concentration of DA.
**Figure 23B** - Elevated HVA levels observed in the amygdala of mice injected with 0.05 LD50 dose of GB \( \text{[F}_{0.05\ \text{LD50}} (2, 17) = 6.53, p = 0.0022] \) * p < 0.05 – significant difference compared to controls. No significant change in concentration of DA.

Similarly, an increase in DOPAC/DA ratio was seen in both the dose groups at 4 weeks because of significantly elevated DOPAC levels (Figure 24A and 24B).

**Figure 24A** - Elevated DOPAC levels observed in the amygdala of mice injected with 0.4 LD50 dose of GB \( \text{[F}_{0.4\ \text{LD50}} (3, 44) = 4.75, p = 0.0013] \) * p < 0.05 – significant difference compared to controls. No significant change in concentration of DA.
Figure 24B - Elevated DOPAC levels observed in the amygdala of mice injected with 0.05 LD50 dose of GB \([F_{0.05 \text{ LD50}} (2, 17) = 9.25, p < 0.001]\) *\(p < 0.001\) – significant difference compared to controls. No significant change in concentration of DOPAC.

In the caudate nucleus the levels of monoamines were mainly analyzed at 4 and 8 weeks. There was no significant change in the ratios of HVA/DA or DOPAC/DA in both the dose groups (Figure 25A and 25B).
Figure 25A - No significant change in the HVA/DA ratio at 4 and 8 weeks in the caudate nucleus.
**Figure 25B** - No significant change in the DOPAC/DA ratio at 4 and 8 weeks in the caudate nucleus.

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**Table 2**

Summary of results for the dopaminergic system

<table>
<thead>
<tr>
<th>Brain region</th>
<th>DOPAC/DA ratio</th>
<th>HVA/DA ratio</th>
<th>DA usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td>Decrease</td>
<td>Decrease</td>
<td>Decreased</td>
</tr>
<tr>
<td>Amygdala</td>
<td>Increase at 4wk</td>
<td>Increase at 4wk</td>
<td>Increased at 4wk</td>
</tr>
<tr>
<td>Caudate nucleus</td>
<td>No change</td>
<td>No change</td>
<td>No Change</td>
</tr>
</tbody>
</table>

The 5-HT levels were also determined in the FC, amygdala and caudate nucleus in both the dose groups. 5-HT levels were significantly elevated at 4 and 8 weeks at both the dose groups in the FC (Figure 26). In the amygdala the 5-HT levels were elevated at 4 and 8 weeks only in the 0.4 LD50 dose group but not in the 0.05 LD50 dose group (Figure 27). Elevated 5-HT levels were also seen in the caudate nucleus in both the dose groups (Figure 28).
Figure 26 - Elevated 5-HT levels in FC at 4 and 8 weeks at both the dose groups $[F_{0.4 \text{ LD}50} (3, 40) = 42.87, P < 0.001; F_{0.05 \text{ LD}50} (2, 16) = 33.59, P < 0.001] \ast p < 0.001$ – significant difference compared to controls.
Figure 27 Elevated 5-HT levels in amygdala at 4 and 8 weeks in the 0.4 LD50 dose group $[F_{0.4 \text{LD50}} (3, 41) = 5.33, P < 0.05]$ * $p < 0.05$ – significant difference compared to controls.

Figure 28 - Elevated 5-HT levels in the caudate nucleus at 4 and 8 weeks at both the dose groups $[F_{0.4 \text{LD50, 4wk}} (2, 16) = 11.53, P < 0.05; F_{0.05 \text{LD50, 4wk}} (2, 14) = 14.40, P < 0.001; F_{0.4 \text{LD50, 8wk}} (2, 16) = 11.53, P = 0.07; F_{0.05 \text{LD50, 8wk}} (2, 14) = 14.40, P < 0.001]$ † $p < 0.05$; * $p < 0.001$; # $P = 0.07$ – significant difference compared to controls.
V. DISCUSSION

The objective of this study was to determine the possible delayed onset effects of low dose administration of GB on monoamine activity, in different brain structures in mice. This study was carried out as a follow-up to earlier studies within this laboratory designed to explain the Gulf War Illness in veterans of the war. There was a significant decrease in the usage of DA in the FC at 1, 4 and 8 weeks after exposure in both the dose groups while there was an increase in the usage of DA in the amygdala, only at the 4 week time point – indicating a reversible effect. There was no change in DA activity in the caudate nucleus which was consistent with no changes in the locomotor activity in the open-field studies. Amongst the other monoamines investigated the levels of 5-HT were significantly elevated in all the brain regions studied. Throughout the experiment there were no signs of cholinergic toxicity such as excessive salivation, urination, tremors or convulsions. There was a slight drop in the body weights and the cholinesterase activity after the administration of GB, both of which recovered within a week.

