NEUROTOXICITY OF CASSAVA CYANOGENS IN RODENTS AND NON-HUMAN PRIMATES

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DEDICATION

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LIST OF ABBREVIATIONS

AAA	All amino acid
AMPA	α-amino-3-hydroxyl-5-methyl-4-Isoxazolepropionic acid; Class of glutamate receptor mediating fast excitatory CNS transmission
CAA	Carbamoyl-amino acids
CAE	Correct arm entries
CDC	Cyanide detoxification capabilities
CN	Cyanide
CNS	Central nervous system
CROET	OHSU Center for research on occupational and environmental toxicology
HCN	Hydrogen cyanide
HPLC	High performance liquid chromatography
KCN	Potassium cyanide
MS	Mass spectrometry
MS/MS	tandem mass spectrometry
NAOCN	Sodium cyanate
NT	Navigation time
OCN	Cyanate, a neurotoxic CN metabolite that increases in SAA deficiency
PNS	Peripheral nervous system
RAM	Radial Arm Maze
RME	Reference memory errors
SAA	Sulfur amino acids
SCN	Thiocyanate, the principal human CN metabolite

WME Working memory errors

DEFINITION OF TERMS

Biomarkers: a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes (disease, infection, environmental exposure) , or pharmacologic responses to a therapeutic intervention

Carbamoylation: a nonenzymatic posttranslational protein modification mediated by cyanate a cyanogenic analog. Carbamoylation reactions alters the structure, charge and functional properties of certain proteins and have been implicated directly in the underlying mechanisms of various diseases namely neurodegenerative conditions, atherosclerosis and inflammation.

Cognition: a Set of all mental abilities and processes related to learning, memory, attention, judgment, evaluation, reasoning, problem solving, decision making and comprehension. In this study, working, long-term and reference memory were evaluated as evidence for cognition

Cyanide detoxification capability (rate): the number of milliseconds required to produce one µmol of thiocyanate per mg of protein (ms/(µmol/mg) in the tested sample.

Cyanogenesis: the ability of a plant to synthesize cyanogenic glycosides which liberate hydrogen cyanide upon hydrolysis.

Konzo: is a non progressive, permanent upper motor neuron disease which occurs in an epidemic pattern among population that staple on cassava in Sub-Saharan Africa (WHO, 1996).

Long term Memory: information that is useful in all exposures to the radial arm navigation task across all the days of testing

Neurodegeneration: term for the progressive loss of structure or function of neurons, including death of neurons. This leads to gradual deterioration of cognitive functions, e.g memory. Neurodegeneration is a key feature of several neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, konzo among others.

Neurotoxicity: alteration of normal activity of the nervous system in such a way as to cause damage to nervous tissue as a result of exposure to neurotoxins (natural or artificial toxic substances). This can eventually disrupt or kill neurons, key cells that transmit and process signals in the brain and other parts of the nervous system.

Pathogenesis: The mechanism and/or development and the chain of events leading to a disease.

Reference memory: During reference (long-term) memory assessment, only four out of the eight arms were baited. The following parameters were measured; correct entry counts into baited arms, entry counts into un-baited arms (reference memory errors (RME), re-entry counts into baited arms (WME) and RAM navigation time.

Rhodanese: is a mitochondrial enzyme that detoxifies cyanide by converting it to thiocyanate

Upper motor neuron diseases: are clinically and pathologically heterogeneous group of neurologic diseases characterized by progressive degeneration of motor neurons. Upper motor neurons originate from the primary motor cortex of the cerebrum (precentral gyrus) and possess long axons forming corticospinal and corticobulbar tracts

Working memory: information that is useful to the rat while navigating the radial arm maze (RAM). The RAM parameters that were assessed included; correct entry counts into unvisited arms, re-entry counts into visited arms (working memory errors (WME)

and RAM navigation time (total amount of time taken by a rat to complete visiting all the arms). Working and short-term memory were used interchangeably.

Abstract

Background: Cassava is staple food to over half a billion people globally. Consumption of insufficiently processed cassava and dietary sulfur amino acid (SAA) deficiency has been implicated in the pathogenesis of Konzo, a paralytic condition prevalent in Sub-Saharan countries. Cyanogenic cassava have also been associated with pervasive cognitive deficits in humans, an overlooked phenomena raising global health concerns. Probable candidate for such neurodegeneration and/or disability include cyanide, or cyanate, sulfur deficiency, or their respective combinations. The susceptibility factors and mechanisms underlying the toxicity of cyanogenic cassava have remained poorly understood partly due the lack of an experimental model.

Objectives: To investigate the neurotoxicity of cassava cyanogens in rodents and non human primates.

Methods: Young 6-8 weeks old male rats were treated intraperitoneally with either 2.5 mg/kg body weight (bw) NaCN, or 50 mg/kg bw NaOCN, or 1 µl/g bw saline, daily for up to 6 weeks and assessed for cognitive performance. Short and long-term memories were evaluated using a radial arm maze (RAM) testing paradigm.

Additionally, young adult male rats (Crl: NIH-Fox1 rnu/Fox 1+, 6-8 weeks old) were fed either a diet rich in all amino acids (AAA) or 75%-deficient in SAA and treated intraperitoneally with either 2.5 mg/kg/body weight (bw) NaCN, or 50 mg/kg/bw NaOCN, or 1µl/g/bw saline, for up to 6 weeks and studied for cyanide detoxification capabilities (CDC) and protein carbamoylation, respectively. Further, male *Macaca fascicularis* monkeys (~12 year-old) (N=12) were exclusively fed on cassava for 5 weeks. CDC was assessed in plasma, spinal cord, and brain of rodents and in plasma of monkeys. Carbamoylation of albumin and spinal cord proteins was analyzed by liquid chromatography mass spectrometry (LC-MS/MS).

Results: Behaviourally, NaOCN impaired short-term working memory with fewer correct arm entries (CAE) ($F_{2, 19} = 4.57$ $p < 0.05$), higher working memory errors (WME) ($F_{2, 19} = 5.09$, $p < 0.05$) and longer RAM navigation time (NT) ($F_{2, 19} = 3.91$, $p < 0.05$) for NaOCN compared to NaCN and saline treatments. Long-term working memory was

significantly impaired by NaCN with fewer CAE ($F_{2, 19} = 7.45$, $p < 0.01$) and increased WME ($F_{2, 19} = 9.35$ $p < 0.05$) in NaCN relative to NaOCN or vehicle treated animals. Reference memory was not affected by either cyanide or cyanate. Further, NaCN induced acute seizures whereas NaOCN induced limb paralysis under SAA-restricted diet. No deficits were observed in non-human primates. Under normal diet, the CDC were up to ~ 80X faster in the nervous system (14 milliseconds to produce one µmol of thiocyanate from the detoxification of cyanide) relative to plasma. The spinal cord CDC was impaired by NaCN, NaOCN, or SAA-deficient diet. In non-human primates, the plasma CDC changed proportionally to total proteins ($r=0.43$; $p<0.001$). The plasma CDC was ~ twice faster compared to the rodent capabilities. Metabolically, there was a time-dependent decrease in BUN/creatinine ratio under the cassava diet ($p<0.001$). Additionally, high levels of carbamoylation relative to NaCN and vehicle ($P<0.001$). At Day 14, we found a diet-treatment interaction effect on albumin carbamoylation ($p=0.07$) at day 14 while there was no effect attributed to diet ($p=0.71$) at day 28. The mean number of NaCN-associated carbamoylated sites on albumin became 47.4% significantly higher relative to vehicle (95% CI:16.7-86.4%) at day 28. Spinal cord proteins were only carbamoylated by NaOCN prominently under the SAA-restricted diet. Differentially carbamoylated proteins included myelin basic protein, myelin proteolipid protein, neurofilament light polypeptide, glial fibrillary acidic protein, and 2', 3' cyclic-nucleotide 3'-phosphodiesterase.

Conclusion: The findings provide an experimental evidence for the biological plausibility that cassava cyanogens may induce cognition deficits. The deficits may result from dual toxicity effect of cyanide and cyanate reflecting their differential toxicity machanisms. The nervous system susceptibility to food (cassava) cyanogenesis and neurotoxic insults seen in konzo subjects may result from a “multiple hit” process including cyanide, cyanate toxicity, deficiency in sulfane sulfur, and cyanate-induced carbamoylation. The multiple hit processes may combine direct mitochondrial insults, protein carbamoylation and a thiol-redox derangement. This level of pathogenetic complexity should be considered in biomarker studies and efforts to prevent neurotoxicity effects of cassava.

CHAPTER ONE: INTRODUCTION

1.1. Background

This doctoral research thesis describes functional (behavioural), biochemical (enzymatic), proteomic (molecular) studies aimed at elucidating the neurotoxicity behaviour of cassava and cyanogens compound, which may be relevant to the biomarkers and pathogenesis of Konzo, a paralytic condition prevalent in Sub-Saharan countries. Cassava cyanogenic neurotoxins namely sodium cyanide (NaCN) and sodium cyanate (NaOCN) in normal and sulphur amino acid (SAA) deficient diets were used to persue the broad objective of investigating the neurotoxicity of cassava cyanogens in rodents and non human primates. To achieve the aforesaid, the following specific objectives were undertaken; (i) to elucidate the differential patterns of neurological deficits associated with cyanide and cyanate intoxication, (ii) to elucidate the impact of protein-restricted diet on cyanide and cyanate toxicity in rodents and non-human primates, (iii) to identify molecular targets of cyanide or cyanate toxicity under SAA-deficient diet in rats.This experimental work combined physical observation, motor (rotarod) and cognitive (radial arm maze) assessments, biochemical (cyanide detoxification capabilities) and the state-of-the-art proteomic (molecular) target determination in rodents and non-human primates to unveil neurotoxicity mechanisms of cassava cyanogens and their role in konzo.

The prevalence of neurological diseases poses a major social, economic and health burden to the world population. In Africa, particularly sub-Saharan Africa, neurodegenerative diseases are a cause of significant morbidity and suffering. The

interaction between environmental, genetic, and socioeconomic factors (Cock, 1985; Rosling and Tylleskar, 1995) may play a major role in the initiation and sustanence of epidemic and non-epidemic neurodegenerative diseases in Africa. Such phenomena have been witnessed following man-driven or natural calamities such as human conflict, political unrest, wars, famine and drought. In this regard, some outbreak of a neurological disease (Beriberi) in Ivory Coast was preceded by political upheaval (MSF, 2011).

1.1.1. Definition of concepts

Konzo is a non progressive, permanent upper motor neuron disease which occurs in an epidemic pattern among population that staple on cassava in sub-saharan Africa (WHO, 1996). There has been an epidemiological association between consumption of insufficiently processed cassava, sulphur amino acid (SAA), (cysteine and/or methionine)-deficiency and neurodegeneration including konzo (Howlett et al, 1990; Tylleskär et al, 1991; Banea-Mayambu et al, 1997; Cliff et al, 1997). The disease affects mainly children and women of child bearing age (Howlett, 1990; Tylleskar et al 1994; WHO, 1996).

Healthy persons are affected with no prodromal phase or triggering illnesses. The onset is characterized by an abrupt muscle weakness and trembling (spasticity) of the legs, during the first days of the illness. The descriptive history is of a healthy person who develops the disease following movents of physical exertion e.g. walking a long distance while coming from the river to collect water for household use. The symptoms

of konzo include heaviness, trembling or weakness of the legs associated with difficulty or inability to walk (Tylleskar et al 1992, Manea-mayambu et al 1997). Additionally, the patient may complain of weakness in the arms or hands, difficulty in articulating speech and blurring of vision. Sensory symptoms of radicular lower back and paraesthesia in the legs may be present but clear in the first weeks or months, however the patient do not have incontinence. The disease may cause significant disability resulting in the use of aids to carry out physiological functions such as walking with a stick in moderate and bed ridden in severe form of konzo (WHO, 1996, Tshala-Katumbay 2001).

