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# Metabonomics, Brain Apoptosis, and Carbon Monoxide

*Review Article*

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**Abstract**

Metabonomics tell us how an organism has responded to a stimulus or challenge and is an indicator of cell physiology and response to stress. This systematic study of unique chemical fingerprints has resulted in quantitative data on a broad range of metabolites reflecting metabolism and/or metabolic shifts associated with brain health, pathology and/or treatment. Complex interactions between nutrition, genetics, and the central nervous system (CNS) determine optimal cerebral energy state and are mediated by changes in energy needs, metabolism, environment and multiple signaling molecules. Evaluation of exogenous administration of normal endogenous neuroprotective molecules may be expeditious and succinct. The normal physiologic effects of one of these agents, carbon monoxide (CO) - a major neurotransmitter and gasotransmitter, is important for multiple neurologic functions and neuroprotection and its role as a neuroprotective and neurotherapeutic agent has been suggested. Metabonomic profiling will provide further insight into the role of CO in CNS health and will add greatly to our ability to improve the quality of life in our patients with neuropathologic, neuropsychiatric, and neurodegenerative disorders.

**Keywords:** Metabonomics of the Brain and CNS; Metabonomic Profiling; Carbon Monoxide as a Neurotransmitter; Gasotransmitters.

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## Introduction

The brain's health and response to stress are dependent on genomics, transcriptomics, and proteomics. Complex interactions between nutrition, genetics, and the central nervous system (CNS) determine optimal cerebral energy state and are mediated by changes in energy needs, metabolism, environment and multiple signaling molecules. Metabolites constitute substrates and products of the biochemical reactions, are the metabolic signature of biochemical activity and reflect the phenotype of the cell and/or organism [1]. Metabolomics, "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" and determined by quantification of the metabolites, tell us how the organism has responded to a stimulus or challenge and is an indicator of cell physiology and response to stress. Metabonomics is the fingerprint of biochemical perturbation caused by disease, drugs, and toxins [2, 3]. Correlation of biochemical changes with phenotype linking cellular pathways to biological mechanisms by using the resulting metabolome provides a powerful tool to assess unexpected biochemical pathways respondent to injury and treatments [4]. This is the most sensitive measure of a cellular phenotype.

Assessed by metabolomic technology, analysis of these small-molecule metabolites can be used to understand the changes in homeostasis in the biological system [5-9]. Single cell metabolomics is being done using mass spectrometry, microfluidics, and capillary separations [10, 11]. Systematic study of these unique chemical fingerprints has resulted in quantitative data on a broad range of metabolites reflecting metabolism and/or metabolic shifts associated with brain health, pathology and/or treatment [12-23]. With brain injury, there is a change in protein and mRNA expression which is observed hours, days, or weeks after the insult, but metabolomes provide the clinician more timely assessment of the extent of injury as well as response to therapy.

Application of metabolomic technologies to assess neurotherapies would improve the understanding of disease and may help to limit progression of disease and/or allow quantification of reversal of injury. Evaluation of exogenous administration of normal endogenous neuroprotective molecules may be expeditious and succinct. One of these molecules, carbon monoxide (CO) - a major neurotransmitter and gasotransmitter, is important for multiple neurologic functions [24-34]. Agents affecting the synthesis, transactions, and disposition of the gas have clinical rel-

evance to neuroprotection [35-46]. Exogenous administration of inhaled CO or carbon monoxide releasing molecules (CORM's) impart similar neurophysiological responses as the endogenous gas and dose and duration of exposure is important. Currently, the drug is under development as a therapeutic agent and safety studies in humans evaluating the safety and tolerability of inhaled doses of CO show no clinically important abnormalities, effects, or changes over time in laboratory safety variables. As an important therapeutic option, CO has entered clinical trials and its clinical role as a neuroprotective and neurotherapeutic agent has been suggested. CO, like nitric oxide (NO) before it, may prove to be a therapeutic option as a new and novel approach to various neuropathologies. The role for CO as a neurotherapeutic based on compelling animal data necessitates further testing in humans. The time has come to assess this simple gas as one cannot ignore the remarkable data that continues to be reported. Metabonomics to assess effectiveness of this gasotransmitter in treating brain pathology and injury would greatly benefit patients by providing timely information on both the extent of damage and effects of treatment.