The measurement of neurotransmitter concentrations to determine the effect of a particular event on that neurotransmitter system is of little value since, the rate of synthesis or the rate of catabolism may have changed. A better approach is to determine the rate of usage of the neurotransmitter. One way of doing this is by determining the ratio of the metabolite to the parent compound e.g. HVA/DA or DOPAC/DA.
In the FC there was a significant decrease in the usage of DA at all time points and dose groups. This decrease in the usage was due to significantly elevated levels of DA, which suggest a decrease in the usage of DA in the FC. The FC mainly receives projections from the A10 group of dopaminergic cell bodies situated mostly in the ventral tegmental area (VTA) of the brain (Oades and Halliday 1987; Robert S. Feldman 1997). The FC region of the brain is mainly associated with attention, planning, long term memory, creativity and drive. Reduced DA activity could account for a number of symptoms of the Gulf War veterans such as problems with memory, problem solving, attention deficits, impaired spontaneity, diminished socialization and decreased creativity. We hypothesize that all these symptoms could be a result of altered function in the FC region of the brain due to low level OP exposures. Further studies at extended time points are necessary to determine if the symptoms worsen with time.

In the amygdala there was an increase in the usage of DA in both the dose groups at 4 weeks but not at 1 and 8 weeks – an indication of a reversible effect. The increased usage was due to elevated metabolites, namely HVA and DOPAC, at 4 weeks. This suggests a transient increase in the use of DA. Like the FC, the amygdala is innervated mainly by the A10 group of cell bodies in the VTA and also partially by the A9 and A8 group of dopaminergic cell bodies located in the substantia nigra, pars compacta and the retrorubral nucleus respectively (Fallon, Koziell et al. 1978; Robert S. Feldman 1997; Inglis and Moghaddam 1999; Hasue and Shammah-Lagnado 2002) – which might account for the difference in results from the FC. Physiologically the amygdala is involved in processing of memory including fear conditioning and emotional responses.
There was no significant change in the DA activity in the caudate nucleus, which in turn receives projections mainly from the A9 group of dopaminergic cell bodies located in the substantia nigra (Robert S. Feldman 1997). The caudate nucleus is mainly associated with the motor activity. The absence of neurochemical change in the dopaminergic system of the caudate nucleus is supported by the results of the locomotor activity in the open field studies. There was no significant change in the locomotor activity between the GB treated mice and the controls. We suggest that the delayed onset effects of low dose OP exposure do not affect the dopaminergic system in the caudate nucleus because impairment of motor activity is not a feature of the Gulf War Illness.

Amongst the central monoaminergic cell groups, the dopaminergic and noradrenergic cell groups are designated with the letter A, the serotonergic cell groups with letter B and adrenergic cell groups with the letter C. According to current nomenclature groups A8 – A15 are the dopaminergic groups. DA containing nerve cells are mainly present in the rostral part of the brain – midbrain, hypothalamus and olfactory bulbs.

In figure 29 the following groups can be distinguished: olfactory bulb (OB) dendritic periglomerular (A16) neurons; the hypothalamic (Hyp; A12, A14 and A15) cell groups, of which A12 group is the largest and provides the tuberoinfundibular and the tuberohypophysial projections involved in neuroendocrine regulation; and the mesodiencephalic tegmental (A8–A10) cell groups. This group of neurons comprises the SNc (A9 neurons), the VTA (A10 neurons) and the retrorubral field (RRF; A8 neurons)(Smidt and Burbach 2007).
The major DA pathways with respect to our study have been briefly described below

1) The Nigrostriatal pathway – which originates mainly from the substantia nigra (A9 cell bodies) and innervates the caudate nucleus, the putamen and the globus pallidus (Figure 30). The nigrostriatal pathway is vitally important in motor control, and degeneration of this system in the main feature of Parkinson’s disease (Robert S. Feldman 1997). Our studies indicate that there was no significant change in the DA usage in the caudate nucleus which was further supported by no impairment in the motor activity in the open field studies. Therefore we suggest that the nigrostriatal pathway does not have an involvement in the long term debilitating symptoms that occur after low level OP exposures including the Gulf War Symptoms.

2) The Mesocortical pathway – originates mainly from the cells in the VTA (A10 cell bodies) and innervates the FC region of the brain (Fuxe, Hokfelt et al. 1974; Oades
This pathway has a very important involvement in the cognitive functions of the prefrontal cortex. We found a long lasting decrease in the DA usage in the FC region of the brain which suggests a very subtle impairment of the mesocortical pathway. This could explain most of the symptoms of low level OP exposure as well as the Gulf War Illness. The mesocortical DA neurons have a relatively low density of DA autoreceptors on their nerve terminals (Jack R Cooper 1982; White 1996). We speculate that an impairment of the long loop forebrain feedback pathway may account for the long term changes in DA activity in this region.