Other neurological disorders notably tropical ataxic neuropathy (TAN), human T-lymphotropic virus type I (HTLV-I) and lathyrism are closely associated with konzo. Tropical ataxic neuropathy has been associated with chronic cyanogenic cassava dietary dependency. The disease is widely seen in the tropics including sub-Saharan Africa notably parts of West, North, East, Central and South Africa (Tylleskar et al, 1991, Osuntokun et al 1969). TAN have also been reported in Caribbean countries in South America and South East Asia (Osuntokun, 1968).

TAN is a syndrome presenting with severe burning pain of the feet sole, dimness of vision, ataxia and increased pigmentation of the skin (Osuntokun, 1968, Osuntokun, 1969, Tor-Agbidye et al, 1998).TAN starts with paraesthesia and dysaesthesia in the distal parts of the lower limbs. Then the blurring or loss of vision sets in, followed by ataxia of gait, tinnitus, deafness, weakness and atrophy of leg muscles. As the disease progresses, defective perception of sensory modalities (often confined to the lower

limbs), bilateral optic atrophy, ataxic gait, and impaired muscular coordination, bilateral perceptive deafness, weakness and wasting of the muscles (usually of the lower limbs) may be common. Symmetrical hyperreflexia of the upper limbs, symmetrical spastic paraparesis, spastic dysarthria, diminished visual acuity, peripheral neuropathy, cerebellar signs, and deafness are among other clinical findings (Osuntokun, 1968).

Human T-lymphotropic virus type I-II (HTLV-I-II) and human immunodeficiency viruses-1-2 (HIV-1-2) have been implicated with endemic/epidemic neurological motor disorders including the typical HAM (HTLV-I Associated Myelopathy) (Bhigjee et al, 1991, Gessain et al, 1986, Goubau et al, 1990). However, studies on konzo have failed to demonstrate the presence of antibodies against these viruses in blood specimens of affected subjects (Carton et al, 1986, Tylleskar et al, 1996).

Lathyrism is a grass pea-induced spastic paraparesis disease found in Ethiopia and Asia. Clinical and neurophysiological studies of human lathyrism and konzo show the diseases to be remarkably similar, with sub-acute onset, association with heavy exercise, and evidence of permanent pyramidal tract dysfunction associated with cortimotorneuronal or corticospinal tract degeneration (Hugon et al 1988)

The similarities between cassava and *Lathyrus sativus* (grass pea) include; both plants tolerate drought and are consumable products that harbor chemical products with potential toxicity, both plants are grown in the tropics and semi-tropics, including sub-Saharan Africa, where very poor populations mainly rely on them as a major source of

food. Under these circumstances, both food products trigger upper motor neuron disease of rapid-onset and poorly reversible spastic paraparesis. The toxic components of these plants differ; grass pea contains amino acid with neuroexcitatory properties known as beta-N-oxalylamino-L-alanine (BOAA) while cassava contains cyanogenic glucosides (linamarin) which is metabolized into thiocyanate (SCN) (Spencer et al 1999). Both SCN and BOAA interfere with fast excitatory synaptic neurotransmission in the mammalian brain. The neurotoxic mechanisms of grass pea (neurolathyrism) and cassava (konzo) may therefore overlap (Bradbury and Lambain 2011).

1.1.2. Epidemiology of Konzo

Epidemiological findings mainly from konzo prevalent areas namely Central Africa Republic, Democratic Republic of Congo, Tanzania, Cameroon, and Mozambique point to a strong association between dietary dependence on cassava, SAA deficiency and konzo (Essers et al 1992, Tylleskar et al 1991). Additionally, the etiological role of SAA-deficient diet and cyanogen intoxication from insufficiently processed cassava has received a broad consensus among scientists and epidemiologists on the pathogenesis of konzo. Indeed, konzo has exclusively been reported in cassava growing and consuming areas (Casadei et al 1990, Cliff et al 1985, Essers et al 1992, Tylleskar et al 1991, Boivin et al 2013).

The prevalence rates for konzo fluctuate depending on the studies, however, it ranges between 1-30 per 1000 (Tylleskar et al 1992). The total number of confirmed konzo cases in reported studies exceeds 4000 (Tylleskar et al 1992, Bradbury and Lambain

2011). The age and sex distribution of konzo show a distinct pattern with women and children bearing the heaviest burden and the highest risk. In addition, no case of konzo has been reported from nearby urban populations (Banea-Mayambu et al 997). There appears to be an association between civil strive, natural calamities with the disease. In this regard, thousands of konzo cases have been reported in DRC especially during outbreaks in some provinces notably, Bandundu and other parts of the country (Tshala-Katumbay 2001, Boivin et al 2013). It is also noteworthy to mention that the konzo affected population constitutes only a fraction of the total of over 500 million cassava consuming population in the tropics.

1.1.2.1. Cassava Use in East Africa

Cassava was introduced to East Africa and Lake Tanganyika regions through the islands of Reunion, Madagascar and Zanzibar, cassava spread to (Nartey 1978; Brough 1991). Since then cassava have been widely grown in Kenya, Tanzania, and Uganda. In Kenya, the major cassava producing areas are in Western, Nyanza, Coast and Eastern (Machakos, makueni) provinces. The coastal areas where cassava is grown include kwale, malindi, Kilifi and Lamu. Whereas in western and Nyanza provinces, Kuria, Migori, Suba districts and Lake Victoria Basin grow cassava substantially (Mugwera et al 2012).

1.1.2.2. Konzo in East Africa

Konzo has been attributed to high dietary cyanogenic exposure from insufficiently processed bitter cassava. The disease has been reported mainly from remote areas of the Great Lakes region (Mozambique, Tanzania, Cameroon, Angola, the Central Africa Republic and Democratic Republic of Congo) (Trolli 1938, Cliff et al 1985, Howlett et al 1990; Banea et al 2000, Tylleskar et al 1992, Boivin et al 2013) figure (Figure 2.4.6-2). Konzo has only been reported in cassava growing and consuming rural areas, while in the urban areas no cases have been reported. It is noteworthy to mention the affected population constitutes only a fraction of the total of over 500 million cassava consuming population in the tropics.

There have been outbreaks of konzo following consumption of high cyanogenic bitter cassava during drought when a person adopts short cuts in processing in Tanzania (Mlingi et al 2011), whereas there have been reports of outbreaks in Uganda (Tylleskar et al 1992). In Kenya it appears no documented cases of konzo, this may be because of misdiagnosis and under reporting since it is a rare case in the country. Additionally, conditions that favour konzo namely dependence on bitter cassava, SAA deficiency, drought, war and conflict are not prevalent in areas with good health surveillance system. Comprehensive research need to be undertaken in the main cassava consuming and growing areas in this field of interest.

1.1.3. On the biomarkers and mechanism of konzo

The mechanisms of cassava-associated neurodegeneration (**k**onzo) have remained unclear. Active research focuses on toxicity, biomarkers of cassava cyanogenesis exposure, and detoxification pathways (Dawson et al 1995, Isom et al 1975) to develop point of care diagnostic and/or therapeutic tools (antidotes) (Marziaz et al 2013, Zottola et al 2009). Current knowledge suggests that cyanide detoxification pathways utilize the enzymes rhodanese (thiosulfate sulfurtransferase, TST; EC: 2.8.1.1), and mercaptopyruvate sulfur transferase (MPST, EC 2.8.1.2) to a lesser extent, in metabolic conversions of cyanide. The TST- or MPST-mediated pathways use sulfur to convert cyanide into the reportedly less toxic thiocyanate (Aminlari et al 1989, Kassa et al 2011, Nagahara et al 1995). The rate of cyanide metabolic conversion appears to be dependent on the bioavailability of sulfur amino acids notably cystein and methionine (SAA) suggesting that SAA and/or protein-deficient diet may increase the individual susceptibility to cyanide-associated diseases (Banea-Mayambu et al 1997, Kassa et al 2011, Tor-Agbidye et al 1999, Tylleskar et al 1995).

Putative candidate for such neurodisability include cyanide, or cyanate, sulfur deficiency, or their respective combinations (Kassa et al. 2011). To date, active research on konzo focuses on biomarkers of cassava cyanogenic exposure and potential pathogenetic mechanisms of related diseases. Limited progress has been made partially due to the lack of an experimental model of cassava neurotoxicity. This study focused on the biomarkers and mechanisms underlying cassava neurotoxicity.

1. 2. Rationale and Significance

1.2.1. Rationale

Experimental modelling of konzo need to utilize the main cassava cyanogenic compound namely linamarin, which is metabolized into cyanohydrins, hydrogen cyanide (HCN), thiocyanate (SCN), cyanate (OCN) and 2- aminothiazoline-4-carboxylic acid (ATCA) as its metabolites (Rosling 1994; Essars et al. 1992; Carlsson et al. 1995). However, conducting experiments using linamarin as test article is extremely expensive to sustain long- term experimentation. Due to the cost implications, we chose to use its analogs notably cyanide and its cyanate metabolite to conduct experimental studies that could possibly provide insights into the biomarkers and mechanism of konzo. The experimental paradigm utilized all amino acid (AAA) and SAA-restricted diets which were isonitrogenous and isocaloric. Under SAA deficiency diet, the product of OCN is increased (Swenne et al, 1996; Tor-Agbidye et al, 1999), therefore we used NaOCN to hypothetically mirror scenarios in human populations prone to konzo.

The study on serum biomarkers included OCN carbamoylation. This is because serum cyanate concentration may be a poor predictor of neurological impairment due to its rapid clearance (Diederich et al 1971) while accumulation of serum carbamoylated proteins provide a more accurate assessment of chronic exposure to cyanate (Crist et al, 1973). We suggest that levels of carbamoylated albumin may serve as predictor of neurological risk associated with cassava cyanogenic intoxications in populations prone to konzo.

We focused on enzymes because of their principal role in the detoxification mechanism of cyanide. We hypothesized that under SAA-deficiency, cyanide detoxification capabilities may be impaired and therefore the susceptibility of CN intoxication may increase. Additionally, the focus on the nervous system was based on the premise that konzo is mainly a neurological disease. We also explored the impact of cyanogenic compounds on cognition because recent findings have suggested that konzo may be associated with cognitive deficits (Boivin et al, 2013).

1.2.2 Significance

The main goal was to identify biomarkers relevant to cassava neurotoxicity and explore their potential relationship to the development of konzo. The significance of this work is; first, cassava is a staple and crop of subsistence for over 500 million people around the globe. It ranks fourth on the list of major food crops in developing countries after rice, wheat and maize. Because of environmental dynamics notably global warming and subsequent changes in climatic conditions, drought and flood-tolerant plants such cassava becomes valuable crop for ensuring food security at community levels. The health impact of this critically important but potentially neurotoxic food plant needed therefore be understood. Second, studies on the neurotoxic effects of cassava cyanogen analogs are relevant to occupational and environmental health. Cyanogen-related environmental and/or occupational health risks may arise from pollution and/or direct exposure resulting from human activities such as gold mining (cyanide), painting (cyanate), and military control (hydrogen cyanide, diphenyl arsine cyanide). Third, the study provide insights into which ingestion of cyanogenic compounds/cyanogenic

cassava may lead to cognitve impairment. In sum, our studies offer insights into biomarkers of cyanogenic exposure and mechanisms of cyanogenic related neurological deficits across the aforementioned fields of interest.