## Metabonomics and the Brain

Cell precursors of the brain begin to develop early in embryogenesis. An ordered sequence of temporally and spatially related morphogenetic events are under the control of specific genes and the molecular mechanisms which underlie normal brain function and development as well as pathology [47-52]. This continues and changes through life. The dynamic and varying metabolomic signatures in the brain reflect both the health of neural tissues at various stages of development and disease [53-61]. These depend on brain vulnerability which relates to whether an agent reaches the nervous system and the time of exposure and may not be apparent immediately. The many chemical modulators of nervous system damage can trigger a sequence of events resulting in neuronal damage and cell death. Resulting injuries depend on brain maturity and cellular health as well as location of the lesion. A chain of events leading to apoptotic DNA fragmentation, cellular fragmentation and engulfment of the cell may result. Salvage and/or the recovery/regeneration of the nervous system, its cells, structure and function may be varied due to the area of the CNS involved, health of the cells/organ, and treatments. Biochemical changes may be normal findings and resulting metabolomes must, therefore, be compared to what is normal for age and development and evaluated in the context of clinical findings [62-70].

However, normal clinical findings do not exclude neuropathology. Neurologic diseases are common as millions of people suffer from neurodegenerative diseases, traumatic brain injury (TBI), and psychiatric illnesses, but timely diagnosis and indicators of outcome have been limited. Diagnosis and successful intervention have been determined by symptoms and scales. Inaccurate and imprecise, these clinical approaches do not address biochemical processes responsible for the recognized pathology. Clinicians have routinely used computed tomography (CT) and magnetic resonance imaging (MRI) to evaluate the extent and type of neurologic disease, but these studies are less sensitive for diagnosing acute injury or response to therapy [71-79]. When changes are identified on CT or MRI, cells have already undergone apoptosis or necrosis. The serious metabolic disorders of neuronal and non-neuronal cells occur earlier than reflected by these studies and specific regions of the CNS show differential vulnerability. Rapid assessment of injury and treatment before clinical manifestations are apparent could improve long-term outcomes and limit pathology by allowing earlier clinical intervention. Transcriptomic approaches provide information on gene expression, but may not reflect physiological processes and proteomics may not be predictive of biological responses [80, 81]. Quantitative analysis of metabolites is able to detect the presence or absence of thousands of small molecules, but no single technique measures the complete metabolome. Mapping of the regulation of neural metabolic pathways using metabonomics will help identify the type of pathology and suggest strategic points of therapeutic intervention in patients with neurologic insults.

Metabonomics provides more timely information and has been applied to the CNS [81, 82-93]. Biofluid evaluation of key metabolites in plasma and whole blood, serum, urine, saliva, cerebrospinal fluid, synovial fluid, semen, and tissue homogenates has characterized clinical status and response to treatment in several diseases [94].

Metabonomic techniques to determine CNS health include *in vitro* technologies as well as *in-* or *ex- vivo* approaches. Approaches include metabolite target analysis (analysis restricted to metabolites of a particular enzyme system that would be directly affected by abiotic or biotic perturbation), metabolite profiling (analysis focusing on a group of metabolites), metabolomics (comprehensive analysis of the whole metabolome under a given set of conditions), metabolic fingerprinting (classification of samples on the basis of provenance of either their biological relevance or origin), metabolic profiling (commonly used in clinical and pharmaceutical analysis to trace the fate of a drug or metabolite), and metabonomics (measure of the fingerprint of biochemical perturbation caused by disease, drugs, and toxins) [95]. Analyses measure a subset of the whole profile with little differentiation or quantitation of metabolites, assess the metabolic profile within or associated with a particular metabolic pathway, and/or focuses on a particular segment of the metabolome by analyzing only a few selected metabolites that comprise a specific biochemical pathway. Results are context-dependent and change depending on the physiology, pathology, and developmental state of the cell, tissue, organ, and/or organism. The types of databases that are useful to interpret this information include databases storing detailed metabolite profiles, single species-based databases, databases storing complex metabolite profile data from many species in many different physiological states, databases listing all known metabolites for each biological species, databases compiling established biochemical facts, and databases that integrate genome and metabolome data with an ability to model metabolic fluxes [95].

