CHAPTER 44

Molecular and Transcriptional Responses to Sarin Exposure

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I. INTRODUCTION

Chemical warfare related agents such as cyclosarin, sarin, soman, tabun, VR, and VX have become the focus of attention of both the international scientific community and the public ever since the end of Gulf War in 1991 (Golomb, 2008), followed by the tragic nerve agent exposure-related deaths and sicknesses in Tokyo in 1995. As a result of the September 11, 2001 terrorist attacks on the World Trade Center, and the ensuing possibility of unexpected war and terrorist attack scenarios that may involve chemical agents, there is a general heightened awareness of the necessity for more detailed studies on the nature of biological effects of these agents, and to improve diagnostic, preventive, and treatment aspects of toxic chemical exposure.

Sarin (*O*-isopropyl methylphosphonofluoridate; GB) is an organophosphorus (OP) ester that belongs to a group of highly toxic nerve agents. Many individuals have been suspected of exposure to various levels of sarin during the Gulf War (Golomb, 2008) as well as the Tokyo subway attacks (Miyaki *et al.*, 2005). Clinical symptoms such as arthralgia, weakness, fatigue, headache, memory loss, and increased susceptibility to infections were recorded in these individuals. There have been speculations about the low level of sarin exposure affecting distinct regions of the brain (Damodaran *et al.*, 2002a). High levels of exposure result in seizures, which increase the release of glutamate and cause toxicity to the surrounding cells (Solberg and Belkin, 1997). However, very little is known about the genomic and related nongenomic effects of sarin exposures at different dosage levels leading to pathological outcome.

II. BACKGROUND

One of the major mechanisms known for sarin-induced toxicity is the irreversible inhibition of the enzyme acetylcholinesterase (AChE), resulting in central accumulation of acetylcholine (ACh) and overexcitation of cholinergic neurons in sarin exposure related neurotoxicity. Besides this, a range of noncholinergic effects have been observed (Ray, 1998). Cholinergic components including ACh, choline acetyltransferase (ChAT), AChE, vesicle acetylcholine transporter (VAChT), muscarinic and nicotinic ACh receptors (mAChRs and nAChRs, respectively) have been identified in numerous nonneuronal cell and tissue types (Kawashima and Fujii, 2008) and thus make the biological response to sarin exposure into a very complex phenomenon. Given the complex levels of physiological alterations, one can expect global changes in RNA and protein species of different genes. Hence, gene expression approaches and other relevant molecular assays will help to identify key pathways that are triggered immediately and showed sustained expression over a long period of time, thus giving a mechanistic view on the possible mode of initiation, modification, persistence or disappearance of distinct molecular lesions that may translate into cellular/tissue level pathology leading to clinically and behaviorally observable disease/syndromic features (Damodaran *et al.*, 2006a).

A. Gene Expression as a Tool of Toxicity Testing

The idea of using gene expression as a tool in toxicology is based on the known fact that all toxic responses require modulation of expression of several key genes involved in various biological pathways to some degree and that no gene is regulated in isolation, even in the simplest biological system. Gene expression studies can be useful for the following reasons: (1) the construction of a large database of gene expression information linked to toxic endpoints, (2) more detailed understanding of the molecular mechanisms of compound toxicity, (3) identifying expression signatures that distinguish toxic effects of closely related chemicals, (4) identification and monitoring of different doses of suspected toxic chemical exposures (such as low vs high level), (5) differentiating acute vs chronic exposures and different combinations of both types, (6) identifying different chemicals in scenarios of suspected exposure to unknown chemical mixtures, (7) therapy or antidote response and effectiveness, and (8) long-term monitoring for any unknown biological effects. The strategic application of information

obtained by the above-mentioned approaches should result in the development of both more rapid, mechanism-based diagnostic screens for toxicity and novel therapeutic targets. Thus the development and use of gene expression-based biomarkers in toxicology are becoming widespread, specifically in the areas of exposure monitoring, and the determination of response and susceptibility to toxins.

B. Application of Genomics Technology for Studying Gene Expression

Two general approaches of gene expression related to toxicology and safety assessment have evolved, either of which can be deployed depending on the nature of the investigation being carried out.

The first (candidate gene or deductive) approach is to use either a single gene or arrays with discrete, often limited number of known (candidates for disease pathology or monitoring either one or several clinical and treatment endpoints related to toxic exposure) genes that have been selected on a rational basis as being associated with one or more toxic endpoints. The advantage here is that the methodology is relatively simple, and data analysis is considerably easier and restricted to those genes that are likely to be ''interesting'' and relevant to the toxicological problem in question. A large body of data has been published regarding expression profiles of few candidate genes for many toxic substances such as sarin and similar chemicals. Very often, the data obtained by this approach can be categorized as ''hypothesis testing'' – for example, the hypothesis that a subacute dose (0.5 LD_{50}) of sarin may affect neuronal and astroglial cells was tested by studying the expression of neuron-specific alpha tubulin (Damodaran *et al.*, 2002b), astrocyte-specific glial fibrillary acetic protein (GFAP), and vimentin (Damodaran *et al.*, 2002a). These time course studies confirmed the hypothesis by yielding expression data indicative of differential (various regions of the brain) and temporal (different days after exposure) modulation of all of these genes. Furthermore, another hypothesis that differential and temporal expression of AChE genes may explain the various molecular, cellular, and behavioral aspects of sarin exposure was tested by this approach (Damodaran *et al.*, 2003) in a time course study which also provided valuable data that confirmed biochemical, cell biological, and behavioral data obtained earlier (Jones *et al.*, 2000; Abou-Donia *et al.*, 2002; Abdel-Rahman *et al.*, 2002).

The second (global gene expression profiling or ''inductive'') approach is to use gene arrays with as many genes as possible, both characterized and otherwise, to maximize the amount of information obtainable from a single experiment. This scenario is applicable when novel genetic markers associated with a particular toxicity are sought or when a gene regulation ''fingerprint'' associated with a particular compound or toxic endpoint is to be generated. Moreover, identification of several novel pathways and gene clusters by this approach makes it a much more attractive option. The amount of data obtained by this approach is enormous and very often requires complex statistical and bioinformatics tools. Other disadvantages include situations where the interaction of cells and tissues with certain compounds such as xenobiotics will result in changes in patterns of gene expression, not all of which will be of significance in terms of toxicity. The data obtained by this approach are generally ''hypothesis generating'' for the most part, while they can be used to verify already known facts or hypotheses. For example, the hypothesis that early molecular events after sarin exposure define long-term pathology was studied by global gene expression approaches (Damodaran *et al.*, 2006a, b). Enormous amounts of data obtained by this study not only verified already known facts and verified the above-mentioned hypothesis, but also generated more hypotheses for future testing. For example, in a recent study, it was shown that signature expression profiles found in lung cancer patients with and without long-term exposure to pesticides were significantly different, thus paving the way to develop novel approaches to classify the patients based on this molecular profile, identify therapeutic targets, and monitor efficacy of treatment (Potti *et al.*, 2005). Hence, ultimately the decision to select an approach depends on the short- and long-term objectives in the effort to elucidate the toxicity of chemicals under study. Very often, both approaches are used simultaneously for better and faster understanding of the biological phenomena associated with toxicity of the chemical being studied.

1. Current Methodologies Available for Studying Gene Expression

Several methods have been developed to screen cells/tissues for differentially expressed genes, such as screening of differential cDNA libraries (Dworkin and David, 1980), screening of subtractive cDNA libraries (Sargentg, 1998), screening by differential display by PCR (Liang and Pardee, 1992), and sequence-based serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995). While these procedures are suitable for identifying few-fold or greater changes in gene expression (i.e. when genes are either turned off or on), they are inadequate for detecting subtle changes in gene expression. High-density cDNA arrays allow one to rapidly screen cells/tissues for differential gene expression as well as quantify changes as small as 0.5-fold or less with statistically significant levels of accuracy (Damodaran *et al.*, 2006b). Recent assessment of the relative impact of experimental treatment and platform on measured expression found that biological treatment had a far greater impact on measured expression than did platform for more than 90% of genes, and thus confirmed that global approaches in gene expression studies are reliable and promising (Larkin *et al.*, 2005).