1.2.3 Hypothesis and objectives

It has been suggested that ingestion of poorly processed bitter cassava (the staple for over 500 millions around the globe), in combination with poor SAA dietary intake, are associated with the occurrence of konzo (Tylleskar et al 1991; Howlett et al 1990; Cliff et al 1985; Mlingi et al 1991). This hypothesis has not, however, been tested. Under normal conditions, ingestion of cassava cyanogenic glucosides (Rosling, 1994; Essars et al 1992; Carlsson et al 1995) leads to the production of other cyanogenic compounds such as acetone cyanohydrin, hydrogen cyanide (HCN), thiocyanate (SCN), trace amounts of cyanate (OCN) and 2-iminothiazolidine carboxylic acid (ATCA). Cyanide is converted into SCN, the main metabolite of HCN, through a SAA-dependent enzymatic (rhodanese *aka* thiosulfate sulphur transferase) pathway. Under conditions of SAA deficiency, oxidative detoxification pathways are favoured and there is increased production of cyanate (Swenne et al 1996, Tor-Agbidye et al 1999).

The identity of the cassava neurotoxic agent(s) that trigger motor-system degeneration remains unknown. Whereas acetone cyanohydrin is neurotoxic to rodent brain (predominantly thalamic), the reported neuropathological findings are not consistent with the marked corticospinal dysfunction observed in konzo (Tshala-Katumbay et al 2013; Soler-Martin et al 2010). Other potential culpable agents include CN (improbable),

SCN (conceivable), ATCA (unlikely), and OCN (likely) (Spencer 1999). SCN is a conceivable candidate because of its chaotropic properties and has the potential to exacerbate glutamatergic neurotransmission thus leading to excitotoxicity (Spencer, 1999). SCN increases glutamate binding to AMPA receptor and potentiates AMPA-mediated responses (Cha et al, 1992; Hall et al, 1993). AMPA receptors are selectively activated by beta-N-Oxalyl amino-L-alanine (BOAA) the culpable agent in lathyrism which is similar to konzo, present with an abrupt onset of a central motor system disease in form of spastic paraparesis (Spencer et al, 1986). OCN is an attractive toxic candidate for the pathogenesis of konzo because repeated treatment with its sodium salt induces a motor-system disease in humans, primates and rodents (Ohnishi et al, 1975; Tellez-Nagel et al, 1977; Tellez et al, 1979). NaOCN induces protein-carbamoylation, a modification that can lead to protein loss of function possibly relevant to the pathogenesis of neurodegenerative diseases (Cocco et al, 1982; Nagendra et al, 1997; Kraus and Kraus, 2001).

We tested the hypothesis that neurological deficits associated with cyanogenic exposure under conditions of SAA deficiency, are mediated by decrease in rhodanese activity and increased cyanate production with subsequent neuroprotein carbamoylation, motor and cognitve deficits. To test the hypothesis, we addressed the following broad and specific objectives:

1.2.3.1. Broad objective

To investigate the neurotoxicity of cassava cyanogens in rodents and non human primates

1.2.3.1.1. Specific objective 1

To elucidate the differential patterns of neurological deficits associated with cyanide and cyanate intoxication

1.2.3.1.2.Specific objective 2

To elucidate the impact of protein-restricted diet on cyanide and cyanate toxicity in rodents and non-human primates.

1.2.3.1.3. Specific objective 3

To identify molecular targets of cyanide or cyanate toxicity under SAA-deficient diet in rats

1.2.4 Research strategy

The experimental paradigm combining animal observation, motor assessment (rotarod), cognitive performance assessment (Radial arm maze) (RAM), state-of-the-art proteomic tools (mass-spectrometry based proteomics notably liquid chromatography-tandem mass spectrometry (LC/MS-MS) to elucidate neuro-cognitive deficits, impact of protein-restricted diet and the molecular targets of either cyanide or cyanate was used.

1.2.5 Premise of the studies

The design of the studies (selection of animal strain, doses and extent of dietary restrictions) were based on the foundation of four published experiments. First, is the demonstration by Mathangi and colleagues that prolonged feeding of a cassava-rich diet (75% cassava, 25% chow) to young male rats induces a slowly developing motor-system disease that is first detectable on the rotarod test after 8 months (Mathangi et al, 1999). Second, is the demonstration that animals fed on a diet deficient in SAA (methionine, cysteine) progressively excreted greater concentrations of cyanate (OCN) when challenged with potassium cyanide (KCN) (Tor-Agbidye et al, 1999). Third, is the demonstration that young adult rats treated with linamarin or NaOCN, under SAA-deficient diet, developed hind limb tremors (linamarin) or severe motor weakness (NaOCN), with differential serum protein-carbamoylation detected in the animals (Kassa et al, 2011). Fourth, is the demonstration that memory impairment had been reported as a delayed effect in individuals who survived cyanide poisoning (Chin and Calderon 2000). We used NaCN and NaOCN as cyanogen analogs of the main metabolite of cassava glucoside i.e linamarin and SAA-dietary restriction to develop a practical model for our experimentation goals. These studies utilized male rodents and non-human primates. Although, the disease konzo mainly affects children and women of reproductive age, we used adult animals because of concern that hormonal cycles decrease the homogeneity of study populations and confound effects of experimental manipulations (Wizemann and Pardue, 2001). This of course has turned out to be an assumption and scientist are urged to include both sexes because respond differently.

Additionally, many donors nowadays fund researches that include both males and females (Beery and Zucker 2011), a lesson learnt.

CHAPTER TWO: LITERATURE REVIEW

2.1 Cassava production and distribution in Africa

Cassava was first brought to Africa by Portuguese in the form of flour or “farinha”. The flour was used for food provision in ships plying between Africa, Europe and South America (Brazil). The cassava production and preparation techniques had been acquired from Tupinamba Indians of eastern Brazil (Jones, 1959). In Africa, it is believed that cassava was originally introduced in the Gulf of Benin around 1562 and along the Congo River in 1611 and thereafter, it spread to the west coast of Africa (Nartey, 1978; Carter et al, 1997). The subsequent introduction of cassava into the islands of Reunion, Madagascar and Zanzibar facilitated its spread to East Africa and the whole of Lake Tanganyika region (Nartey, 1978; Brough, 1991). Currently, cassava is widely grown in East, South, Central and West of Africa. Cassava is nutritionally and economically important crop in Africa, with Nigeria the most populous country in the continent producing the largest quantities of cassava roots in the world (FAO/IFAD, 2000; FAO, 2001).

Cassava is drought-tolerant tropical shrub that is cultivated for its carbohydrate rich roots as well as leaves for some limited source of proteins. Cassava (*Manihot esculenta* Crantz) is the English name given to the manioc plant, a hardy perennial shrub belonging to the family of *Euphorbiaceae.* The plant grows to a height of between 1-3 m depending on the environmental conditions (Brough, 1991). There are several varieties of cassava which are classified into sweet or bitter cultivars. The classification, however, is not based on any morphological or other taxonomic characteristics (Nweke and

Bokanga, 1994). Botanically, cassava is classified under the genus Manihot, which include over 200 species, of which *Manihot esculenta* is the most important nutritionally and economically (Nartey, 1978). Cassava stems have single or multiple branches of various colours such as grey, green or brown, with large green leaves (**Figure 2.1-1**).

Figure 2.1-1. Cassava plant (*Manihot esculenta* Crantz). The leaves are used to make side dishes that are consumed with fufu (a cassava-derived stiff porridge).

The cassava roots (**Figure 2.1-2**) are consumed as food by over 500 million people in the tropics and subtropics (Africa, Asia and Latin America), majority of whom live in Africa. The leaves are an important source of proteins consumed as a vegetable (Nartey, 1978). Cassava ranks fourth on the list of major food crops in developing countries trailing rice, wheat and maize (FAO, 1990). It is an important food source in Africa with its productivity in terms of calories per unit land area significantly higher than other staple food crops (Koch et al, 1994; Scott et al, 2000, Bradbury and Lambain 2011). The cassava cultivars are produced for food to make fufu (thick porridge), animal feeds and/or industrial uses (starch for fabrics) and therefore will continue to be relevant for domestic as well as commercial use.

Figure 2.1-2. Harvested and unprocessed cassava roots used to make cassava flour. A single root may contain levels of cyanogenic glucosides that are lethal to a whole family.

Cassava is extensively distributed in the whole of Africa probably due to its favourable characteristics. The characteristics include the ability to withstand drought, locust attack, poor soil, weeds and relatively low cost of production. These characteristics, together with its flexibility in-terms of timings of planting and harvesting, make it useful during periods of famine, war and therefore, very important for food security (Nartey, 1978). Geographically, cassava is grown on a wide scale between latitudes 30^{o} north and south, an ecological zone referred to as “cassava belt”, which coincides with many of the less developed countries where cassava has adapted to the prevailing conditions (Nartey, 1978).

2.2 Cassava and food security systems in Africa

The growing of cassava is widespread in tropical and subtropical regions (Africa, Asia and Latin America) for its carbohydrate rich tuberous roots and leaves for proteins as a

source of food and food security in adverse periods. It has major dietary importance, because it yields higher calories per unit land area relative to other staple food crops (Koch et al, 1994; Scott et al, 2000). It can withstand harsh climatic conditions, pestilence and therefore, appears to be an excellent crop for food security during famine, drought and war. The crop is less costly in terms of labour requirement, and has a flexible schedule in terms of when to plant or harvest. The viability span of the roots in the ground is long and the roots can stay for a lengthy period of time, up to 3 years or more after the formation of the edible roots is complete without harvesting (Koch et al, 1994; Scott et al, 2000). The environmental dynamics, especially global warming and subsequent changes in climatic conditions make drought and flood-tolerant plants such as cassava valuable crops for ensuring food security. The global warming phenomenon means that crops such as maize, rice and wheat which mainly rely on rain water will no longer be sustainable in sub-Saharan Africa and therefore populations may be forced to rely more on cassava, which is more resistance to harsh climatic conditions.

Cassava is an important food source especially in Sub-Saharan Africa. In the Democratic Republic of Congo (DRC) for example, cassava is a major food crop. Congolese are among the highest per capita consumers of cassava in the world (FAO, 1990). Approximately 60% of the total daily energy intake is from this crop, while the leaves contribute for about 20% of the protein intake. Cassava farming is not only a subsistence activity but also a commercial venture. It is an important source of cash income for poor as well as the large scale farmers (Nweke and Bokanga, 1994).

Economically, at the time when the prices of oil are soaring and global reserves are diminishing, the focus will turn to bio-fuels which can be obtained from ethanol from fermented cassava starch. This may help reduce bills on fuel and levels of pollution. In addition, cassava may be used as livestock fodder, which offers an interesting economic potential for Africa.

The majority of people who rely on cassava are very poor, live in the drought prone rural areas, have little or no formal education (Bradbury and Lambein, 2011), and are ravaged by war and diseases. Drought, war, diseases and other causes of food insecurity makes families and communities to shift to drought tolerant and low labour cost crops such as cassava. However, cassava has its down side because of potential intoxication from its cyanogenic glycosides linamarin and lotaustralin. During drought the concentration of cyanogens (linamarin, lotaustralin) can increase more than two-fold. Drought and war can trigger epidemics of konzo because they reduce the post-harvest processing period of cassava roots required to ensure safe consumption. They also reduce the number of different food items available in the diet precipitating protein malnutrition. Because of food insufficiency, cassava consuming communities do not adhere to rigorous post harvest processing procedure, resulting in inadequate cyanogen detoxification. Children over 2 years and women in reproductive age are more susceptible to the toxicity of these afore-mentioned cyanogens (Bradbury and Lambein, 2011).