Systems-biology approaches to the normal and abnormal CNS using metabolomics purports a metabolism-based mechanistic understanding of the metabolic and resulting physiologic function of disease and response to therapy [89]. Approaches quantify data on a broad range of metabolites in order to understand shifts in metabolism and biochemistry in different CNS pathologies. Instruments are able to quantitate thousands of small molecules and mathematical tools identify molecular signals for a specific disease. The two most accepted methods used in the measurement of metabolites are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). The former consists of the absorption and re-emission of electromagnetic radiation by atomic nuclei in a magnetic field and is applicable to analysis of biofluids, cell extracts, and cell cultures and requires almost no sample preparation. The standard approach using pa-

tient's samples is using proton NMR (1H NMR) although $^{2}H$, $^{13}C$, $^{31}P$, $^{15}N$, and $^{19}F$ may be employed. MS-based metabolomics may provide a targeted or large-scale metabolome analysis and is usually combined with three types of prefractionation techniques – gas chromatography (GC), high-performance liquid chromatography (HPLC), or capillary electrophoresis (CE). GC is highly efficient, sensitive, and reproducible, but can only be performed with volatile compounds or those that can be made volatile. Although HPLC separation may reach a wider range of substrate that can be analyzed, its resolution is poorer. CE is applicable to charged substrate and is superior in performance regarding separation than HPLC.

Application has allowed discrimination of metabolic markers noninvasively *in vivo*. In animal models of asphyxia/hypoxia, accumulation and delayed recovery of Krebs cycle intermediates have been described, changes in amino acid profiles shown, and disturbances of the cell membrane illustrated [96-99]. NMR spectroscopic metabolic profiling of cerebral spinal fluid and serum identifies differences between idiopathic intracranial hypertension, multiple sclerosis, cerebrovascular disease, and mixed neurological diseases in humans. The authors concluded that metabolomics may be a clinically useful tool for diagnosis and may help identify biochemical pathways unique to a neurodisease [100]. In infants with perinatal asphyxia, Huang and colleagues measured the ratio of lactate to creatinine in urine by proton nuclear magnetic resonance spectroscopy. The authors concluded that measurement soon after birth may help identify infants at high risk for hypoxic-ischemic encephalopathy (HIE) [101]. Reinke et al characterized the NMR-derived umbilical cord serum metabolome and concluded that 4 metabolites (3-hydroxybutyrate, glycerol, O-phosphocholine, and succinate) predicted HIE severity [102]. Untargeted metabolomic LC/MS analysis on plasma of patients who were sleep deprived showed that 27 metabolites (tryptophan, serotonin, taurine, 8 acylcarnitines, 13 glycerophospholipids, and 3 sphingolipids) showed significantly increased levels compared with during sleep [103].

Neuronal and non-neuronal cells are assessed. Morphologic differences include size, number of dendrites, complexity of the dendritic tree, number and types of synapses, axonal length, and degree of axonal myelination. Differences are expressed by the chemical specificity of the neurotransmitters and by their electrical properties. Critical assessment provides knowledge of mental health and well-being, mental disorders and schizophrenia, neurological (neurodevelopmental and neurodegenerative) disorders, Alzheimer's disease and traumatic brain injury (TBI). With aging, normal changes in metabolomic profiles and signatures are seen. Expression of genes results in metabolomic signatures unique to the metabolic properties of normal and abnormal CNS metabolism and differ in patients with neurodegenerative diseases, Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, and traumatic brain injury as neuronal vulnerability and markers are diverse but specific to pathologies. Identification of the unique metabolome associated with neurologic diseases and/or injury would greatly help clinicians to understand the biochemical processes, possible treatment options, and response to treatment.