2. Latest Genomic Technologies that Hold Promise for Future Investigations

Although most of the gene expression studies on sarin and related compounds address the changes in transcriptomes,

there have been recent developments in microarray technology such as: (1) global analysis of changes at DNA level such as deletions, inversions, duplications, and translocations in monogenic diseases, complex diseases, and various types of cancers, possibly due to exposure to chemical and physical agents, using comparative genomic hybridization (CGH) methodology, (2) genotyping single nucleotide polymorphism (SNP) or other point mutations as a factor in susceptibility to certain disease development and/or as an adverse or protective effect against certain chemical exposures or drug treatment, (3) level of methylation changes due to chemical exposure or other disease states using global epigenetic profiling, and (4) microRNA expression profiling as an adjunct technology to address specific effects of chemical exposure on molecular regulation of differentiation and proliferation-related changes in various target tissues. It is noteworthy to mention here that there are studies using sister chromatid exchange (SCE) analysis of peripheral blood lymphocytes from victims of sarin exposure from the Tokyo train terrorist attack (Li *et al.*, 2000) and scoring of various cytogenetic anomalies such as mitotic chromosome and chromatid breaks, gaps, and other anomalies of anaphase such as lagging of chromosomes have been employed in studies of *in vitro* peripheral blood culture from humans exposed to pesticides (Das *et al.*, 2006) to assess genomic effects on somatic cells. Furthermore, the use of multicolor fluorescence *in situ* hybridization (FISH) on germ cells (sperms) of individuals exposed to OPs was also attempted (Recio *et al.*, 2001). There is an excellent Prospectus for identifying subtle DNA level changes which could be either megabase deletions of large contiguous regions of genome or minor deletions of a few kilobases of genomic regions of instability and/or recombination due to exposure to warfare chemicals by CGH technology. This also corresponds to the identification of the modifying effect of inherited and acquired interactions between AChE and paraoxonase1 (PON1) polymorphisms modulating AChE and plasma paraoxonase enzyme activities both within and between genotypes on the susceptibility of individuals, in their response to OPs exposure warrants, such as genotype studies and other SNP-based screenings at population level (Bryk *et al.*, 2005; Sirivarasai *et al.*, 2007). This kind of population data will be of great advantage in situations where there is a need to screen soldiers, medical personnel, and members of other support organizations during warfare or a terrorist attack for assessing their increased or decreased susceptibilities to warfare agents. The modifying effects of methylation and its importance in transmitting abnormally methylated alleles to future generations are also gaining widespread attention. Besides these latest developments, other established and emerging technologies such as proteomics and global analysis of post-translation changes (such as phosphorylation), respectively, may address several biological end points relevant to sarin-induced toxicity, its treatment and management as well as long-term monitoring. Tissue arrays and high throughput validation (such as high content confocal analysis) methodologies related to genomic technologies make it a very promising future for prompt and precise detection of toxic exposure that can lead to better treatment options, thus reducing overall mortality and morbidity associated with any such scenarios.

III. SARIN-INDUCED CLINICAL CHANGES AS A FUNCTION OF CELLULAR AND MOLECULAR ALTERATIONS

A. Acute Sarin Exposure and Clinical and Molecular Symptoms

A high dose of acute exposure results in marked muscle fasciculation, tachycardia, high blood pressure (nicotinic responses), sneezing, rhinorrhea, miosis, reduced consciousness, respiratory compromise, seizures, and flaccid paralysis (Yokoyama *et al.*, 1998). High-dose acute exposure targets several pathways such as cholinergic, cyclic nucleotide signaling, catecholaminergic signaling, GABAergic signaling, glutamate and aspartate signaling, nitric oxide signaling, and purinergic, serotogenic, as well as cholinergic signaling, within the first 15 min to 2 h of sarin exposure (Damodaran *et al.*, 2006a, b). Other signal transduction pathways such as calcium channels and binding proteins, ligand-gated ion channels, neurotransmission and neurotransmitter transporters, neuropeptides, and their receptors are also altered. Inflammation-related pathways such as TNF pathways, chemokines and their receptors, and cytokines are also altered within 15 min to 2 h after sarin exposure (Damodaran *et al.*, 2006a).

B. Sarin-Induced Delayed Neurotoxicity

High doses of sarin exposure can lead to delayed onset of ataxia, accompanied by wallerian-type degeneration of the axon and myelin of the central nervous system (CNS) and peripheral nervous system (PNS), also known as both organophosphorus ester-induced delayed neurotoxicity and OPIDN (Smith *et al.*, 1930; Abou-Donia, 1990). After the Tokyo subway incident, a 51-year-old man who survived the initial acute toxicity later died (after 15 months), with neurological deficits and histopathological lesions consistent with OPIDN (Himura *et al.*, 1998). This observation strongly suggests that humans are more sensitive to OPIDN caused by sarin than the hen (a commonly used experimental model organism to study OPIDN), as it required 26–28 daily doses of sarin LD_{50} (25 μg/kg, i.m.) to produce OPIDN in this species (Davies and Holland, 1972). It has been shown that the anomalous increase in phosphorylation and altered expression of mRNA and proteins of cytoskeletal genes is the central mechanism in the pathogenesis of OPIDN in model systems (Abou-Donia, 1995; Gupta *et al.*,

1998, 1999, 2000a, b, 2001; Gupta and Abou-Donia, 1999; Damodaran and Abou-Donia, 2000; Xie *et al.*, 2001). Sustained hyperphosphorylation of cytoskeletal proteins is not only a feature of OPIDN, but also a mediator of axonal dysfunction that perturbs the dynamics of the cytoskeleton. There are several kinases [such as protein kinase A (PKA)] associated with cytoskeletal proteins [such as neural filaments (NFs), tau, tubulin] shown to be altered in OPIDN (Gupta and Abou-Donia, 1999). Abnormal axonal transport is one of the main reasons for the degeneration of axons in OPIDN. Microtubules form the base for transport of axonal constituents, which are essential for the maintenance of axons. Microtubules interact with microfilaments, neurofilaments, kinesin, dynein, and microtubule-associated proteins (e.g. MAP-1, MAP-2, tau). Altered mRNA expression of several major cytoskeletal genes such as beta tubulin (Tubb1), Nestin (Nes) at immediate early (15 min), and neurofilament heavy (NEFH), GFAP at early (2 h), as well as MAP2 (microtubule associated protein 2) at late (3 months) time points after sarin exposure in rats indicates the existence of OPIDN-related molecular changes (Damodaran *et al.*, 2006a, b). Similar changes were noted in studies on hens showing clinical and histopathological features typical of OPIDN (Gupta *et al.*, 1999; Damodaran and Abou-Donia, 2000).

C. Sarin-Induced Chronic Neurotoxicity

The category is postulated as organophosphorus-induced chronic neurotoxicity (OPICN) which is characterized as long-term neurological deficits accompanied by brain neuronal cell death (Abou-Donia, 2003). Exposure to large toxic or small subclinical doses of OPs can result in such syndrome. Some of the Tokyo subway incident's victims developed long-term, chronic neurotoxicity characterized by central nervous system deficits and neurobehavioral impairments (Masuda *et al.*, 1995). Rescue workers and some victims who failed to show any early symptoms of neurotoxicity exhibited a chronic decline in memory 3 years and 9 months after the Tokyo incident (Yokoyama *et al.*,1998). Several genes (Bax, Bok, Bcl2l1, Casp6, Bcl-X, Dedd) related to cell death phenomena showed alterations at various time points after sarin exposure (Damodaran *et al.*, 2006a, b). Differential alteration of mRNA expression of AChE in rat brain regions exposed to sarin has been reported (Damodaran *et al.*, 2003) and is postulated to be related to the cell death observed in earlier studies on the brain of sarin-treated rats (Kadar *et al.*, 1995; Abdel-Rahman *et al.*, 2002).

D. Sarin-Induced Molecular Changes

Because of the complexity of the nervous system and the multiplicity of neurotoxic effects (sensory, motor, cognitive, etc.), it is apparent that several mechanisms of neurotoxicity exist; some may be specific to the nervous system, while others may be specific to other target organs and represent general cytotoxicity processes. Some of the major mechanisms include:

1. direct effect on a particular step in cell signaling and associated gene expression (e.g. protein kinases)
2. effect on second messenger responses activated by an endogenous compound (e.g. a neurotransmitter-induced activation of phospholipid hydrolysis) and related gene expression
3. indirect effect due to other toxic actions (e.g. induction of oxidative stress) and related gene expression.

The complexity of the pathways altered by sarin can be very well understood by the schematic pathway diagram (Figures 44.1–44.3).

1. Sarin-Induced Changes in Cell Signaling

Cell signaling is the fundamental process by which specific information is transferred from the cell surface to the cytosol and ultimately to the nucleus, leading to changes in gene expression. Since these chains of biochemical and molecular steps control the normal function of each cell, any disruption of these processes will have a significant impact on cell physiology. The phosphoinositide/calcium/PKC system, with a focus on the cholinergic system, plays a prominent role in several CNS- and PNS-related functions. ACh, by activating muscarinic receptors, may play a fundamental role in the regulation of synaptogenesis, neurocytomorphogensis, and glial cell proliferation (Costa, 1993) and exposure to sarin may alter its effect on the above-mentioned biological aspects. In astrocytes, ACh stimulates the metabolism of phosphoinositides and the hydrolysis of phosphatidylcholine increases intracellular calcium levels. These effects linked to muscarinic receptor stimulation may be modulated by sarin exposure. Other pathways important for glial cell proliferation (e.g. the phosphoinositide 3-kinase, MAP kinase, etc.) may be activated by ACh in these cells and may represent significant targets for sarin's action.

Neurotransmitters, hormones, and growth factors serve as first messengers to transfer information from one cell to another by binding to specific cell membrane receptors. This interaction results in activation or inhibition of specific enzymes and/or opening of ion channels, which leads to changes in intracellular metabolism and, in turn, to a variety of effects, including activation of protein kinases and transcription factors. These intracellular pathways can be activated by totally different receptors and are very interactive, or crosstalking, so they can control and modulate each other. The principal systems that link membrane receptors to second-messenger systems are represented by G-protein coupled receptors (GPCRs) and by receptors that possess intrinsic tyrosine kinase activity (Costa, 1998).