3) The Mesolimbic pathway – originates from the cells in the VTA (A10 cell bodies) as well as the substantia nigra (A9 cell bodies) and retrorubral nucleus (A8 cell bodies) and projects to the amygdala, hippocampus and some regions of the medial prefrontal cortex. (Fallon, Koziell et al. 1978; Robert S. Feldman 1997; Inglis and Moghaddam 1999; Hasue and Shammah-Lagnado 2002). In our investigations we found a transient increase in DA usage at 4 weeks but not at 1 and 8 weeks. The difference in responses between the FC and the amygdala could be accounted by the differential innervations of these two regions – A10 group of cell bodies project to the FC and the amygdala; in addition to this the amygdala input also arise from the A8-A9 dopaminergic cells (Fallon, Koziell et al. 1978; Inglis and Moghaddam 1999). The amygdala is associated with memory with respect to emotional events such as in fear conditioning. The FPS results at 4 weeks were inconclusive. The controls did not show any signs of associative learning making the data for the GB treated mice of little use. The FPS paradigm in mice has not been well established, not only in this lab but also in other
labs and needs further development so that neurochemical changes in the brain could be correlated with the learning behavior in FPS. The amygdala is a major part of the brain implicated in anxiety disorders. We did not find any signs of anxiety in the EPM test. One of the major reasons for this could have been the fact that the C57BL/6 mice have very high basal anxiety. The changes in the brain neurochemistry must not have been enough to cross the threshold and induce visible signs of anxiety (increase time in the open arms). Anatomically the amygdala is in a position to influence many neuronal systems therefore, further behavioral studies with the use of different paradigm are necessary to see any visible signs of the subtle changes in brain neurochemistry. The mesolimbic pathway, with amygdala being a part of it, is involved in a number of dopaminergic abnormalities such as addiction and schizophrenia (Breiter, Gollub et al. 1997; Haber and Fudge 1997) and it is also very likely to be involved in the neuropsychological symptoms of the Gulf War Illness.
Figure 30 - Diagrammatic representation of dopamine pathways in the human brain. The systems are derived from animal data. The neuronal pathways represent a bilateral system on either side of the brain. (Zygmunt L. Kruk 1979)

Apart from the significant changes in the activity of DA obtained in the various brain regions, another very important point of discussion is the manner in which the DA pathways are affected. The differential effects on the various dopaminergic cell bodies are an important result of the study. Further investigations with respect to the different inputs of the various DA cell bodies could answer a lot of questions pertaining to the mechanisms of many psychological disorders as well as the Gulf War Illness.
With respect to the serotonergic system, the 5-HT levels were transiently elevated in all the brain structures at later time points i.e. at 4 and 8 weeks. Our findings are consistent with previous reports which indicate that cholinesterase inhibitors cause an increase in 5-HT usage (Rausch, Janowsky et al. 1985). However without the measure of the 5-HT metabolite – 5-hydroxyindolacetic acid (5-HIAA) it is difficult to come to a definitive conclusion on the effect of low dose OP on the serotonergic system. During the optimization of the HPLC the voltage of the detector could be adjusted in a manner such that it is was possible to get a measure of the usage of DA (by getting a measure of HVA and DOPAC) but we could not get a measure of the usage of 5-HT (by measuring 5-HIAA) because 5-HIAA oxidizes below 0.7V. Further studies involving the measurement of 5-HT and its metabolite – 5-HIAA are necessary to come to a general conclusion on the effect of low dose OP on the serotonergic system.

In conclusion, the change in DA activity in the FC (decreased) and amygdala (increased – reversible) point towards a long term neurochemical alteration in the neurotransmission function of the CNS after OP exposure. The caudate nucleus did not show any changes in the dopaminergic system which was consistent with the normal locomotor activity. These data suggest a differential effect in the different dopaminergic cell bodies and their projections. The effect seen in the different pathways opens up a new approach to the neurochemical investigations. It is quite possible that other groups of cell bodies such as serotonergic, noradrenergic and adrenergic cell bodies might be affected in a similar manner. Further investigations with focus on different cell bodies and their projections are necessary. In addition to this, our studies strongly indicate that asymptomatic doses of nerve agents cause long-term subtle changes in the brain neurochemistry seen even weeks
after the exposure. These neurochemical changes could account for the delayed onset
neuropsychological symptoms that occur after OP exposure. However, how the effects of
OP nerve agent such as GB, on the neurotransmitter systems relate to the delayed onset
neuropsychological symptoms (Gulf War Illness) is still not clear. Further behavioral
studies and studies at extended time points are necessary to relate the neurochemical
changes to the behavioral dysfunctions.
VI. REFERENCES


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