## Metabonomics and Brain Apoptosis

Morphological traits have been used to classify cell death modalities [104]. Subtypes include accidental cell death where there is immediate loss of structural integrity in a completely uncontrollable manner and regulated cell death which is initiated by a genetically encoded pathway. The former, necrosis, is a passive process resulting in cell swelling, membrane rupture, inflammation, and depends on toxicant-specific biochemical mechanisms. Apoptosis, the latter, is an active process resulting in cellular shrinkage with an intact membrane, nuclear condensation, no inflammation and is an evolutionarily conserved pathway. Programmed cell death refers to regulated cell death that occurs as part of a developmental program or to preserve physiologic adult tissue homeostasis and the terms apoptosis and programmed cell death have been used interchangeably [105]. The system defines extrinsic apoptosis to be apoptotic cell death induced by extracellular stress signals that are sensed and propagated by specific transmembrane receptors. Caspase-dependent and caspase-independent "intrinsic apoptosis" refers to apoptotic demise of cells triggered by intracellular stress. Regulated necrosis has also been shown to be important in multiple physiological and pathological settings and is dependent on specific signaling modules. Autophagic cell death indicates cell death accompanied by massive cytoplasmic vacuololization. Mitotic catastrophe refers to cell death triggered by aberrant mitosis and executed either during mitosis or in the subsequent interphase. Anoikis describes the absence of cell-to-matrix interactions resulting in cell death. Entosis is a cell death mechanism linked to cell-in-cell phenotype frequently exhibited by non-phagocytic cells in clinical tumor samples. Parthanatos is a cell death mode involving DNA damage-responsive enzymes poly (ADP-ribose) polymerases (PARPs). Pyroptosis describes a specific type of death of macrophages infected by Salmonella typhimurium. Netosis cell death subroutine is restricted to granulocytic cells, insensitive to caspase inhibition, insensitive to necrostatin, dependent on NAPDH oxidase-mediated superoxide generation, and dependent on the autophagic machinery. Difficulties in defining the various biochemical pathways resulting in specific morphologic findings of a specific type of cell death had impaired the discovery of therapies for pathology. Biochemical methods of classifying cell death subroutines has been suggested for systematic classification of cell death. However, there must be physiopathological relevance, must correlate with genetic studies, must be specific to signaling of cell death, must reflect crosstalk between the different cell death subroutines, must define programmed versus regulated versus accidental cell death, and must be specific to a particular signaling pathway. And while cellular heterogeneity is normal in the CNS - cells may be exposed to a different microenvironment, may be in a different cell-cycle stage, may be genetically different, etc. - single neuron metabolomic analysis will improve our knowledge of similarities and differences between cells, functioning of the CNS, response to stress, and improvement with treatment.

Correlation of accepted technologies to determine the presence of apoptosis with metabonomic studies has resulted in an underestimation of pathology by the former. In a mouse model of Parkinsons' disease using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), there was no evidence of apoptosis in all areas of the mesencephalic dopaminergic network by TUNEL assay, but metabolomic profiling revealed 17 metabolites which were significantly altered relative to controls and an additional 13 metabolites narrowly missing the $p<0.05$ cutoff. Variations of the metabolites resulted in five major groups based on role and mapping of a set of metabolites into metabolic pathways. The authors concluded that there was significant modulation of metabolic pathways in the brain without causing significant nigrostriatal cellular changes

with acute MPTP exposure [106]. Tsang and colleagues evaluated biochemical changes in 3-Nitroprionic acid rat model of Huntington's Disease. Comparative morphology of neurological tissues control and study groups using H&E and GFAP brain sections showed no difference and changes indicative of apoptotic and excitotoxic cells were equally abundant in both groups. However, in the study group, metabolic changes were seen in all brain regions examined by $^{1}$H NMR spectroscopy. They concluded that the metabolic profiling technique provided a more sensitive characterization of the toxicity of 3-NP than standard histopathological criteria [107]. In a rat model of focal cerebral ischemia-reperfusion, apoptosis of hippocampus nerve cells were similar in sham versus optimized rhubarb aglycone treated rats. However, metabonomic analysis on plasma and urine metabolites showed that principle component analysis scores plots were not the same as the sham operation group [108]. Further evaluation of metabonomic profiling diagnosing apoptosis of neural cells is needed to improve our understanding of the biochemical changes resulting in pathology and possible cell death and more timely interventions resulting in improved outcomes.