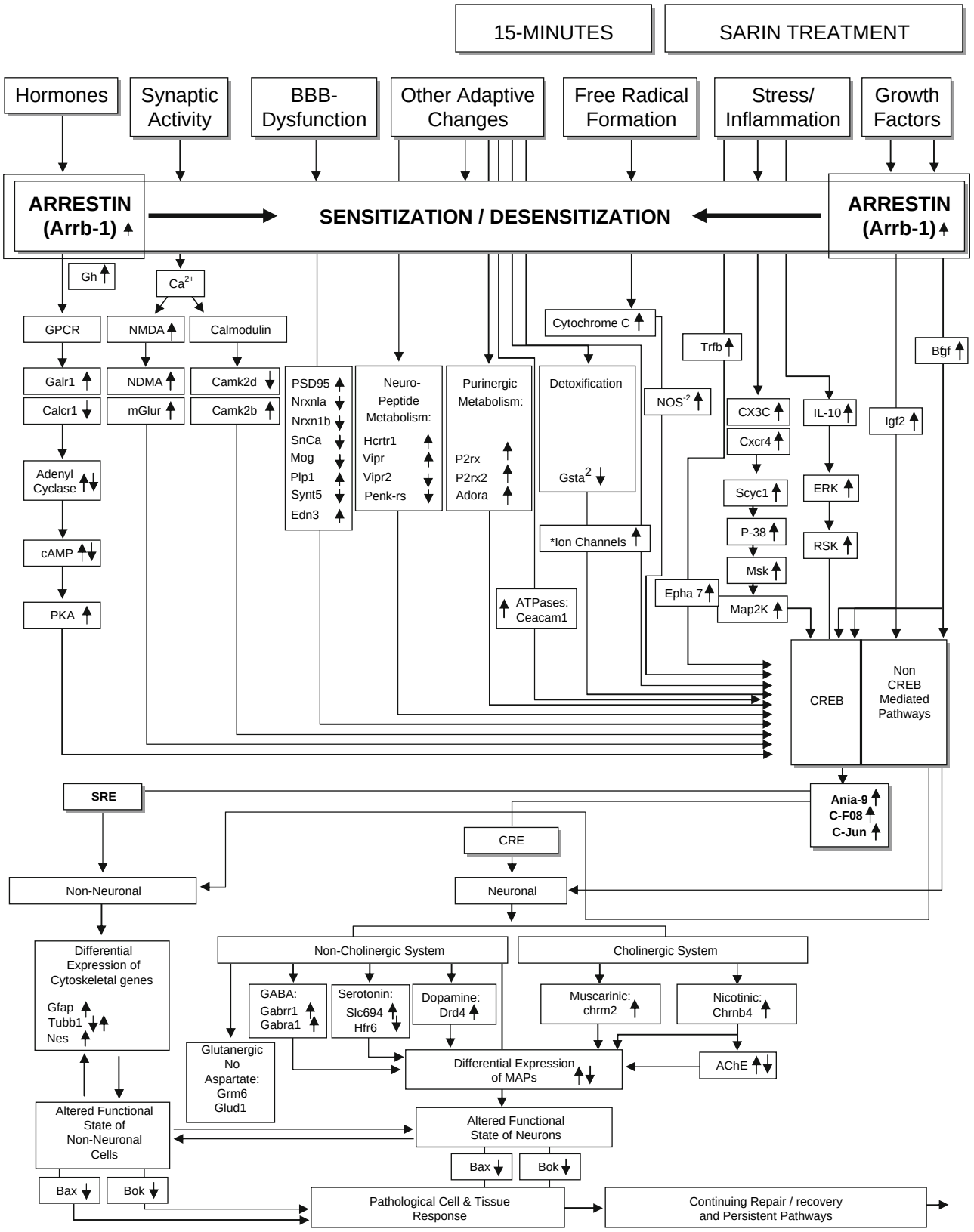

FIGURE 44.1. Similar phenomena as shown in Figure 44.1, at 2 h post-sarin exposure (0.5 LD_{50}) in rat brain, to much lesser complexity, as indicated by the fewer number of genes (pathways) altered. Major upstream events include beta arrestin/GPCR pathways followed by CREB/non-CREB pathways. In spite of the same dose (0.5 LD_{50}) used in both, 15 min (Figure 44.1) and 2 h (Figure 44.2), there are far fewer genes altered at 2 h, which strongly suggests initiation of prompt and effective recovery, repair, and adaptive processes early on. This figure is reproduced with permission from Springer Pvt Ltd.

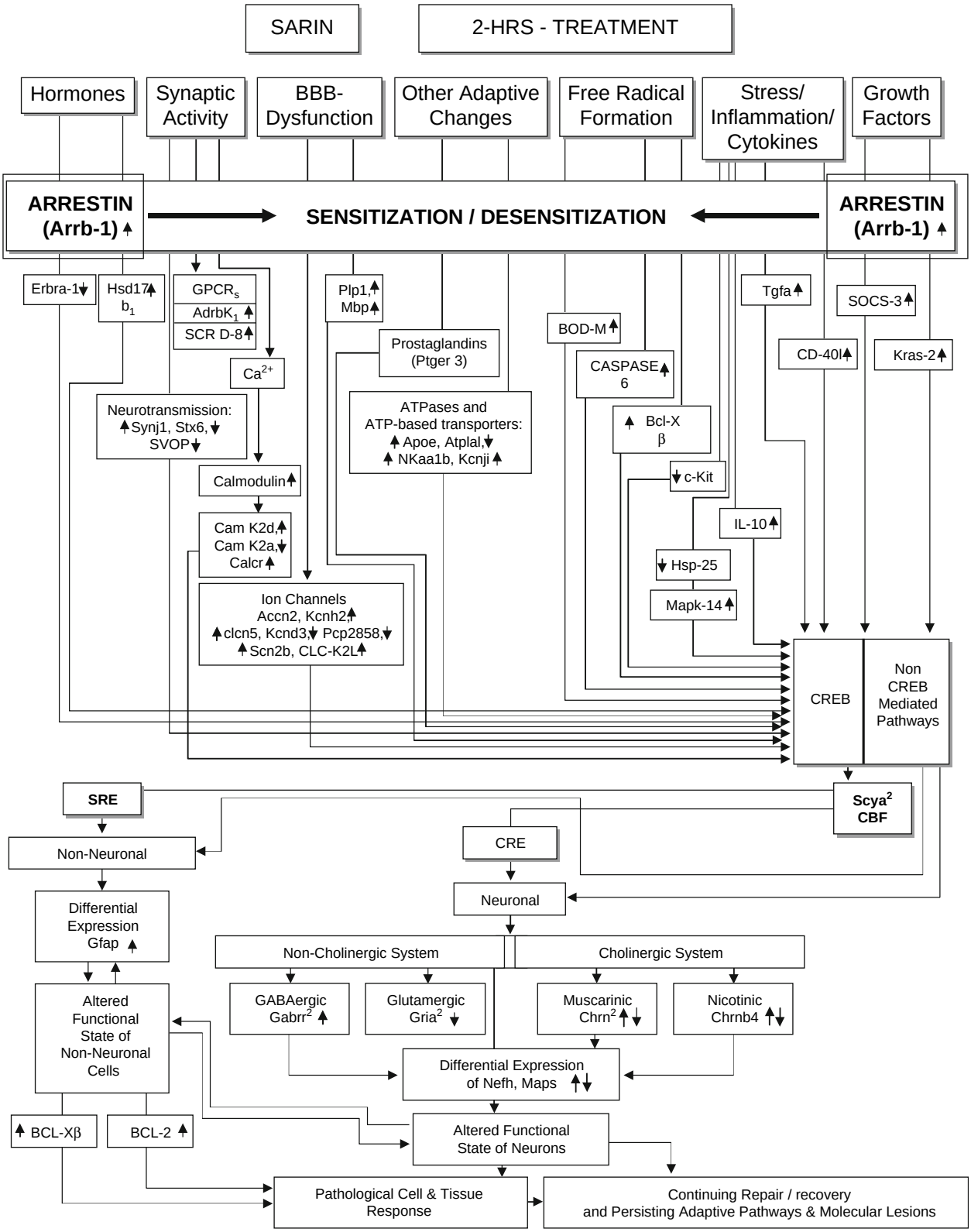

FIGURE 44.2. The complex gene expression changes initiated by sarin exposure (0.5 LD_{50}) in rat brain. Various factors such as hormones, altered synaptic activity, BBB dysfunction, free radical formation, stress/inflammation, alteration in the level of growth factors, and other adaptive pathways are found to be triggered by these changes. Major upstream events are all mediated through altered sensitization/desensitization mechanisms of beta arrestin/GPCR pathways. Another major upstream event which is downstream to beta arrestin/GPCR is the CREB (phosphorylated and/or unphosphorylated) and non-CREB mediated pathways. The signals processed at these two levels cause further changes in cholinergic and noncholinergic pathways in neuronal and nonneuronal cells. Thus, there are alterations in several mechanisms related to cell and tissue homeostasis, repair, and recovery pathways, thus leading to several adaptive changes. This figure is reproduced with permission from Elsevier Pvt Ltd.