2.3 Cassava cyanogenesis and threats to human health

2.3.1 Cassava cyanogenesis

Cyanogenesis is the ability of a plant to synthesize cyanogenic glycosides which liberate hydrogen cyanide upon hydrolysis (Conn, 1969). Cassava cultivars are classified as either “bitter” or “sweet”, the former generally containing a higher content of the toxic cyanogens linamarin and lotaustralin in the ratio of 93:7, respectively, (Bokanga, 1993) (**Figure 2.3.1-1**). Cyanogenic glucosides are part of a chemical defense system against predators. The levels of cyanogenic glucosides depends on environmental conditions, including season, soil fertility and moisture, age of the plant and genetic factors (Sundaresan, 1987; Yeoh and Oh, 1979; Mahungu, 1994; Conn, 1969; Dixon et al., 1994). The glucosides are stored in cell vacuoles while a cyanogen-cleavage enzyme (β-glucosidase, synonymous to linamarase) exists in the cell wall (Du et al., 1995; Joachim and Panditteseker, 1991; Mkpong et al., 1990).

The interaction between cyanogenic glycosides and linamarase following disruption of physical integrity of the root tissue leads to hydrolysis, as in root processing during food preparation, resulting to the formation of glucose and cyanohydrins. Higher pH (>5) makes the cyanohydrins to spontaneously break down into ketones and hydrogen cyanide (HCN) gas, which evaporates. Lower pH, on the other hand, leads to persistence of cyanohydrins in the finished food product, with the resultant HCN release by bacterial enzymatic cleavage in the gut of the consumer (Obrien et al, 1992).

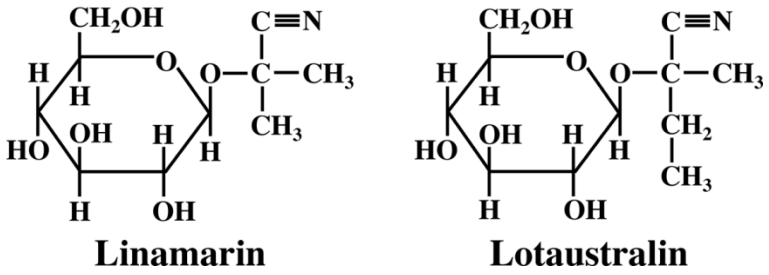


Figure 2.3.1-1. Chemical structure of cassava cyanogenic glucosidesLotaustralin has one more methyl group on the cyanogenic side chain. The concentration of linamarin is approximately10 times more than lotaustralin.

Linamarin is present in large amounts in the leaves (Bokanga, 1994) and the peel of cassava roots (900-2000 mg HCN equivalents/kg fresh weight equivalent to parts per million have been reported) (Nambisan and Sundaresan, 1994). The interior of the roots (parenchyma) have been reported to have 1-1550 ppm of linamarin (Cardoso et al, 2004). The parenchymas of some varieties of cassava contains small amount of linamarin and are referred as to sweet cassava, but majority of varieties have large amounts of linamarin which has bitter taste (King and Bradbury, 1995) and these are referred to as bitter cassava.

Bitter as well as sweet cassava need to be processed before consumption. There are many processing methods that have been developed to remove cyanogenic glucosides and their degradation products from cassava food products. These methods include; soaking or grating followed by drying or heating (Bokanga, 1995; Nweke, 1994; Oke, 1994). The cassava processing is time consuming. The time necessary for proper processing may not be available because of factors such as food shortages induced for example by armed conflict or drought (Cliff et al., 1997; Tyleskar et al., 1994; Benea et al., 1997). In some circumstances, a minor change in sequence or short-cut in one of

the different processing steps can lead up to 100-fold build up in the level of cyanogenic compounds in the final food product (Rosling and Tylleskar, 1999). Cyanogenic exposure arises in populations where adherence to established effective processing techniques is no longer possible.

2.3.2 Human metabolism of cassava cyanogens

In normal nutritional state, ingestion of cassava cyanogenic compounds leads to the production of “cyanogens” which include acetone cyanohydrins and hydrogen cyanide (HCN), thiocyanate (SCN), trace amounts of cyanate (OCN), and 2-iminothiazolidine carboxylic acid (ATC) (**Figure 2.3.2-1**) (Rosling, 1994; Essars et al, 1992; Carlsson et al 1995). Cyanide (CN) is metabolized to thiocyanate (SCN) by a rhodanese-regulated pathway that is reliant on dietary intake of SAA methionine and cystein (Spencer, 1999). Under SAA deficiency, protein catabolism maintains the sulfane sulfur required for the thiocyanate pathway and there is increased production of cyanate which may irreversibly carbamoylate proteins, inducing changes in their structure and function (Kraus and Krauss, 1998; Kuckel et al., 1993; Swenne et al., 1996; Kassa et al 2011).

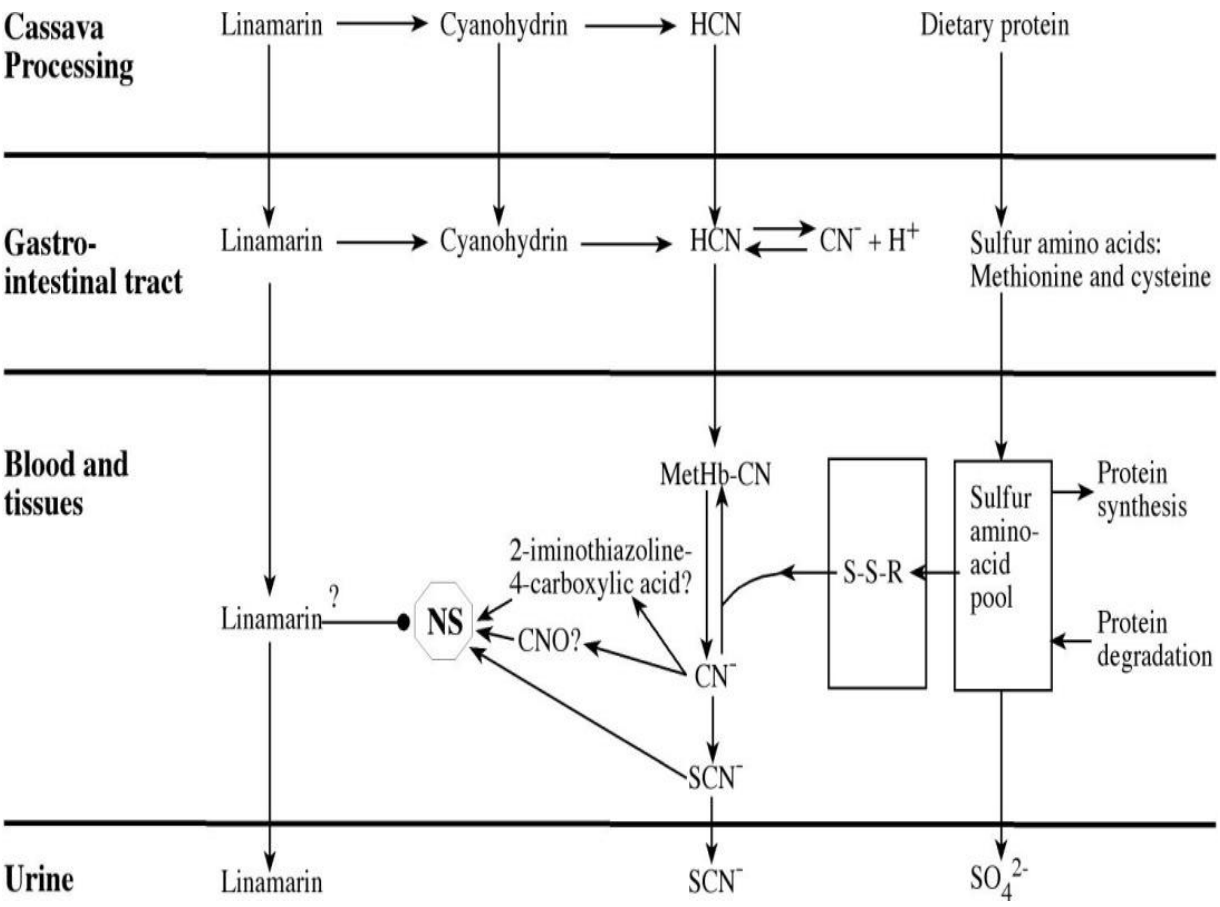


Figure 2.3.2-1. Pathways illustrating the metabolism of cassava cyanogens in the human body. Thiocyanate (SCN) is the major detoxification metabolite of cyanide (CN) and it is presumed to cause non neurotoxicity. However, cyanate (CNO) and 2-iminothiazoline-4-carboxylic acid (ATC) are neurotoxic, with the former capable of inducing a motor system disease while ATC induces seizures in experimental animals (Adapted from Tshala-Katumbay, 2001).

The ingestion of even a single cassava root may yield enough hydrogen cyanide with the risk of fatalities within an hour of consumption (Diasolua Ngudi et al 2002). Sub-acute to chronic dependency on incompletely detoxified cassava leads to outbreaks of acute spastic paraparesis (konzo). Konzo is an upper motor neuron disease reported only from poor rural communities in Africa (Rosling et al, 1988, Rosling and Tylleskar, 1995). Konzo is a yaka (DRC tribe in the province of Bandundu) word meaning ‘tied legs,’ a typical description of the resulting spastic gait. Epidemiological studies have demonstrated an association between consumption of poorly processed cyanogen-

containing cassava, a low intake of SAA needed to detoxify cyanide, and the development of konzo that mostly affects children and women of child-bearing age (Tylleskar et al., 1991; Banea et al., 2000; Howlett et al., 1990; Cliff et al., 1985; Mlingi et al., 1993).

2.3.3 Cyanide and rhodanese enzymatic activity

Living organisms have evolved innate mechanisms for detoxifying cyanogens. The defense mechanisms against cyanide in humans are two-fold (Way, 1984). First, when cyanide enters the blood stream from the lungs or the gastrointestinal tract, it is trapped by the methemoglobin fraction in the red blood cells. The fraction constitutes approximately 1% of all hemoglobin, and it can reversibly bind about 10 mg of HCN as cyanomethemoglobin (Schultz, 1984). Second is the mechanism catalyzed by the enzyme rhodanese (thiosulfate sulfur transferase (TST) EC: 2.8.1.1) (Sorbo, 1953). Rhodanese is a mitochondrial enzyme that is widely distributed in both prokaryotes and eukaryotes (Jarabak and Westley, 1978; Nagahara et al., 1998). Rhodanese has been detected in many tissues of various animals (Aminlari and Shahbazi, 1994; Aminlari et al., 1994, 2002; Al-Qarawi et al., 2001; Nazifi et al., 2003), and its distribution pattern appears to be highly species- and tissue-specific (Nazifi et al., 2003). For example, the liver and kidneys exhibit high TST activity in rats (Nagahara et al., 1999). The enzyme catalyses the transfer of a sulfur atom from thiosulfate to sulfur acceptors like cyanide and thiol compounds to produce less toxic molecules, thiocyanate and persulfides, respectively (Schlesinger and Westley, 1974) (**Figure 2.3.3-1, Figure 2.3.3-2**). Similarly, a homologous enzyme, the mercaptopyruvate sulfurtransferase (MPST; EC:

2.8.1.2) catalyses the same type of transsulfuration reaction using 3-mercaptopyruvate as the sulfur donor (Sorbo, 1957). The rate limiting factor for rhodanese activity is the availability of SAA substrate (Lundquist, 1992).