## Carbon Monoxide and Metabonomics

The predominant source of endogenous CO in the brain is from oxidative degradation of heme by the hemeoxygenases. Other sources include degradation of other heme proteins (myoglobin, catalase, peroxidases, and cytochromes) and lipid peroxidation. Interactions of CO with macromolecules in the brain is the result of signaling cascades which determine biologic activity (neurotransduction, transcription, vascular resistance, and metabolism). Heme proteins are the key to the generation, signal transduction and interaction of CO, nitric oxide (NO), and hydrogen sulfide ($H_2S$) neurobiology. Functions of these proteins include gas transport, transfer of electrons, facilitation of reduction-oxidation reactions that occur at catalytic sites of specific enzymes, and sensing of gases. CO modulates ATP production, glucose metabolism, energy balance, and cellular respiration [110]. Determination of biochemical mechanisms of CO have been difficult to evaluate due to its pleiotropic nature, ability to contact rapidly with functional groups of different molecules, and change of redox state of metal centers of prosthetic groups of proteins. *In vitro* analysis using purified enzymes to correlate structure of heme binding pockets with catalytic reactions have helped determine gas-sensing and gas-transduction mechanisms [110-115]. *In vivo* studies though have been limited. Mass spectrometry with quantitative metabolomics is now able to determine metabolic footprinting in animal models with deletion of specific gas-producing enzymes to determine sites of action of the gas.

Presence of the gas is required before biochemical actions result. Permeability across membranes is by simple diffusion and/or transport and detection is by gas sensors usually heme-based sensor proteins. Signaling is determined by oxidative states of the central iron of the prosthetic heme and the binding affinity of CO, ligand binding and base affinity, conformational changes within the protein arising from ligand binding and structural changes and protein functions. Heme iron is important in defining ligand discrimination. The ferrous oxidation state of hemoglobin preferentially binds CO. This ligand-binding causes positional changes of the distal histidine group and is a step in the signal-transduction mechanism of heme-protein sensors. Differential metabolomics suggest that CO upregulate metabolites in the remethylation cycle and downregulate those in the transsulfuration cycle. The hemeoxygenase (HO)/CO and cystathionine β-syntase (CBS) systems interface and CO can regulate the activity of CBS, an $H_2S$-producing enzyme. The HO/CO biochemical pathway is between the tricarboxylic acid (TCA) and methionine/thiol pathway. Changes impact remethylation, transsulfuration, methionine salvage and polyamine metabolism which alters the cells response to oxidative stress [116]. Treatment directed toward these changes would impact cellular and subsequent brain physiology.