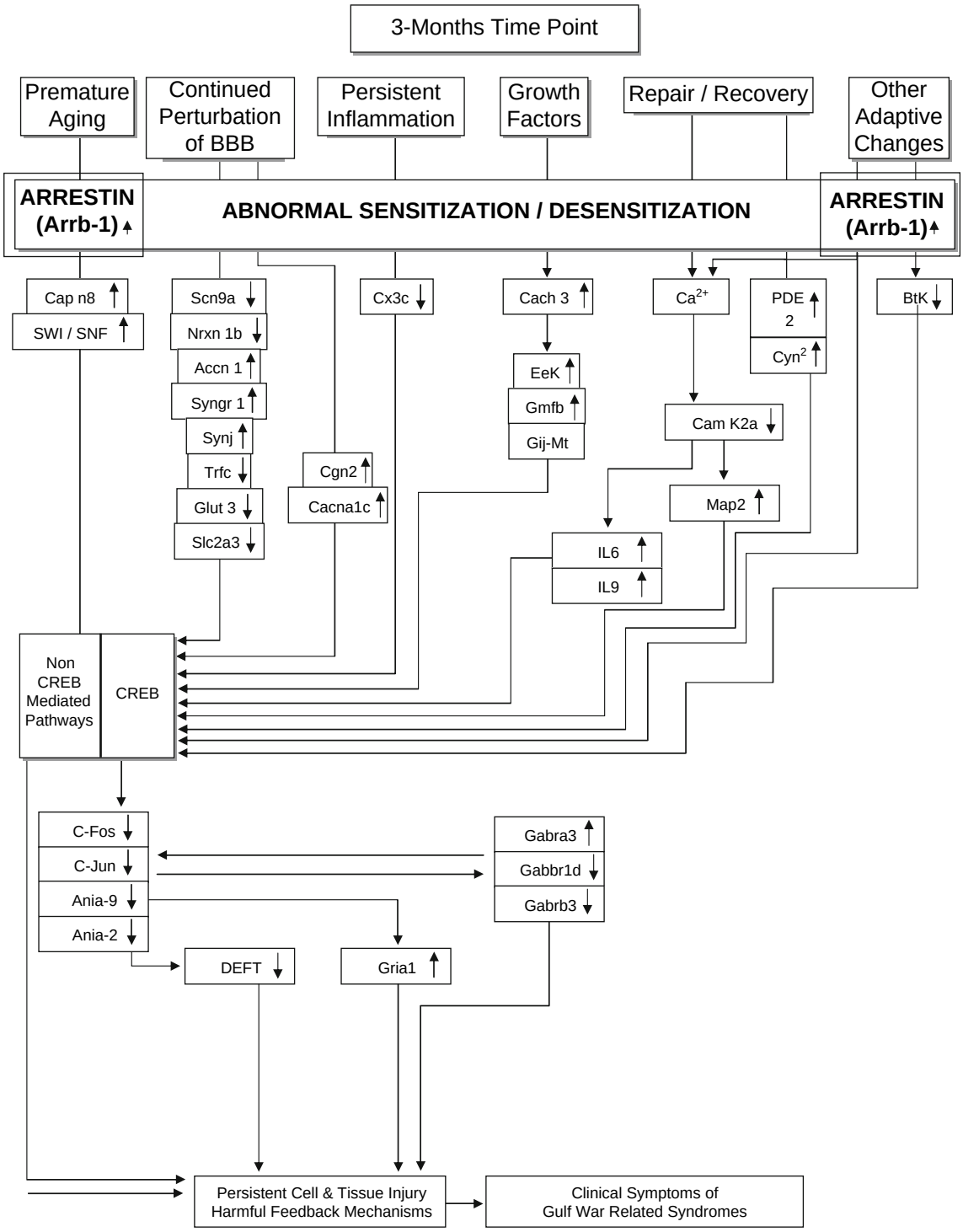

FIGURE 44.3. Pathways (genes) triggered at 3 months post-sarin exposure ($1 \times LD_{50}$) in rat brain. Various factors such as abnormal or premature aging process, continued BBB perturbations, persistent inflammation, and other adaptive, repair, and recovery pathways are found to be altered by upstream events such as beta arrestin/GPCR. This is followed by CREB/non-CREB pathways leading to changes in other downstream mechanisms of cell and tissue damage which lead to observable clinical symptoms as noted in Gulf War Syndrome patients and Japan subway terrorist incident-related victims. This figure is reproduced with permission from Elsevier Pvt Ltd.

2. Sarin-Induced Changes in G-Protein Coupled Receptors (GPCR) and Arrestin Mediated Pathways

G-protein coupled receptors (GPCRs) are one of the largest gene families of signaling proteins. Residing in the plasma membrane with seven transmembrane domains, GPCRs respond to extracellular stimuli that include catecholamine neurotransmitters, neuropeptides, larger protein hormones, lipids, nucleotides, and other biological molecules. They activate cellular responses through a variety of second messenger cascades (such as PKA and PKC signaling pathways). These receptors provide rapid responses to a variety of stimuli, and are often rapidly attenuated in their signaling. Failure to attenuate GPCR signaling can have dramatic consequences. One method to attenuate GPCR signaling is by removal of the stimulus from the extracellular fluid. At the synapse, removal of neurotransmitter or peptide signaling molecules is accomplished by either reuptake or degradation. ACh is removed from synapses through degradation by the enzyme AChE. Inhibition of AChE results in prolonged signaling at the neuromuscular junction (NMJ), and uncontrollable spasms in humans caused by sarin and other OPs. Transporters for serotonin, dopamine, GABA, and noradrenaline remove these neurotransmitters from the synapse to terminate signaling. The attenuation of GPCR signaling is receptor desensitization, in which receptors are modified to terminate signal transduction, even if the stimulus is still present.

The overexpression of either arrestin 1 or arrestin 2 has been demonstrated to augment the desensitization and internalization of several GPCRs (Pippig *et al.*, 1993; Oakley *et al.*, 1999). Thus, overexpression of arrestin at 15 min, 2 h, and 3 months post-sarin exposure, along with continued overexpression of several other genes belonging to several classes (Camk Ib, Camk2d, SCR D-8, M2-AchR; GALR1; CRLR) at 15 min, 2 h or 3 months, clearly indicate that sarin-induced hyperphosphorylation and related molecular changes persist until a later time point, thus causing defects in the tissue repair process (Damodaran *et al.*, 2006a, b).

3. Sarin-Induced Immediate Early Genes (IEGs)

In sarin-induced Ania-9 (activity and neurotransmitter-induced early gene 9) at 15 min post-exposure, the levels were down-regulated to 42% of the control value at 3 months post-exposure. Other genes such as c-fos, c-jun, and Ania-2 were also severely down-regulated at 3 months (Damodaran *et al.*, 2006a). Scya2 (immediate early serum responsive JE) was the only gene induced (285% of control levels) at 2 h post-sarin exposure (Damodaran *et al.*, 2006b). It is a well-known fact that ITFs (immediately induced transcription factors) and IEGs are among the most rapidly degraded of all proteins suggesting breakdown by the calpain and ubiquitin systems operating through lysosomes and proteosomes (Jariel-Encontre, 1997). Thus, down-regulation of these IEGs at a late time point can be caused by (1) successful regeneration, or, if this cannot be achieved, (2) a resting atrophic state, or (3) cell death (Damodaran *et al.*, 2006a, b).

4. Cholinergic System as the Primary Target of Sarin-Induced Toxicity

Sarin, like other OPs, elicit its toxic effects by irreversibly inhibiting AChE in the central cholinergic system, and excessive stimulation of postsynaptic cholinergic receptors. The overstimulation of the central cholinergic system is followed by the activation of other neurotransmitter systems including glutamate receptors leading to an increase in extracellular levels of the excitatory amino acid glutamate (McDonough and Shih, 1997). Numerous studies have shown that excessive accumulation of ACh leads to activation of ligand-gated ion channels, nicotinic ACh receptors (nAChRs), and muscarinic ACh receptors (mAChRs), which activate diverse kinds of cellular response by distinct signaling mechanisms (Abou-Donia *et al.*, 2002; Damodaran *et al.*, 2006a). Immediate and overexpression of the M2 receptor (214%) of the control value (100%) confirms biochemical data from several studies, showing consistent alterations in receptor density at 1, 3, 6, 15, and 20 h post-treatment (Abou-Donia *et al.*, 2002; Damodaran *et al.*, 2006a, b). nAChRs are essential for neuromuscular signaling, apoptotic signaling, neuroprotection, and synapse formation. The induced levels of nAChRs (150%) probably indicate its role in all of the above-mentioned capacities (Damodaran *et al.*, 2006a).

It is a well-known phenomenon that the cholinergic system interacts with various CNS neurotransmitters including serotonin (Quirion *et al.*, 1985), dopamine (Lehmann and Langer, 1982), catecholamines (Mason and Fibiger, 1979), GABA (Scatton and Bartholini, 1980), and neuropeptides (Lamour and Epelbaum, 1988). The cholinergic neurotransmitter system also controls cerebral vasculature (Armstrong, 1986). Modified gene expression of genes belonging to various systems such as serotonin [Slc6a4 (141%), Htr6 (47%)], dopamine [Drd4 (225%)], GABA [Gabbr-1 (179%), Gabra1 (130%)], and neuropeptides [Vipr (219%), Edn3 (236%), Hcrtr1 (139%)] from the control value (100%) confirms such an active interaction (Damodaran *et al.*, 2006a, b).