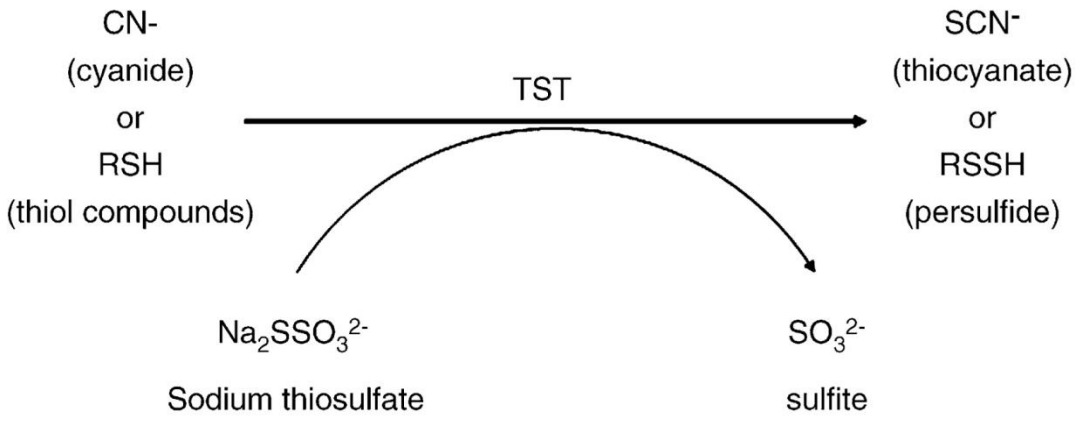


Figure 2.3.3-1. Transsulfuration reaction catalyzed by rhodanese (TST) enzyme adapted from Billaut-Laden et al, 2005.

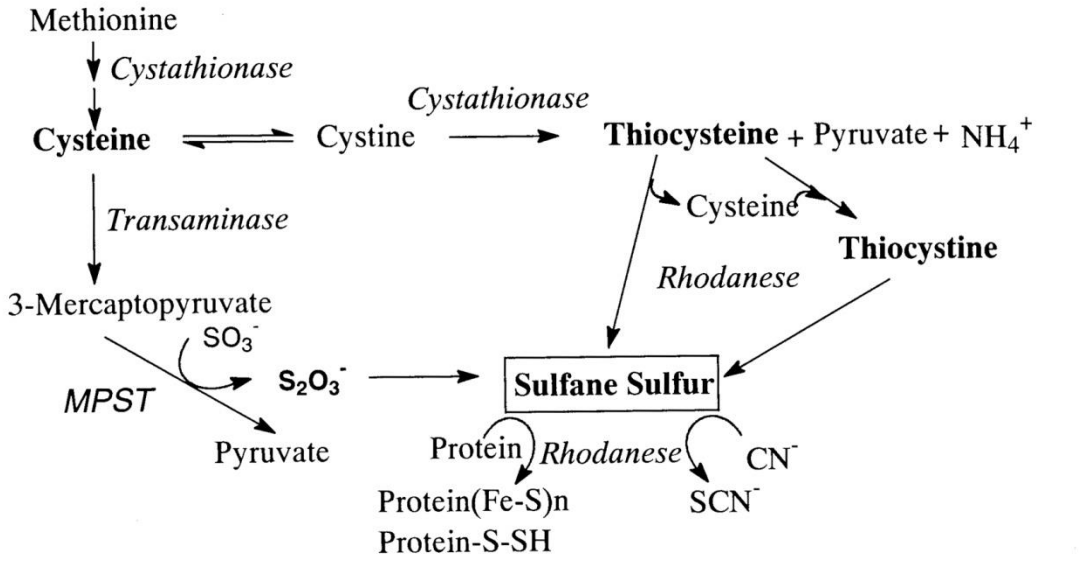


Figure 2.3.3-2. The disulfuration pathways of L-cysteine metabolism, adapted from Wrobel et al, 2000.

In case of very high levels of cyanide beyond the conversion rate, the methemoglobin pool gets saturated resulting in cyanide intoxication. This leads to accumulation of cyanide in plasma with resultant intoxication of vulnerable organs among them the brain (Lundquist et al 1985). Cyanide toxicity emanates from cellular energy failure because of inhibition of cytochrome *c* oxidase, the terminal enzyme of the mitochondrial electron transport chain which also blocks the oxidative phosphorylation (Pettersen and Cohen 1993). Cyanide has very high affinity for iron in ferric state and when absorbed, it readily reacts with the trivalent iron of cytochrome *c* oxidase in mitochondria. This leads to inhibition of cellular respiration resulting in lactic acidosis and cytotoxic hypoxia (Isom and Way 1974). This may result in a vicious cycle where other enzyme process are inhibited exacerbating the toxicity, for example, antioxidant defense enzymes (catalase, superoxide dismutase and glutathione peroxide among others) (Ardelt et al 1989).

2.3.4 Cyanide and cytochrome *c* oxidase enzyme activity

Cytochrome c oxidase [EC 1.9.3.1.] is the principle terminal oxidase of high affinity oxygen in the aerobic metabolism of all animals, plants, yeasts, and some bacteria (Lemberg 1969). The enzyme is present in mitochondria of the more highly developed cells. This enzyme provides the energy for the cell by coupling of the electron transport through the cytochrome chain with the process of oxidative phosphorylation. Cytochrome c oxidase is located on the inner mitochondrial membrane that divides the mitochondrial matrix from the intermembrane space and it has been used for many years as a marker for this membrane (Hovius et al 1990; Rasmussen and Rasmussen 2000; Musatov et al 2000). Cyanide (as hydrogen cyanide), originating from cyanide

salts, and other cyanogenic compounds or arising from catabolism of cyanogenic glycosides, exerts its acute toxic effects by complexing with the ferric iron atom in metalloenzymes, resulting in histotoxic anoxia through inhibition of cytochrome c oxidase (Rieders 1971; Way 1984). Metalloenzymes function as the terminal oxidase of the inner mitochondrial membrane respiratory chain (Rieders 1971; Way 1984). A two-step process has been proposed; cyanide as hydrogen cyanide first penetrates a protein crevice of cytochrome c oxidase and binds to the protein (Stannard and Horecker 1948). Hydrogen cyanide then binds to the trivalent iron ion of the enzyme, forming a relatively stable (but reversible) coordination complex. One mole of hydrogen cyanide is bound to one mole of cytochrome c oxidase (Van Buuren et al 1972). As a result, the enzyme becomes unable to catalyze the reactions in which electrons would be transferred from reduced cytochrome to oxygen. Cellular oxygen utilization is thus impaired, with resultant reduction in or cessation of aerobic metabolism (Rieders 1971; Way 1984). Glucose catabolism then shifts from the aerobic pathway to anaerobic metabolism including the pentose phosphate pathway, resulting in increased blood glucose, pyruvic acid, lactic acid, and nicotinamide adenine dinucleotide (NADPH) levels, and a decrease in the adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio (Rieders 1971; Way 1984). Wilson et al. (1975) proposed that it is the binding of cyanide to oxidized CuB, the copper ion that is part of the dioxygen binding-site that leads to the inhibition of cytochrome c oxidase.

The inhibition of oxygen use by cells (termed histoxic hypoxia) causes oxygen tensions to rise in peripheral tissues (Smith 1996). This results in a decrease in the unloading gradient for oxyhemoglobin; thus, oxyhemoglobin is carried in the venous blood

(Rieders 1971). Inhibition of oxygen utilization is thought to occur rapidly after cyanide exposure. Tadic (1992) established that inhibition of cytochrome c oxidase activity in rat brains was most remarkable between 15 and 20 minutes after administration of sodium cyanide.

2.3.5 Clinical features of cyanide toxicity

Cyanide acts rapidly in the body. The health effects are due to inhibition of cellular aerobic respiration resulting in buildup of lactic acidosis, cytotoxic hypoxia and vicious inhibition of other enzymatic processes (Johnson et al 1986). The respiratory system is stimulated by chemoreceptors responsive to reduced oxygen. A transient stage of central nervous system stimulation with hyperpnoea and headache is witnessed**.** Hypoxic convulsions results, while coma and death results from respiratory arrest. Most of the victim of cyanide poisoning die quickly or recover fully with neurological sequelae which include extrapyramidal syndromes, personality changes and memory deficits (Marrs and Maynad 1995).

2.3.6 Nutrition and cyanide detoxification

Sulfur amino acids deficiency is a common occurrence among malnourished populations and may affect the metabolism of cyanide in cassava subsisting populations. In well nourished individuals, cyanide is primarily metabolized to non-neurotoxic thiocyanate which is excreted in urine. However, in the malnourished person, cyanide detoxification may be impaired resulting to preferential metabolism into

neurotoxic cyanate (Tylleskär et al 1991; Swenne et al 1996; Tor-Agbidye et al 1999a). Under normal conditions, the conversion of cyanide to thiocyanate is facilitated by the sulfur amino acids cysteine and metionine and catalyzed by the enzyme rhodanese (Wood and Cooley 1956). Cyanide is absorbed gastrointestinally and distributed systemically. In the blood, cyanide is sequestered to cyano-methemoglobin by cyanocobalamin (B12) in a reversible reaction (methemoglobin trapping). Cyanide may also react with cysteine to form 2-amino thiazoline carboxylic acid, an excitatory amino acid (Ansell and lewis 1970) (**Figure 2.3.6-1**)

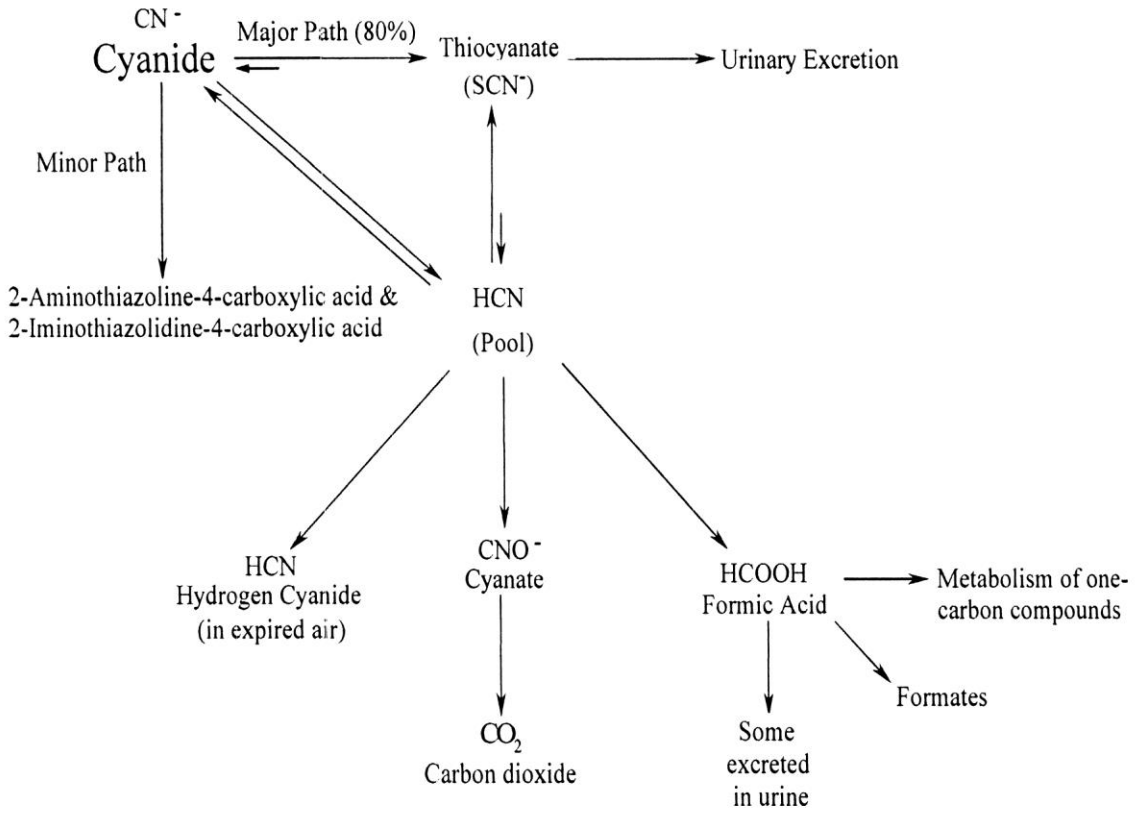


Figure 2.3.6-1. Cyanide primary metabolic pathways. Adapted from Ansell and Lewis (1970)

2.3.7 Cyanate (OCN) and neurotoxicity

The use of sodium cyanate (NaOCN) as an effective drug in the treatment of sickle cell anaemia has been documented, however; it was discontinued due to its neurotoxic properties (Peterson et al 1974). Prolonged treatment with NaOCN induces neuronal damage in cerebral cortex, basal ganglia, spinal cord and peripheral nerves of primates (Shaw 1974) and a sensorimotor neuropathy in humans and rats (Peterson et al 1974; Ceremi et al 1973). Chronic administration of OCN in high doses causes hind limb paralysis in rats (Alter et al 1974) and spastic quadriplegia in monkeys (Shaw et al 1974). Polyneuropathy manifested by severe motor weakness has also been observed in sickle cell anaemia treated orally with cyanate (Ohnishi et al 1975). In addition to nerve fiber damage, cyanate may inhibit nerve conduction velocity and elicit blockage of axonal transport (Samson and Hinkley 1972).