## Relationship of Carbon Monoxide to Apoptosis

Involvement of CO in several aspects of neuron biochemistry and physiology suggests relevance to apoptosis and clinical outcomes. The hemeoxygenase/CO axis has been suggested as a therapeutic target for several neurodegenerative diseases. A product of heme degradation catalysed by hemeoxygenase, CO is considered important as a neuroprotective agent [30, 117-121]. Potential mechanisms of action are redox control, modulation of proliferation, and modulation of the immune system. Neuronal and non-neuronal CNS cells up-regulate HO-1 in response to stress [122]. Reactive oxygen species (ROS), involved in the development of neurodegenerative disorders, are affected by the end products of the hemeoxygenases. Upregulation of HO-2 or HO-1 induction correlates with increase in cerebral blood flow during seizures, hypoxia, and/or hypotension. HO-2 maintains autoregulation of cerebral blood flow and is a defense mechanism that blocks oxidant formation preventing cell death. Intrinsic production for homeostasis may be upregulated by different stresses and may have important roles in cellular antioxidant defense. In a bicuculline model of seizures in piglets, Parfenova and colleagues found that an HO inhibitor potentiated seizures whereas an HO-1 inducer blocked seizure activity [123]. Hung and colleagues injected adenovirus containing human HO-1 gene into rat substantia nigra concomitantly with 1-methyl-4-phenylpyridinium. The authors found that overexpression of HO-1 increased the survival rate of dopaminergic neurons, reduced the production of tumor necrosis factor alpha and interleukin-1 beta in substantia nigra, antagonized the reduction of striatal dopamine content induced by 1-methyl-4-phenylpyridinium, and up-regulated brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor expression in substantia nigra. The authors concluded that HO-1 induction exerts neuroprotection [124]. Doré and colleagues demonstrated increased neuronal death in cerebellar granule cultures of HO2 (-/-) mice with a selective augmentation of apoptotic death and that HO2 transfection rescued apoptotic death [125]. $P2Y_{13}$ receptor mediated activation of the Nrf-2/HO-1 axis also results in neuroprotection. The former binds antioxidant response elements and regulates transcription of detoxification genes. Nrf2 activation induces HO-1, important to cellular defense against oxidative stress [126, 127]. In human immunodeficiency virus (HIV) infection of the brain, HO-1 expression is decreased and is associated with the release of neurotoxic levels of glutamate. HO-1 induction has been suggested as a therapeutic strategy for neuroprotection against HIV infection [128]. However, up-regulation of hemeoxygenase has also been associated with apoptosis [37, 129-134]. Why this occurs is not clear. Determination of metabonomic signalling due to hemeoxygenases will improve our understanding of the different pathways associated with the hemeoxygenase/CO axis and, thus, improve our clinical treatment of the biochemical derangements seen with the neuropathologies.

## Carbon Monoxide and Nutrition

Calorie restriction and diets rich in antioxidants may delay aging and neurodegenerative diseases. Nutritional manipulation of vitagenes and, therefore, the HO/CO axis, may promote activation of cytoprotective genes and down-regulation of proinflammatory and pro-oxidative genes. Nutrition impacts intracellular NAD/NADH ratio regulating a group of proteins linked to metabolism and stress intolerance in multiple organisms. Multiple metabolic abnormalities with excessive production of reactive oxygen species (ROS) and oxidative stress can result in "protein conformational diseases" including amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and Friedreich ataxia [135]. Nutritional antioxidants have been shown to activate vitagenes which encode for HO-1 and are important for counteracting oxidative and nitrosative stress [136]. The HO-CO axis modulates the neuroendocrine mechanism of stress. Increases in CO production exerts biological effects through the activation of the cytosolic form of guanylyl cyclase (sGC) resulting in increased intracellular cGMP [137]. Activation of cyclooxygenase (COX), large-conductance $Ca^{2+}$ -activated $K^+$ ($K_{Ca}$) channels, and modulation of the p38 MAPK-signaling pathway are alternative intracellular signal transduction pathways resulting in neuroprotection [138-140]. Further study is necessary before developing recommendations for diets to prevent aging and neurodegeneration by nutritional activation of vitagenes and the HO/CO axis.

## Effect of Extrinsic Carbon Monoxide on Neuropathology

Carbon monoxide is neuroprotective in numerous small animal models of brain injury [141-145]. Safety and tolerability studies using inhaled CO have been completed in adults and are in progress in neonates. Clinical application could be as a preconditioning agent, postconditioning agent, or periconditioning agent. Inhaled CO has entered clinical trials (www.clinicaltrials.gov) and is an important therapeutic option. Although the role of CO in the brain has historically been negative, current data suggests that the drug may be an important therapy for patients with neuropathology, psychiatric diseases, and neurodegeneration. Clinical trials evaluating its role in treating patients are needed. CO, like nitric oxide before it, may prove a novel treatment approach to CNS disease and health.

## Summary

By evaluating the biochemical reactions during apoptosis in the brain, prevention of clinically defined disease may be attainable. Metabonomics is becoming more widely used in defining the presence of neuropathologies and response to treatment. Use in further refining our understanding of biochemical pathways should result in the development of better therapies and improvement in outcomes. CO therapy for neurodegenerative diseases is being evaluated and response is dependent on dosing and timing. Metabonomic profiling will provide further insight into the role of CO in CNS health and will add greatly to our ability to improve the quality of life in our patients with neurodegenerative disorders, psychiatric diseases, and neuropathologies.

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