In addition, neurons express two pools of AChE: active and inactive (Choi *et al.*, 1998). It is not known how these two pools of AChE are regulated post-translationally.

Moreover, Kaufer *et al.* (1999) suggested that in addition to ACh, an autologous feedback response could regulate transcriptional elevation from the AChE gene through AChE-antiChE complexes on signaling intracellular pathways. Excess muscarinic activation induced either by agonist application or by inhibition of the AChE results in long-lasting modifications of gene expression and protein levels of key cholinergic proteins (Meshorer and Soreq, 2006). Specifically, such changes involve transcriptional accentuation as well as shifted alternative splicing of AChE

pre-mRNA transcripts. This yields elevated levels of the stress-induced ''readthrough'' AChE-R mRNA transcript, translated into soluble AChE-R monomers rather than the primary ''synaptic'' AChE-S membrane-altered tetramers (Meshorer and Soreq, 2006). These two AChE variants can both hydrolyze ACh with similar efficiency, yet their different C-terminal sequences affect their subcellular localization as well as their protein partners (Meshorer and Soreq, 2006). Thus, multilevel changes initiated by sarin exposure on the cholinergic system have a major effect on several downstream processes of cell metabolism.

5. Sarin-Induced Changes in Protein Kinase C (PKC) Pathway

The protein kinase C (PKC) signaling pathway has been associated with modulation of *N*-methyl-D-aspartate (NMDA) receptor activity, motor behavior, learning, and memory, all of which are severely impaired in intoxication with sarin and similar OPs. There was a reduction in the immunoreactivity levels of betaII-PKC and Zeta-PKC in the frontal cortex (up to 24 h), and in the striatum (up to 5 days) post-sarin exposure, in contrast to the increase in the immunoreactivity of both enzymes in the hippocampus or thalamus, following a $1\times LD_{50}$ exposure to sarin. These observations suggest a role for both conventional and atypical PKC isozymes in OP-induced neuropathy in the rat and further support their role in cell death (Bloch-Shilderman *et al.*, 2005).

6. Sarin-Induced Alterations in Gene Expression Related to Electrophysiological Activities of CNS

Abnormal electrophysiological recordings following a single large dose or repeated subclinical dose of sarin in rhesus monkeys (Burchfiel *et al.*, 1976; Burchfiel and Duffy, 1982) and neuropathy in rats (Kadar *et al.*, 1995) have been reported. High concentrations of OPs have been reported to affect excitable membranes directly, by blocking peripheral nerve conduction (Woodin and Wieneke, 1970). A very large group of ion channel-related genes, belonging to almost every possible type (sodium, potassium, chloride, and proton-gated) such as Scn4a (338%), Kcnma1 (331%), Kcnj3 (199%), Clns1a (177%), Clcn5 (146%), Kcnd (138%), and Accn1 (129%), were induced to various degrees from the control levels (100%) as well as down-regulation of Kcnn2 to 64%, and clearly support severely altered electrophysiology at 15 min post-exposure (Damodaran *et al.*, 2006a). Similarly, several genes encoding mRNA for ion channels remained induced although to a lesser extent at 2 h after sarin exposure (Damodaran *et al.*, 2006b). The list includes Clcn5 (155%), Scn2b (138%), Cncg (136%), Clc-k21 (130%), Accn2 (122%), Kcnh2 (113%). However, the overall trend seems to be that of returning to control levels. Two genes, namely Kcnd3 (76%) and Pcp2858 (72%), showed down-regulation. On the other hand, there were only two genes (Accn1: 130% and Scn9a: 47%) showing altered expression at 3 months (Damodaran *et al.*, 2006a). The fact that two genes such as voltage-gated sodium channel (Scn9a) and neuronal degenerin channel MDEG (Accn1) showed persisting alterations at 3 months clearly indicates that sarin-induced alterations in the electrophysiological changes initiated at an early time point were minimized, but not completely normalized in rat animal models. Some degeneration and other problems related to memory and other defects in sarin-exposed GWS-related clinical features could be due to such a phenomenon. In a similar way, the highest expression of Ecto-ATPase at 15 min (Damodaran *et al.*, 2006a) and the persistence of altered levels of several other ATPases and ATP-based transporters at a 2 h time point (Damodaran *et al.*, 2006b) as well as at 3 months (other transporters like transferrin and neuron glucose transporter) clearly support the idea that perturbations in electrophysiological parameters of CNS persisted for a long time after the initial acute sarin exposure event (Damodaran *et al.*, 2006a, b).

7. Sarin-Induced Calcium Channels and Binding Proteins

Ca^{2+} is involved in the control of neuronal membrane excitability, neurosecretion, synaptic plasticity, gene expression, and programmed cell death. In physiological conditions, neuronal stimulation induces transient increases in the $[Ca^{2+}]$. Basal Ca^{2+} levels in the cytoplasm are principally maintained by efflux of Ca^{2+} through a membrane-associated Ca^{2+}-ATPase and also through a membrane Na/Ca^{2+} exchanger. In addition, cytosolic Ca^{2+} concentration is controlled by active Ca^{2+} sequestration into intracellular stores (endoplasmic reticulum and mitochondria) and by Ca^{2+} binding to intracellular proteins. An increase in the cytosolic concentration of Ca^{2+} can be the result of either influx of extracellular Ca^{2+} or the release of Ca^{2+} from internal stores. Ca^{2+} can enter the neuron through two major classes of Ca^{2+} channels, such as voltage-sensitive Ca^{2+} channels (VSCCs), which are sensitive to alteration in membrane potential and receptor-operated Ca^{2+} channels (ROCCs).

A sustained increase in cytosolic Ca^{2+} concentration, different from the rapid and transient changes occurring in physiological conditions, is invariably associated with neural damage (Nicotera, 1992). Sarin may induce alterations in the physical integrity of the plasma membrane, mitochondrial impairment, and consequent ATP depletion (Komulainen and Bondy, 1988). In addition, $[Ca^{2+}]$ overload is postulated to play an important role in hypoxic–ischemic brain damage, as a consequence of excitatory amino acid excessive stimulation and enhanced Ca^{2+} influx through membrane channels (Kristian and Siesjo, 1996). Moderate induction of CamK1b, Cacna1g, and down-regulation of Cacna1d and Camk2d were noted at 15 min post-sarin exposure (Damodaran *et al.*, 2006a). Induction of several genes (Calcr: 207%; Camk2d: 159%;

Calm: 132%; S100b, calcium binding protein: 130%) and down-regulation of CamK2a (56%) was found at 2 h post-sarin exposure (Damodaran *et al.*, 2006b). Induction of CaCh3 (neuroendocrine calcium channel alpha 1) to 220% and Cacna1c (L-type VDCC alpha unit) to 149%, accompanied by down-regulation of Camk2a (21% of the control levels) and Atp2a3 (Ca^{2+} transport ATPase 2) to 79%, suggest expression changes persisted at 3 months post-sarin exposure (Damodaran *et al.*, 2006a).

8. CaMKII as the Key Molecule in the Development of Neurotoxicity

The processing of a Ca^{2+} signal requires its union with specific intracellular proteins. Calmodulin is a major Ca^{2+} binding protein in the brain, where it modulates numerous Ca^{2+}-dependent enzymes and participates in relevant cellular functions. Among the different calmodulin-binding proteins, the Ca^{2+}/calmodulin-dependent protein kinase II and the phosphatase calcineurin are especially important in the brain because of their abundance and their participation in numerous neuronal functions. Studies on the acute effects of sarin on microsomal and cytosolic components of the calmodulin system in the rat striatum indicated CaM/calmodulin levels in both cell fractions were significantly increased by sarin (Hoskins *et al.*, 1986). Decreased cyclic AMP-PDE and adenylate cyclase activities and cyclic GMP-PDE and guanylate cyclase activities were noted after sarin exposure. Sarin administration caused significant increases in microsomal protein kinase activity (Hoskins *et al.*, 1986). Recent gene expression studies also confirmed the prominent role of the CAMK system by the altered mRNA levels of CaMK 2 alpha, CaMK 2 delta, calmodulin, and CaMK1. Persistent down-regulation of CamK2a at early (2 h) and late (3 months) time points clearly indicates its important role in sarin-induced neurotoxicity (Damodaran *et al.*, 2006a, b).