In malnourished subjects, subsisting on cassava in which SAA intake is deficient, cyanide conversion to thiocyanate may be impaired and may be converted to cyanate a known cause of neuronal degeneration in rodents, primates and humans (Ceremi et al 1973; Alter et al 1974; Tellez-Nagel et al 1977, Shaw et al 1974, Ohnishi et al 1975). Even in severe protein malnutrition, available sulfur is preferentially utilized for cyanide detoxification (Swenne et al 1996). Recent studies have shown that young adult rats treated with linamarin or NaOCN, under SAA-deficient diet, developed hind limb tremors (linamarin) or severe motor weakness (NaOCN), with differential serum protein-carbamoylation detected in the animals suggesting that serum protein carbamoylation

may be used as a marker of cyanogenic exposure in relation to risk for neurodegeration (Kassa et al 2011).

2.4 Cassava related neurotoxicity syndromes

Neurological toxicity following ingestion of cyanogenic compounds depends on the amount, the duration, cumulative doses of exposure, and the ability of the body to detoxify. In addition, intrinsic factors such as age, nutritional status, and genetic background are important factors (Rosling et al, 1988, Rosling and Tylleskar, 1995). Several human diseases have been attributed to the acute or chronic effects of cassava dietary dependence. The brain metabolism is predominantly aerobic, and as a result, the central nervous system appears to be the primary target for cyanide toxicity in humans and animals (Way, 1984) because it triggers anaerobic metabolism with its resultant effects. Acute inhalation of high concentrations of cyanide provokes a brief central nervous system stimulation followed by depression, convulsions, coma, and death in humans and in animals (Peden et al 1986**,** Singh et al 1989, Purser et al 1984, ATSDR 1997). These effects are probably associated with rapid biochemical changes in the brain, for instance, ion flux, neurotransmitter release, and possibly peroxide formation (Johnson and Isom 1985, ATSDR 1997). Other likely cyanide poisoning mechanisns in addition to inhibition of cytochrome *c* oxidase activity include the following. Cyanide is a strong nucleophile with multiple effects including release of secondary neurotransmitters, catecholamines from adrenal glands and adrenergic nerves, and inhibition of antioxidant enzymes in the brain (Smith 1996).

2.4.1 Acute cyanogenic exposure and neurotoxicity

The Symptoms of acute cyanide intoxication may include constriction of the throat, nausea, vomiting, giddiness, headache, palpitations, hyperpnoea, dyspnoea, bradycardia, unconsciousness and violent convulsions followed by death. Similar effects occur more slowly following exposure to cyanide salts (Sax, 1984). Acute cassava cyanogenic poisoning, sometimes lethal, may occur within hours of consumption (Rosling and Tylleskar, 1999).

In humans, if the hydrogen cyanide exceeds the limit an individual is able to detoxify/tolerate, death may occur (Rosling and Tylleskar, 1999). The acute oral lethal dose of hydrogen cyanide for humans is reported to be 0.5-3.5 mg/kg bodyweight. Tremors have been observed in a patient who accidentally ingested an unknown amount of cyanide (Chen and Rose, 1952). Additionally, children who ingested a large number of apricot pits which contain cyanogenic compounds experienced various neurological effects ranging from headaches to coma (Lasch and El Shawa, 1981). The severity of the effects corresponded with the amount of ingested pits. Cyanide appears to alter the physiology as well as the structure of nervous system. For example, histopathological effects in the brain were noted with potassium cyanide poisoning (Riudavets et al, 2005), which included, autolysis in several brain regions (basal ganglia, thalamus, hypothalamus, and cerebellum), acute hypoxic/ischemic changes (neuronal necrosis) in the cerebellum (Purkinje and granule cells), basal ganglia, hypothalamus, and deep cortical layers (manifest as pseudolaminar necrosis), and apoptosis of glial cells in the white matter (Riudavets et al, 2005).

2.4.2 Cassava growth related impact on children and other effects

Chronic oral exposure to cyanide in humans who eat cassava as a main carbohydrate source of their diet has been associated with thyroid toxicity. The exposure resulted in high serum levels of thiocyanate (SCN) the main CN metabolite. SCN is a pseudohalide that interferes with iodine uptake in the thyroid gland and may aggravate iodine-deficiency disorders (Spencer, 1999; Rosling and Tylleskar, 1999). The effect of cyanide is proportional to the body weight and therefore children tend to be more susceptible to its poisoning than adults. In regions where there is iodine deficiency, which causes goiter and cretinism, cyanide intake from cassava exacerbates these conditions (Delange et al, 1994).The result is that children will have arrested growth and stunting. In sum, cassava dependency has also been associated with thyroid dysfunction, growth stunting and diabetes type 3 (tropical) diabetes mellitus (Ihedioha and Chineme, 1999, Banea Mayambu et al, 2000).

The incidence of endemic goiter correlated with cassava intake in the Congo, where thyroid uptake of radioiodine was decreased in the goitrous area, compared with the controls (Delange and Ermans, 1971). Similarly, altered thyroid hormone parameters were measured in a village in Mozambique where an epidemic of spastic paraparesis was found and it correlated with ingestion of cassava (Cliff et al, 1986). Increased thyroid stimulating hormone levels and the ratio of triiodothyronine to thyroxine (T3/T4) were detected in serum, consistent with these measurements, the authors calculated a decrease in the index of free thyroxine and an increase in free triiodothyronine. However, the incidence of endemic goiter was not elevated in this village. The

examined individuals also had very high levels of thiocyanate in serum and urine (Cliff et al. 1986). Similarly, rats fed on a diet containing potassium cyanide for 4 months had a significant decrease in plasma thyroxine levels and thyroxine secretion rates at 11 months, while treated rats showed no significant decreases in thyroxine concentrations, but had significant increases in relative thyroid weight (Philbrick et al. 1979).

Cassava have low protein to energy ratio (P: E) compared to other staple crops. The protein content among common cassava cultivars is approximately 1-4% (Stupak et al, 2006, Ceballos et al, 2006). Therefore, populations that consume large amounts of cassava may be at risk for inadequate dietary protein intake (Nunn et al 2011) and further predisposing them to the risk of cyanogenic toxicity.

2.4.3 Cerebellar-Parkinson-dementia syndrome and cassava neurotoxicity

There is suggestion to another cassava associated neurological disorder that develops in older subjects reliant heavily on incompletely detoxified cassava. This disorder, called cerebellar-Parkinson-dementia syndrome presents with a pattern of slowly evolving ataxic neuropathy with or without evidence of pyramidal deficits with occasional presence of visual auditory sensorineural deficits. The disease was first reported in Nigeria (Osutonkuton 1973; Osuntokun 1981).

Furthermore, cassava dependency has also been reportedly associated with other neuromuscular syndromes including proximal myopathy and a movement disorder resembling ballism among Indians (Madhusudan 2008). Extrapyramidal disorder of this

type are seen in subjects with mildewed sugarcane poisoning, which raises the question of fungal contamination of food in cassava-associated cases with ballistic movement disorders.

The effects of chronic oral exposure of humans to cyanogenic glucosides have been extensively studied in African regions with populations that consume a high level of cassava roots (Howlett et al 1990, Ministry of Health, Mozambique, 1984; Osuntokun 1968, 1972; Osuntokun et al 1969, Tylleskar et al 1994). In some cases, the diet consisted mainly of cassava roots, due to failure of other food crops (Howlett et al 1990). Various neuropathies have been observed in these regions and the findings correlated with increased blood and urinary SCN levels (Howlett et al 1990). Biomarker studies on serum linamarin, cyanide and SCN have yielded non-conclusive results as to what is the best biomarker for konzo (Tylleskar et al 1994). There are no studies that have been conducted on these biomarkers in relation to rhodanese activity and the production of OCN or that of carbamoylated proteins which appear to have a longer half time relative to OCN (Kraus and Kraus 1998, Kassa et al 2011).

Cyanide toxicity has been implicated in parkinsonism-like signs after ingestion of potassium cyanide. Such toxicity resulted in extrapyramidal signs such as drooling, marked micrographia, masked facies, mild intention tremors, and cogwheel rigidity. Magnetic resonance imaging of the brain revealed bilateral, symmetrical abnormalities of the basal ganglia, particularly the globus pallidus (Feldman and Feldman 1990, Chin and Calderon 2000). Additionally, inhalation and oral studies using animals have shown that acute or chronic cyanide exposure leads to encephalopathy in both white and gray matter. Damage has been observed in the deep cerebral white matter, corpus callosum,

hippocampus, corpora striata, pallidum, and substantia nigra (Chin and Calderon 2000). The white matter may be more sensitive because of its relatively low cytochrome *c* oxidase content. Memory impairment has also been reported as a delayed effect in individuals who survived a cyanide poisoning incident with antidotal treatment. In this regard, a female patient developed difficulties with short-term memory 5 months after ingesting an unknown amount of an unspecified cyanide compound (Chin and Calderon 2000).

2.4.4 Tropical ataxic neuropathy

Tropical ataxic neuropathy (TAN) was first reported in 1888 by Strachan among the Jamaicans as a syndrome presenting with severe burning pain of the feet sole, dimness of vision, ataxia and increased pigmentation of the skin (Tor-Agbidye 1998). Syndromes similar to that described by Strachan (comprising lesions of the skin, mucus membranes and the nervous system) and putatively related to dietary deficiencies, toxins or infections, have been described in communities on poor nutrition, especially in tropics and in prisoner of war camps (Tor-Agbidye 1998). TAN or variants thereof have been reported in different parts of West, North, East, Central and South Africa, Caribbean countries and South East Asia (Osuntokun 1968).

The clinical picture of TAN commences with paraesthesia and dysaesthesia usually starting in the distal parts of the lower limbs. Then the blurring or loss of vision sets in, followed by ataxia of gait, tinnitus, deafness, weakness and atrophy of leg muscles. As the disease progresses, defective perception of sensory modalities (often confined to

the lower limbs), bilateral optic atrophy, ataxic gait, and impaired muscular coordination, bilateral perceptive deafness, weakness and wasting of the muscles (usually of the lower limbs) may be common. Symmetrical hyperreflexia of the upper limbs, symmetrical spastic paraparesis, spastic dysarthria, diminished visual acuity, peripheral neuropathy, cerebellar signs, and deafness are among other clinical findings (Osuntokun 1968). Cassava has been implicated because the condition improved when its component of the diet was reduced. In 1935, Clark attributed the cause of TAN to the high content of cyanogens in cassava (chronic cyanide intoxication) (Clark 1936). Thereafter, considerable circumstantial evidence have incriminated chronic cyanide intoxication of dietary origin as the major etiologic factor in TAN among Nigerians (Osuntokun 1981). The essential neurological components of the disease are signs of myelopathy, bilateral optic atrophy, bilateral perceptive deafness and polyneuropathy (Osuntokun 1968).