9. Sarin-Induced Blood–Brain Barrier (BBB) Damage and Related Alterations in Gene Expression

The entry of molecules to the CNS is regulated by the BBB, which is the interface between blood and the CNS, and this entry is based on the size, charge, hydrophobicity, and/or affinity to carriers. MOG (myelin oligodendrocyte glycoprotein), PSD-95, and neurexin were also altered, indicating disturbances to the BBB-related function. Large doses of sarin caused BBB damage and BBB disruption plays an important role in sarin-induced cell death (Abdel-Rahman *et al.*, 2002; Damodaran *et al.*, 2003). Kinase cascades such as CaMKII and MAP kinase kinase are altered in cell types with compromised BBB, within 15 min, which probably received transducing signals due to changes seen in several upstream molecules (Damodaran *et al.*, 2006a, b). Tyrosine phosphorylation is the next logical step, which may increase tight junction permeability (Staddon *et al.*, 1995). Up-regulation of tyrosine kinase receptor EHK-3, altered levels of transporters such as sodium-dependent neurotransmitter transporter, and ATP-based transporters were suggested to support such a possibility (Damodaran *et al.*, 2006a). A primary mediator of injury-induced BBB disruption is nitric oxide (NO). Induction of nitric oxide synthase along with changes in EDN 3 expression clearly support such a scenario (Damodaran *et al.*, 2006a). Agents that regulate vasoactive processes, such as vasoactive intestinal receptor-1 (Vipr 1) and 2 (Vipr 2), may be involved in the biochemical opening of the BBB (Damodaran *et al.*, 2006a), as other vasoactive genes such as bradykinin and angiostensin have been shown to (Black, 1995).

Other changes noted in the NMDA receptor function-related genes such as PSD-95 as well as PSD-95/SAP-90-associated protein were up-regulated at 15 min post-sarin exposure, along with NMDA and NO synthase levels (Damodaran *et al.*, 2006a). PSD-95 participates in the anchoring of the NMDA receptor and interacts with neuronal NO synthase at the postsynaptic membrane, and thereby plays a fundamental role in synaptic transmission and memory formation (Kim *et al.*, 2002) about the role of NOS/NMDA mediated apoptosis in DFP (a structural analog of sarin)-treated rat CNS. Altered levels may explain an early abnormal event that may initiate a complex process leading to memory loss observed in sarin-exposed rats (Kassa *et al.*, 2001). Besides, persistence of altered expression of BBB-related molecules such as neurrein-1 adds support to the important role of BBB damage in initiating and amplifying neurotoxicity-related phenomena.

10. Effect of Sarin on Hormones and Growth Factors

Hormones play an important role in the cholinergic neurotransmission at pre- and postsynaptic levels in the basal forebrain. The neurobiological mechanisms that underlie these effects are not fully understood, but most likely reflect effects of hormones on the survival, connectivity, and function of specific neural systems in the hippocampus and may trigger a variety of intracellular signals (Foster, 2005). All of the growth factor-related genes such as rat growth hormone, basic fibroblast growth factor, and insulin-like growth factor-II were found to be induced at 15 min, while at 3 months growth factors related to glial function (Gmfb) were induced along with eek (eph and elk-related kinase) and growth inhibitory factor-metallothionein homolog (Gif-Mt) (Damodaran *et al.*, 2006a, b). C-erb-A-Thyroid hormone receptor and 17 betahydroxysteroid dehydrogenase showed down- and up-regulation, respectively, 2 h after sarin exposure (Damodaran *et al.*, 2006b). DFP (structural analog to sarin) has been shown to reduce serum prolactin, thyropton, luteinizing hormone, and growth hormone, and increase adenocorticotropin and corticosterone in rats and it was concluded that cholinesterase inhibition evokes a multiplicity of effects on anterior pituitary function (Smallridge *et al.*, 1991). A similar mechanism of action can be expected for the effect of sarin exposure on

endocrine systems. A statistically significant correlation between body weight loss, plasma cholinesterase levels, and hormonal control of hydration levels in sarin-dosed animals established that the satiety center controlling the hypothalamus and CNS might be affected by sarin treatment (Young *et al.*, 2001).

It is known that cells that are stimulated by bFGF also showed robust expression of nestin, which plays a role in the neuronal glial differentiation of bipotential cells (Cameron *et al.*, 1998). Both nestin and bFGF are induced in the sarin-treated CNS at 15 min, thus confirming their protective role either inducing the proliferation or increasing the differentiation of bipotential cells into neurons or glia as and when required at distinct regions of damaged CNS (Damodaran *et al.*, 2006a, b).

11. Effect of Sarin on CREB Pathway

CAMP-response element binding protein (CREB) is a member of a large family of structurally related transcription factors which has the transcriptional control of numerous genes, many of which are rapidly expressed in response to an elevation of cytoplasmic cAMP or Ca^{2+} levels. The persistent phosphorylation of CREB in the CNS, in response to OPs like diisopropyl-phosphorofluoridate (DFP), has been shown to be involved in the initiation of delayed neurotoxicity (Damodaran *et al.*, 2002c). There are numerous adaptive changes that can be broadly divided into (a) cell survival and (b) cell death that may be mediated through CREB pathways. Altered gene expression for several genes (Camk2, Pka, c-Fos, c-Jun) was shown in DFP-treated hen CNS (Gupta *et al.*, 1997, 1998, 2000a, b; Gupta and Abou-Donia, 1999; Damodaran *et al.*, 2003). Similarly, expression profiles for several genes (Cam2a, Camk2d, Calm, Cbf-C, and Scya2) were also shown to be altered in sarin-treated rats (Damodaran *et al.*, 2006a, b). Transducing signals of several other kinases also converge on the CREB pathway leading to the activation of several downstream processes. Activation of c-Jun N-terminal kinase (JNK) and slight activation of mitogen-activated protein kinase (MAPK) in the cytosol fraction of the brain homogenate was observed after exposure to sarin-like organophosphate BIMP (bis isopropylmethyl phosphonate), which increased tyrosine phosphorylation of several proteins in the cytosol fraction (Niijima *et al.*, 2000). Altered expression of Camk2a, Camk2d, Calm, JNKs, and MAPKs by sarin exposure clearly supports such a phenomenon mediated through CREB pathways.

12. Sarin-Induced Alterations in Gene Expressions Related to Astroglial Marker Genes

Astrocyte activation or reactive gliosis involves proliferation, recruitment to the site of injury (Walton *et al.*, 1999), and release of numerous cytotoxic agents including proteolytic enzymes, cytokines, complement proteins, reactive oxygen intermediates, NMDA-like toxins, and nitric oxide (Weldon *et al.*, 1998). Sarin exposure activated astrocytes and resulted in the altered mRNA expression profile of GFAP and vimentin in a time course study (Damodaran *et al.*, 2002b). Global gene expression studies showed an alteration in the expression levels of S-100 beta, an astrocyte-specific Ca^{2+}-binding protein (neurotropic factor), at 2 h post-treatment, thereby confirming the fact that sarin-induced calcium changes resulted in alterations in several downstream molecules. The alteration in S-100 levels also indicates changes taking place in Schwann cells of the CNS. In rats, S-100 beta has been shown to present in about 35% of the neurons (Landgrebe *et al.*, 2000). Hence up-regulation at 2 h probably indicates that both neurons and glial cells are affected by sarin treatment. Another gene of special interest induced by sarin is apolipoprotein E (apoE), which may play a role in various CNS disorders, and its altered levels indicate modified astrocyte and/or neuronal response and functions (Damodaran *et al.*, 2006b). Altered expression of apoE has been associated with chronic reactive gliosis (Martins *et al.*, 2001) and is also suggested to be neuroprotective (Boschert *et al.*, 1999). ApoE has been shown to stimulate the transcriptional activity of CREB by activating the extracellular signal-regulated kinase (ERK) cascade in rat primary hippocampal neurons (Ohkubo *et al.*, 2001).

13. Gender-Based Differences in Gene Transcript Expression

Overall, female rats were more sensitive to sarin vapor toxicity than male rats over the range of exposure concentration and duration studied (Mioduszewski *et al.*, 2002). In a recent global gene expression study, male and female Sprague-Dawley rats exposed to low-level doses (0.010 to 0.033 mg/m^3) of the aerosolized sarin via whole body inhalation for 4 h showed differential gene expression response between male and female rats (Sekowski *et al.*, 2002). Many of the altered genes participate in cellular processes critical to detoxification pathways and neurological homeostasis which may reflect the difference in the sensitivities as demonstrated by the differences in the miosis EC_{50} levels. Transcripts of UDG glycolase (Uracil DNA glycosylase) were shown to be significantly up-regulated at the highest dose point (0.033 mg/m^3) for females but not for males. UDG glycolase is involved in base excision repair (Scharer and Jiricny, 2001). The transcripts for the gene Cyp2A and other Cyp2 family members also showed differential expression pattern. While the expression pattern of all Cyp2 family members in females was not altered by any of the doses of sarin tested, the Cyp2A family of enzymes is variably, but significantly influenced by the doses of sarin tested in males. Furthermore, it appears that the constitutive levels of these enzymes are higher in males than in females. It is well known that the Cyp2 family of enzymes is involved with primary metabolism and bioconversation of many toxicants (Sekowski *et al.*, 2002).

DNA polymerase alpha was up-regulated following low-level sarin exposure. Since mammalian DNA replication (and possibly DNA repair) mechanisms utilize DNA

polymerase alpha, the DNA replication machinery may be turned on or up-regulated to synthesize DNA and/or repair lesions in the DNA (Sekowski *et al.*, 2002). Sarin exposure also down-regulated endothelin-1, which plays a critical role in vascular tension, as it interacts with the sympathetic nervous system and the renin–angiotensin system (Sekowski *et al.*, 2002).

14. Sarin-Induced Changes in Cytokine Profile

Sarin exposure resulted in prolonged central neuro-inflammatory processes in rat brain tissues (Damodaran *et al.*, 2006a, b; Chapman *et al.*, 2006). Gene expression analysis at various time points (15 min, 2 h, and 3 months) in sarin-treated rats indicated up-regulation of 1L-10 at early time points, while IL6 and IL9 were noted at late (3 months) time points (Damodaran *et al.*, 2006a, b). Biochemical evaluation of rat brain tissues revealed a significant increase in the level of the proinflammatory peptides starting at 2 h and peaking at 2–24 h following sarin exposure (Chapman *et al.*, 2006). Hippocampal values of IL1-beta increased one-fold at 2 h and nine-fold at 8 h. PGE2 (prostagladin E2) levels in the hippocampus increased up to four-fold to six-fold at 2 and 8 h. In addition, a second increase in inflammatory markers was observed 30 days following sarin exposure only in the rats with 30 min of prolonged seizures. Marked histological damage to the brain was demonstrated following 30 min of seizure activity, consisting of severe damage to the hippocampus, piriform cortex, and some thalamic nuclei as compared to rats with 5 min seizures.

Subacute doses of sarin on wistar rats led to suppression of cellular and humoral immune reactions and to a decrease in blood concentrations of cytokines (IL-2, IL-4, IFN-gamma), with a reduction of the IFN-gamma/IL-4 and IL-2/IL-4 ratios, which attests to a more pronounced decrease in Thy1 lymphocyte function in comparison with Th2 cells (Zabrodskii *et al.*, 2007). Grauer *et al.* (2008) studied the long-term neuronal and behavioral deficits after a single whole body exposure to sarin vapor. Neuronal inflammation was demonstrated by a 20-fold increase in prostaglandin (PGE2) levels 24 h following exposure that markedly decreased 6 days later. An additional, delayed increase in PGE2 was detected at 1 month and continued to increase for up to 6 months post-exposure. Glial activation following neural damage was demonstrated by an elevated level of peripheral benzodiazepine receptors (PBR) seen in the brain 4 and 6 months after exposure. Six week, 4 and 6 month post-exposure behavioral evaluations were performed. In the open field, sarin-exposed rats showed a significant increase in overall activity with no habituation over days. In the working memory paradigm in the water maze, these same rats showed impaired working and reference memory processes with no recovery. Thus, the data suggest long-lasting impairment of brain functions in sarin-exposed animals. The PGE2-dependent, ERK (extracellular signal-regulated kinase), 1/2-regulated, microglia–neuron signaling pathway may mediate the interaction between microglia and neurons in pain maintenance after injury (Zhao *et al.*, 2007) in sarin-exposed animals.

15. Sarin-Induced Oxidative Stress and Cell Death/Neuroprotection-Related Gene Expression

Oxidative stress mediated free radical generation and alterations in the anti-oxidative scavenging system have been implicated in OP-induced neurotoxicity (Abu-Qare and Abou-Donia, 2001; Abu-Qare *et al.*, 2001). Alteration in the levels of brain Acyl-CoA synthetase and leptin accompanied by alterations in the mRNA levels of key mitochondria associated proteins such as Bax (apoptosis inducer) and BOK (BCL-2-related ovarian killer protein) in sarin-exposed rats clearly supports oxidative stress-induced cell death (Damodaran *et al.*, 2006a). Induction of both proapoptotic (Bcl2l11, caspase 6) and antiapoptotic (Bcl-X) genes, besides suppression of p21, suggests complex cell death/protection-related mechanisms operating early on. Moreover, persistent alteration in the cell death-related molecule DEFT at 3 months adds support to the notion that oxidative stress-related changes can cause long-term complications in cell physiology and pathology (Damodaran *et al.*, 2006a, b).

Reactive oxygen species (ROS) have been shown to initiate a number of signaling events that lead to endothelial cell ‘‘activation’’ and up-regulation of cell adhesion molecules and chemoattractants. Strong induction of tumor necrosis factor (TNF)-beta along with down-regulation of CX3C at 15 min and further down-regulation at 3 months post-sarin exposure strongly suggest such a mechanism (Damodaran *et al.*, 2006a). Many cell adhesion and cytoskeleton and BBB-related molecules (neurexin 1-beta; neurexin 1-alpha, PSD-95/SAP90-associated protein 4), and cytoskeletal genes (nestin, beta tubulin) showed prominent alterations in mRNA expression (Damodaran *et al.*, 2006a, b).

Lipid peroxidation has been shown to be present in the CNS of rats treated with sarin (Abou-Donia *et al.*, 2002). Altered expression levels of lipophilin supports to the notion of sarin exposure initiated lipid peroxidation as an important by-product of oxidative stress. The glutathione system is an important protective mechanism responsible for removing H_2O_2 and maintaining protein thiols in their appropriate redox state in the cytosol and mitochondria for minimizing oxidative damage. Altered expression of glutathione, dopamine (Drd4), and GABA (Gabbr-1, Gabra1, Gabra3, Gabbr-1d, Gabrb3)-related genes may potentiate the cell death and injury to these populations (Damodaran *et al.*, 2006a).

16. Effect of Sarin on DNA and Protein Content

Kassa *et al.* (2000) showed that not only symptomatic level 3 but also asymptomatic levels 1 and 2 of sarin were able to significantly decrease the incorporation of radiolabeled thymidine without changing total concentrations of DNA or

protein at 3 months following exposure. On the other hand, the significant decrease in total contents of DNA and protein in liver without the changes in the incorporation of tritiated thymidine was noted in liver 6 months following sarin exposure. No significant changes in the metabolism of DNA and protein were observed at 12 months following sarin exposure. Thus, not only clinically manifested intoxication but also low-level, asymptomatic exposure to sarin altered nucleic acids and protein metabolism, several months following exposure.

Repeated low-dose exposure to sarin produced a dose-dependent response in leukocytes at 0 and 3 days post-exposure. There was a significant increase in all measures of DNA fragmentation at 0.2 and 0.4 LD_{50}, but not at 0.1 LD_{50}. There was no significant increase in DNA fragmentation in any of the groups at 17 days post-exposure. Sarin did not produce systematic dose-dependent response in parietal cortex at any of the time points. However, significant increases in DNA fragmentation at 0.1 and 0.4 LD_{50} were observed at 0 and 3 days post-exposure and levels were back at control level by day 17 (Dave *et al.*, 2007). Analysis of phosphor-carrying metabolites of sarin and its by-products by Li *et al.* (2000) in urine samples from the victims of the Tokyo train terrorist attack suggested that they were exposed not only to sarin, but also to by-products generated during sarin synthesis, i.e. diisopropyl methylphosphonate (DIMP) and diethyl methylphosphonate (DEMP). Sister chromatid exchange (SCE) was significantly higher in the victims than in a control group. Both DIMP and DEMP significantly inhibited NK and CTL activity in a dose-dependent manner. The inhibition induced by DIMP was stronger than that by DEMP. The effect of DIMP and DEMP on the splenic NK activity of mice was stronger than on the splenic CTL activity, and the human lymphocyte is more sensitive to DIMP and DEMP than the splenocytes of mice.

17. Effect of Sarin on Immune System

There is increasing evidence that the immune, endocrine, and nervous systems communicate with each other through hormones, neurotransmitters, and cytokines (Tracey, 2005). Immune function is not only regulated by the cytokine system, it is also under the control of an independent lymphoid cholinergic system (Kawashima and Fujii, 2008). Immunosuppressive effects of sarin are mediated through the central and peripheral mechanisms (Sopori *et al.*, 1998). Interestingly, serum corticosterone levels of the sarin-treated animals were dramatically lower than the control animals (Kalra *et al.*, 2002). Subclinical doses (0.2 and 0.4 mg/m^3) inhibited the anti-sheep red blood cell antibody-forming cell (AFC) response of spleen cells without affecting the distribution of lymphocyte subpopulations in the spleen (Kalra *et al.*, 2002). Moreover, sarin suppressed T cell receptor (TCR) antibody-induced T cell proliferation and the rise in the intracellular calcium following TCR ligation. These concentrations of sarin altered regional but not total brain acetylcholinesterase activity (Kalra *et al.*, 2002). Pretreatment of animals with the ganglionic blocker chlorisondamine abrogated the inhibitory effects of sarin on spleen cell proliferation in response to Con A and anti-TCR antibodies. These results suggest that effects of sarin on T cell responsiveness are mediated via the autonomic nervous system and are independent of the HPA (hypothalamus–pituitary–adrenal axis). Furthermore, sarin-induced inhibition of T cell dependent functions such as the AFC response and the TCR mediated rise in intracellular Ca^{2+} is lost 2 weeks after sarin exposure. Low-level (single or repeated) inhalation exposure of sarin on inbred BALB/c mice to evaluate the effect of sarin on immune functions indicated that sarin is able to alter the reaction of the immune system at 1 week after exposure to sarin (Kassa *et al.*, 2004). While the number of CD3 cells in the lung was significantly decreased, a slight increase in CD19 cells was observed especially in the lungs after a single sarin inhalation exposure. Lymphoproliferation was significantly decreased regardless of the mitogen and sarin concentration used and the number of low-level sarin exposures. The ability of peritoneal and alveolar macrophages to phagocytose the microbes was also decreased regardless of the number of low-level sarin exposures. The production of N-oxides by peritoneal macrophages was decreased following a single low-level sarin exposure but increased following repeated low-level sarin exposure. However, the change in the production of N-oxides that reflects a bactericidal activity of peritoneal macrophages was not significant. The natural killer activity was significantly higher in the case of inhalation exposure of mice to low concentrations of sarin regardless of the number of exposures. Generally, repeated exposure to low concentrations of sarin does not increase alteration of immune functions compared to the single low-level sarin exposure, with the exception of phagocyte activity of alveolar macrophages and natural killer cell activity.