2.4.5 Cassava and other nervous system impairments

Cassava cyanogens may impair brain development and possibly induce a higher incidence of late-life neurodegenerative disease (Rosling and tylleskar 1999, Osuntokun 1969, Osuntokun 1973). A study on EEG findings in konzo patients showed significant EEG abnormalities. The abnormalities depicted generalized slowing of the background activity consisting of theta activity (Tshala-Katumbay et al 2000). In severely affected konzo subjects, besides slowing of background activity, non-specific paroxysmal activity and decreased frequency of the postcentral background was seen with dominant distribution in the frontal areas; a finding that suggests that konzo may be associated

with a diffuse lesion of the nervous system. The slowing of EEG activity could, however, also be due to hypothyroidism as a consequence of the cyanogens exposure (Tshala-Katumbay et al 2000).

2.4.6 Konzo

Background

Konzo *aka* Mantakassa in Mozambique is characterized by the abrupt onset of an isolated and symmetric spastic paraparesis which is permanent but non-progressive. Konzo is an upper motor neuron disease reported only from poor rural communities in Africa (Rosling et al 1988, Rosling and Tylleskar 1995). Konzo is a yaka (D.R.C tribe in the province of Bandundu) word meaning 'tied legs', a description of the resulting spastic gait (Rosling et al 1988, Rosling and Tylleskar 1995, Tor-Agbidye 1998) as illustrated (**Figure 2.4.6-1**). Konzo was first reported following its outbreaks in the former Belgium Congo *aka* Zaire the present day D.R.C (Trolli 1938). Subjects affected by the disease present with a spastic paraparesis or tetraparesis in severely affected subjects (Tshala-Katumbay et al 2001, Boivin et al 2013).

Figure 2.4.6-1. Severe form (child left) and moderate form (woman with walking stick) of konzo. (Photo courtesy of Tshala-Katumbay 2013)

Epidemiology of konzo

Konzo has been attributed to high dietary cyanogenic exposure from insufficiently processed bitter cassava. The disease has been reported mainly from remote areas of Mozambique, Tanzania, Cameroon, Angola, the Central Africa Republic and Democratic Republic of Congo (D.R.C) (Trolli 1938, Cliff et al 1985, Howlett et al 1990; Banea et al 2000, Tylleskar et al 1992, Boivin et al 2013) figure (**Figure 2.4.6-2**). Konzo has only been reported in cassava growing and consuming areas, however, the affected population constitutes only a fraction of the total of over 500 million cassava consuming population in the tropics. Similarity of konzo with neurolathyrism has been put forward; however, there is no geographical overlap between the two disease regions (Howlett et al 1990, Tylleskar et al 1994).

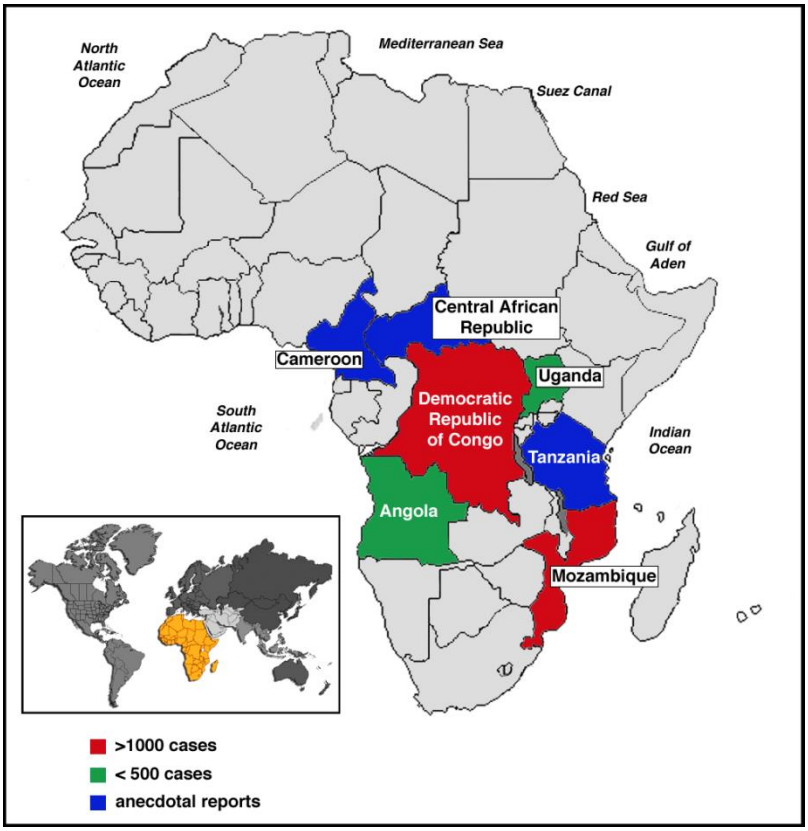


Figure 2.4.6-2. Map of Africa showing areas affected by konzo. Heavily (red), moderately (green) and sparsely (blue) affected sub-saharan countries.

The prevalence rates for konzo vary as the number of studies, however, it varies between 1-30 per 1000 (Tylleskar et al, 1992). The total number of confirmed konzo cases in reported studies exceeds 4000 (Tylleskar, 1994, Bradbury and Lambain, 2011). The age and sex distribution of konzo show a distinct pattern. No child of below 2.5 years (breast feeding) has ever been seen to contract konzo. Women of child bearing age and children 3-13 years of age run the highest risk of contracting konzo. No case of konzo has been reported from nearby urban populations (Banea-Mayambu et al, 1997). Thousands of konzo cases have been reported in DRC especially during

outbreaks within some provinces notably Bandundu and other parts of the country (Tshala-Katumbay 2001, Boivin and Tshala-Katumbay et al 2013).

There have been outbreaks of konzo following consumption of high cyanogenic bitter cassava during drought when a person adopts short cuts in processing in Tanzania and Uganda (Mlingi et al 2011, Tylleskar et al 1992). No documented evidence of konzo cases in Kenya. This could probably be because of under reporting, in addition to the absence of favourable conditions that favour konzo namely dependence on bitter cassava, SAA deficiency, drought, war and conflict are not prevalent in the country. In some instances, fatal cases of acute toxicity after consumption of cassava have been reported. There may be cases of chronic toxicity which may be elicited after rigourous clinical and biochemical evaluation of the consumers of the crop. This approach will unravel non overt and sub-clinical cases of konzo through neurological and well as psychological testing (Boivin et al 2013). Comprehensive research need to be undertaken in the main cassava consuming and growing areas in this field of interest.

Clinical manifestations of konzo

The disease typically occurs in an apparently healthy person and there is no prodromal phase or triggering illnesses. The onset is characterized by an abrupt muscle weakness and trembling (spasticity) of the legs, occurring the first days of the illness. A common history is that of a healthy person develops the disease following moments of physical exertion e.g. walking a long distance. Initial symptoms are often described as heaviness, trembling or weakness of the legs associated with difficulty or inability to

walk. Other complaints that may appear with time include weakness in the arms or hands, difficulty in articulating speech and blurring of vision. Sensory symptoms of radicular lower back and paraesthesia in the legs can also be present but these usually clear in the first weeks or months. However, the patients do not present with incontinence. The disease affects mainly the children and women of child bearing age (Howlett 1990, Tylleskar 1994, WHO 1996). Although the main clinical picture of konzo consists of the sudden onset of a non progressive and symmetrical spastic paraparesis of the legs in affected subjects, the diagnosis of konzo is based on the WHO criteria (WHO 1996) (**Table 2.4.6-1**). The degree of physical disability caused by konzo was classified by Lucasse (1952) and later amended by WHO (1996). The criteria are easy to use in the field while screening the population. However, Tshala-Katumbay (2001) suggested a new version with more operational criteria in comparison with the WHO criteria, (**Table 2.4.6-1**). The following are the criteria for konzo severity; Mild form: when the patient does not need regularly to use any walking aid; Moderate form: when the patient regularly uses one or two stick(s) or clutches; Severe form, when the patient is bed ridden or unable to walk without support.

Table 2.4.6-1. WHO criteria for konzo versus newly suggested criteria (with permission from Tshala-Katumbay, 2001)

Criteria	WHO	New version
1	Visible symmetric spastic abnormality of gait while walking or running	Sudden onset of a non-progressive bilateral and symmetric abnormality of gait while walking or running
2	History of onset less than one week followed by a non-progressive course in a formerly healthy person	Bilaterally exaggerated knee or ankle jerks
3	Bilaterally exaggerated knee or ankle jerks without signs of disease of the spine	Absence of objective sensory and genitourinary symptoms
4	Absence of grass pea (Lathyrus sativus) consumption	Living under conditions of sub-acute or chronic exposure to cyanogens and under-nutrition at the onset

A study on impairments, disabilities and handicap pattern in konzo showed that the overall clinical picture consisted of muscle power impairment in lower limbs, spasticity walking activity limitations, mobility restrictions, and exaggerated tendon reflexes, and hip mobility impairment due to adductor spasms. The subjects may also present with speech problems of pseudobulbar type, hyperlordosis as a compensatory phenomenon to the spastic gait, nystagmus, and blurred vision at the time of the clinical examination. Most subjects have normal mental, sensory, and genitourinary functions (WHO 1996, Rosling and Tylleskar,1999, Tshala-Katumbay et al, 2001) with the exception of few subjects who showed signs of mental retardation suggesting that chronic reliance on toxic cassava may have adverse effects on cognition. The retardation could be due to the effect of cyanogens on the endocrine system or it could be because of the effect on cyanate modification of brain proteins. This hypothesis is consistent with the fact that suggests a long-term deleterious effect on learning due to carbamoylation (Crist et al 1973).

CHAPTER THREE: MATERIALS AND METHODS

3.1. Experimental set up

Experiments on elucidating the differential patterns of neurological deficits associated with cyanide and cyanate intoxication in rodents (study I) was conducted in Toxicology laboratory at the department of Pharmacology and Pharmacocognosy, University of Nairobi, Kenya. Studies to elucidate the impact of protein-restricted diet on cyanide vs. cyanate toxicity in rodents relative to non-human primates (study II); together with, experiments to identify molecular targets of cyanide or cyanate toxicity under SAA-deficient diet (study III) were conducted at the Center for Research on Occupational and Environmental Toxicology (CROET) and proteomic core facility at Oregon Health and Science University (OHSU), USA, respectively. Non-human Primates studies were conducted at the Haman Ranch (The Mannheimer Foundation, Inc., LaBelle, FL, USA).

As regards the rodent studies, a controlled entry experimental room measuring about 4.5 X 3.5 meters at OHSU was utilized. The room was maintained on light and dark cycle by an automated system that turn on and off the lights at 0600 and 1800 hrs daily, respectively. The rodents were caged in groups of three. The cages were placed on raised surface using racks and had corn beddings which were changed daily. The rats were fed with special all amino acid (AAA) or sulphur amino acid (SAA) restricted diet (Harlan laboratories, Madison, Winscousin, USA) and water *ad libitum* in the home cages. With regard to rodent study on cognitive performance done at University of Nairobi, the animals were fed on ordinary rat pellets (Unga Feeds, Nairobi, Kenya) that

contained the necessary nutrients. Assessment and testing on rotarod and Radial arm maze were carried out in the experimental room.

3.2 STUDY I: DETERMINATION OF DIFFERENTIAL PATTERNS OF NEUROLOGICAL DEFICITS ASSOCIATED WITH CYANIDE AND CYANATE INTOXICATION

3.2.1 Chemicals

NaCN (CAS No. 143-33-9, 97.2% purity) and NaOCN (CAS No. 917-61-3, 96% purity) were bought from Sigma–Aldrich (St. Louis, MO) and stored at room temperature. All other laboratory reagents were of analytical or molecular biology grades.