Sarin exposure also up-regulated the mRNA expression of proinflammatory cytokines in the lung, which is associated with the activation of NFκB in bronchoalveolar lavage cells (Pena-Philippides *et al.*, 2007). These effects were lost within 2 weeks of sarin inhalation. While the effects of sarin on T cell function were on the autonomic nervous system (ANS), the decreased corticosterone levels by sarin might result from its effect on the HPA axis. Sarin induces lung inflammation resulting from neutrophilic and eosinophilic infiltration (Levy *et al.*, 2004; Pant *et al.*, 1993). Subclinical doses of sarin did not cause signs of lung inflammation, but left molecular imprints of lung inflammation. Sarin promotes the nuclear translocation of NFκB in BAL cells. Therefore, increased gene expression of IL-1, TNF-alpha, and IL-6 observed in BAL cells from sarin-treated animals may result through activation of NFκB. In higher doses, sarin may elevate the transcription, as observed by others (Levy *et al.*, 2004; Pant *et al.*, 1993; Damodaran *et al.*, 2006a).

18. Studies on the Long-Term Effect of Sarin Exposure to Human Subjects Support Data from Animal Models

Golomb (2008) summarized the findings from several epidemiological studies, and suggested that AChE inhibition by various OP nerve agents and other OP and carbamate pesticides could be linked to Gulf War Syndrome-related illness. Thirty-eight victims of the Tokyo subway sarin attack exhibited smaller than normal regional brain volumes (of the matched controls) in the insular cortex and neighboring white matter, as well as in the hippocampus, all of whom had been treated in an emergency department for sarin exposure in a long-term study, using T1-weighted and diffusion tensor magnetic resonance imaging (DTT), the voxel-based morphometry.

Furthermore, an extensively lower than normal fractional anisotropy was also noted in the victims. The reduced regional white matter volume correlated with decreased serum cholinesterase levels and with the severity of chronic somatic complaints related to interoceptive awareness (Yamasue *et al.*, 2007). Similarly, another study of Gulf War veterans showed subtle but persistent central nervous pathology such as reduced white matter and increased right and left lateral ventricle with higher levels of exposure to sarin and cyclosarin (Heaton *et al.*, 2007). Moreover, electrophysiological deficits of controlled attention, accompanied by lower P300 amplitude and smaller anterior cingulated cortex volume, were observed in patients (victims of the Tokyo subway sarin attack), and suggested a link to brain morphological changes (Araki *et al.*, 2005). Similarly, the patients from subway sarin attack also performed significantly less well in the psychomotor function test (tapping) than the referent group. There were dose-dependent effects for these observations and thus these results indicate a chronic decline of psychomotor function and memory function 7 years after sarin exposure (Miyaki *et a*l., 2005). Investigations on the neurobehavioral task performance of Gulf War veterans categorized as having received high, moderate, or low-to-no exposure dose levels to sarin and cyclosarin indicated that sarin and cyclosarin exposure was significantly associated with less proficient neurobehavioral functioning on tasks involving the psychomotor dexterity and visuospatial abilities 4–5 years after exposure (Proctor *et al.*, 2006). All these observations from human studies strongly correlate with the data from gene expression and other molecular studies in animal models.

19. Sarin-Induced Altered Expression Profile Provides Biomarker Panels and Therapeutic Targets

Molecules that show persistent altered expression over a period of several time points strongly suggest that they play important roles in preserving, amplifying, and transmitting the altered expression as and when needed, which could be either degenerative or regenerative in nature. There are several molecules such as Arrb-1, Nrxn-1b, Nos-2a, Ania-9, PDE2, Gabab-1d, CX3C, CamK2a, Camk2d, Clcn5, IL-10, c-Kit, and Plp1 that showed such a pattern (Damodaran *et al.*, 2006a, b). Beta-arrestin (Arrb-1) maintained its altered state at three different time points such as immediate early (15 min), early (2 h), and late (3 months), thus confirming persistent pathological changes due to altered signal transduction pathways mediated through beta arrestin. Continued down-regulated mRNA levels of neurexin (Nrxn-1b) and persistent up-regulation of Nos2a clearly support an idea that BBB-related perturbations were still persistent at 3 months after the treatment, and might lead to persistent cell death in susceptible brain regions. Immediate early up-regulation of Ania-9 at 15 min followed by significant down-regulation at 3 months strongly indicates either successful regeneration or a continuing cell death/atropic state. Initial down-regulation at 15 min, followed by up-regulation of PDE 2 at 3 months, probably indicates recovering cyclic nucleotide metabolism at 3 months from its sarin-induced suppression. Initial up-regulation (15 min) followed by severe down-regulation of GABAB-1d (3 months) confirms the persistent defective GABAerginic metabolism. Induction at early time points followed by down-regulation at late time points makes CX3C one of the potential biomarker molecules. Clcn5 showed persistent alterations at both immediate early (15 min) and early (2 h) suggesting altered ion-channel-related functions. Persistent overexpression of IL-10 at early time points may indicate an underlying anti-inflammatory response. Continued alteration of kinase pathways was suggested by the persistent alterations in the levels of Camk2a, Camk2d, and c-Kit. Hence early events such as BBB damage (neurein 1-beta, Plp1, Nos2a), IEGs alteration (Ania-9, probably c-Fos and cJun and other Ania members), modified GPCR mediated signaling pathways (mediated by beta arrestin), altered cyclic nucleotide metabolism (PDE 2), and inflammation and injury-related changes (CX3C), probably play major roles along with other known mechanisms as shown in Figures 44.1–44.3. These persistently altered genes can become candidate biomarkers to monitor sarin exposure as well as to develop therapeutic targets. For example, the Camk2 family of genes can be a good therapeutic target of intervention, as it seems to be central to the upstream and downstream signal transduction processes. Large amounts of data are already available for Camk2 regarding its structure, expression, inhibitors, and potential target genes (pathways). Similarly, AChE is a good candidate for such intervention, as already several studies attempted for that purpose show promising results at least at the level of ''proof of hypothesis'' (Curtis *et al.*, 2005, 2008; Li *et al.*, 2006). Time course studies on both Camk2 and AChE have shown that they are differentially altered in various regions of the brain (Gupta *et al.*, 1998; Damodaran *et al.*, 2003, 2006a). Beta arrestin can be a good candidate for monitoring any interventions, as its expression was consistently up-regulated. Different doses of sarin exposure can be identified by monitoring abundant yet

dose-dependent expression of selected genes, representative of various subregions (such as cerebellum, hippocampus, etc.) and cell types (neuronal, astroglial, microglial, oligodendrocytes, endothelial, etc.) which can be identified by further testing on several published genes (Damodaran *et al.*, 2006a, b). Other approaches include measuring the levels of tyrosine adducts which are relatively long-lived and are not degraded by oxime therapy, after exposure to sarin and other warfare chemicals (Williams *et al.*, 2007).

IV. CONCLUDING REMARKS AND FUTURE DIRECTION

Sarin exposure results in significant levels of transcriptional changes (either activation or suppression) of several genes (pathways) in the brain of animal models like the rat, mouse, and, potentially, humans. Global and candidate gene expression studies in model systems yielded data that confirm the toxic effect of sarin exposure on different cell types of the central nervous system such as neuronal, astroglial, microglial, and endothelial as well as oligodendrocytes. Sarin exposure also causes damage to structural components of the brain such as BBB at gross level as well as to the cytoskeletal framework at the molecular level as indicated by the altered expression data and histopathological data specific to such structures. There seem to be gender-specific gene expression profiles as a response to sarin exposure in rats, suggesting higher susceptibility in females than in males. The changes indicate that persistence of both altered degenerative and regenerative-related gene expression patterns activated early on may contribute to the level of neurodegeneration and neuropathology at a later time point. Sarin exposure also affected the molecular profiles of immune and endocrine systems besides the nervous system, as it is a well-known fact that they communicate with each other through cytokines, hormones, and neurotransmitters. Exposure to sarin can initiate (1) acute response-related clinical changes, (2) OPIDN, and (3) OPICN. Altered profiles of gene expression, protein expression, hormonal and other biochemical parameters in response to sarin exposure confirm the highly toxic nature of the nerve agent leading to significant amounts of nervous system pathology and end organ system pathology. Various neurological abnormalities observed in the victims of sarin exposures may be due to the above-mentioned molecular changes. These data thus provide a wealth of novel insights into the mechanisms of toxicity, so that newer approaches can be initiated in the diagnosis, prevention, and treatment aspects of toxic exposures to warfare chemicals like sarin.

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