3.2.2 Animals

Young adult Sprague-Dawley male white albino rats, 6-8 weeks old (N = 22), weighing 120-230 g upon arrival were used. The rats were acquired from the Department of Zoology, School of biological sciences, University of Nairobi, which maintains animals for experimental studies. These are the commonly bred and used strain of rats in the laboratory and have also been extensively used in neurobiological research (Kuramoto et al. 2012). The animals were caged in an experimental room in the toxicology laboratory at the department of Pharmacology and Pharmacognosy, University of Nairobi. The rats were used as per institutional regulations on colony handling and efficient use of laboratory animals.

3.2.3 Diet

A normal rodent pellet diet was purchased from Unga Feeds, (Nairobi, Kenya). This was commercially available balanced diet for rats with all the necessary vitamins and minerals. Animals were fed and given tap water *ad libitum* for 6 weeks during acclimatization and treatment phases. During both short and long-term memory testing rats were fed on 85% food and water *ad libitum.* The 85% food restriction was based on the fact that when rodents are partially starved, they get motivated to explore and search for food in the arms of the RAM therefore encouraging navigation (Hodges 1996).

3.2.4 Dosing regimens

Rats were acclimated for a 5-day period on a diet consisting of normal rodent pellet, Unga Feeds (Nairobi, Kenya). On day 6, animals were assigned to experimental groups (N = 7-8/group) and treated intraperitoneally (i.p) (one injection per day) for 6 weeks as follows: (1) 2.5 mg/kg body weight (bw) NaCN; (2) 50 mg/kg bw NaOCN; (3) equivalent amount of vehicle (1 µl/g bw saline). The rats were weighed daily to assess changes in body weight and to adjust the dose of the test articles.

3.2.5 Animal observations

Rodents were examined daily for physical signs of disease including tremors and the hind limb extension reflex which is elicited by gently raising the animal by the tail.

3.2.6 Radial arm maze assessment

The assessment was based on performance of rats on custom-made wooden radial arm maze (RAM), a paradigm that has been used for testing spatial working and reference memory (Olton and Samuelson 1976; Olton 1985; Wenk et al. 1986). The assessment was carried out in two phases, namely; working (short-term) memory (defined as information that is useful to the rat during the current experience with the task) and reference (long-term) memory (information that is useful in all exposures to the task across all the days of testing) (Olton 1985; Wenk et al. 1986). Short-term (working) memory was tested for 8 consecutive days with one trial per day that was preceded by 3 days of habituation (15 min trial per day). The reference (long-term) memory was assessed thereafter for 5 days with two trials per day.

During the testing, rats were individually placed on the RAM made of 8 arms measuring 50 cm length, 10 cm width and 5 cm height. The RAM had a central octagonal platform measuring 25 cm with eight arms extending from it like the spokes of a wheel. RAM was fitted with guillotine doors that were opened and closed individually; it also separated the central platform from the arms. The arms had drilled holes where food pellets were kept to motivate the animals. The maze was raised one meter above the floor of the room that was enriched with cartoons to serve as spatial cues. The investigator assumed the same position throughout the experimentation. Rodent urine and feces were cleaned immediately after each animal trial to minimize intra-maze cues that may influence cognitive performance.

During long-term memory testing, rats were allowed 60 seconds after the first trial for the commencement of the second trial. The RAM assessment parameters namely; correct entry counts into unvisited arms, re-entry counts into visited arms (working memory errors (WME) and RAM navigation time (total amount of time taken by a rat to complete visiting all the arms) were used to assess working memory (short-term memory) during each trial (10 minutes) for 8 days. During reference (long-term) memory assessment, only four out of the eight arms were baited. The following parameters were measured; correct entry counts into baited arms, entry counts into un-baited arms (reference memory errors (RME), re-entry counts into baited arms (WME) and RAM navigation time.

3.3 STUDY II: DETERMINATION OF THE IMPACT OF PROTEIN-RESTRICTED DIET ON CYANIDE AND CYANATE TOXICITY IN RODENTS AND NON-HUMAN PRIMATES.

3.3.1 Animals

Young adult male heterozygous rats (Crl: NIH-Fox1 rnu/Fox 1+, 6-8 weeks old) (N= 52), weighing 140-210 g upon arrival were donated by Professor Neuwelt, Department of Neurology, Oregon Health & Science University (OHSU). These rats are known to have a normal phenotype (Charles River technical data sheet 2009). Animals were caged in an animal room maintained on a 12/12-h light dark cycle in the Oregon Institute of Occupational Health Sciences for the experimental studies. Food and water were given

ad libitum. Experimental protocols were approved by the OHSU Institutional Animal Care and Use Committee (IACUC).

3.3.2 Diets (AAA and SAA)

Custom-synthesized isonitrogenous rodent diet with either all amino acids (AAA-diet; code TD09460) or lacking 75% of the SAA-content relative to the control diet (SAA-deficient diet; code TD09463) were purchased from Harlan (Madison, WI) and stored at 4^{o}C until use (**Table 3.4.2-1**). Previous experimental studies showed animals fed with SAA-free chow had dramatic weight loss and muscle weakness (Tor-Agbidye et al., 1999). We chose a 75%-deficient but not free SAA to allow for animal survival and cyanide detoxification.

Table 3.4.2-1. Diets (AAA and SAA) composition

Ingredients	AAA g/kg	SAA g/kg (25% cysteine/methionine)-deficient
L-Alanine	3.5	3.8
L-Arginine HCl	12.1	12.1
L-Asparagine	6	6
L-Aspartic Acid	3.5	3.8
L-Cysteine	**4**	**1**
L-Glutamic Acid	40	40
Glycine	23.3	23.8
L-Histidine HCl, monohydrate	4.5	4.5
L-Isoleucine	8.2	8.2
L-Leucine	11.1	11.1

L-Lysine HCl	18	18
L-Methionine	**4**	**1**
L-Phenylalanine	7.5	7.5
L-Proline	3.5	3.5
L-Serine	3.5	3.8
L-Threonine	8.2	8.2
L-Tryptophan	1.8	1.8
L-Tyrosine	5	5
L-Valine	8.2	8.2
Sucrose	355.28	355.28
Corn Starch	150	150
Maltodextrin	150	150
Soybean Oil	80	80
Cellulose	30	30
Mineral Mix, AIN-93M-MX (94049)	35	35
Calcium Phosphate, monobasic, monohydrate	8.2	8.2
Vitamin Mix, AIN-93-VX (94047)	13	13
Choline Bitartrate	2.5	2.5
TBHQ, antioxidant	0.02	0.02
Coloring	0.01	0.01
Total	1000	1000
kcal/g	3.94	3.94

Pro. (N x 6.25)	15.1	15.1
CHO	65.3	65.3
Fat	8	8

3.3.3 Dosing regimens

Rats were first acclimated for a 5-day period on a diet consisting of 4:1 portions of normal rodent chow (PMI Nutrition International, NJ) and either AAA-diet (N = 28, group 1) or SAA-deficient diet (N = 24, group 2). On the 6th day, animals were assigned to experimental groups (N = 7-10/group) and treated intraperitoneally (one injection per day) for up to 6 weeks (till first occurrence of physical signs notably motor deficits) as follows: (1) AAA-diet, 2.5 mg/kg body weight (bw) NaCN; (2) AAA-diet, 50 mg/kg bw NaOCN; (3) AAA-diet, equivalent amount of vehicle (1 µl/g bw saline); (4) SAA-deficient diet, 2.5 mg/kg bw NaCN; (5) SAA-deficient diet, 50 mg/kg bw NaOCN; (6) SAA-deficient diet, equivalent amount of vehicle (1 µl/g bw saline). Dose selection for cyanide was informed by findings that indicate the existence of sublethal and/or lethal cyanide poisoning in konzo-affected areas (Banea-Mayambu et al., 1997). Cyanate was given at doses similar to those known to induce neuropathy in humans (Ohnishi et al., 1975). The rats were weighed daily to assess changes in body weight and adjust the dose of the test articles accordingly.

3.3.4. Primate studies

3.3.4.1. Animals

Adult male *macaca fascicularis* monkeys (N = 12, mean aged 12.7 years) were used and housed at the Haman Ranch (The Mannheimer Foundation, Inc., LaBelle, FL). Animal protocols were approved by the Mannheimer Foundation's Animal Care and Use Committee (IACUC) (The Mannheimer Foundation, Inc., LaBelle, FL).

3.3.4.2 Diet

Animals were randomly assigned to two dietary conditions (N = 6/diet), namely, control monkey diet (Teklad 2050, Teklad Diets, Madison, WI) or cassava (*Manihot dulcis*). Cassava was purchased from Sysco Foods (Ft. Myers, FL, USA) in frozen form and maintained at -10 ºC till use. Animals under cassava diet first received a transition diet for a week (starting with 100% control diet for 2-3 days and then, control/cassava (1/1) diet for 2-3 days). Thereafter, they were fed the control/cassava diet in a 1/3 ratio for 2-3 days and, finally, 100% cassava; or the control diet, for 5 weeks. Animals were fed fresh daily thawed cassava and/or regular diet under the supervision of an experienced veterinarian to provide 70 kcal/kg of body weight per day while water was given *ad libitum*. Animals were weighed weekly to assess changes in body weight.

3.3.5 Specific protocols

3.3.5.1 Animal observations

Physical and behavioral changes in rodents were observed. Rats were observed for overt convulsions as manifestation of seizures following administration of cyanogenic toxins. Each convulsion episode was defined as one event per day and tabulated

accordingly. In addition, rodents were examined daily for physical signs, including tremors and the hind limb extension reflex, which is elicited when the animal is gently raised by the tail. Motor functions were assessed by animal performance on an accelerating rotating rod. Animals were individually placed on rotating rods in a software-driven rotarod apparatus (AccuScan Instruments, Inc., Columbus, OH) set in an accelerating mode. The rotation speed was gradually increased from 5 to 25 r.p.m. The apparatus had an automatic system for fall detection *via* photobeams. Animals were tested on alternate days and each session consisted of two consecutive trials of 90s. The latency to fall was recorded and compared across treatment-groups. Rotarod testing was carried out only in animals maintained on SAA diet to determine whether the dietary restrictions had an impact on the motor performance in rats treated with NaCN.

For the non-human primate studies, animals were first moved to single housing units. Physical examination and biological samples were collected (blood, feces, and food) for baseline evaluations. Thereafter, physical examination and sample collection were conducted weekly, on the same day, for 5 weeks. Blood was collected for complete blood count (CBC); liver function test (AST (aspartate transaminase), ALT (alanine transferase), ALP (alkaline phosphatase, GGTP (gamma glutamyl transpeptidase), total bilirubin), trypsin, as well as, plasma protein (total protein, globulin, and albumin); kidney function test [(blood urea nitrogen (BUN), creatinine, BUN:Creatinine ratio)]; metabolic profiles (glucose, cholesterols and triglycerides); pancreatic function test (amylase, lipase); CPK (creatinine phosphokinase), as well as electrolytes (phosphate, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, Cl^{-}). Animals were monitored daily for attitude, alertness, responsiveness,

appetite, food consumption, and clinical signs for abnormal gait and deficits in arm and hand coordination.

3.3.5.2 Tissue preparation for the measurement of cyanide detoxification rates

In the rodent studies, plasma samples were collected on the last day of the experimentation. Rats were deeply anesthetized with 4% isofluorane (1liter oxygen/min), and the blood (1.5–3.5 ml/rat) collected via cardiac puncture in vacutainer tubes with anticoagulant and kept overnight at 4^{o}C. Thereafter, the plasma samples were centrifuged at 15,000 rpm for 15 min at 4^{o}C. The samples were then aliquoted in cryotubes and stored at -80^{o}C until later for protein assay using the Pierce BCA (bicinchoninic acid) protein assay kit (Thermo Scientific, Rockford, IL, USA). Spinal cord and brain tissues were sonicated in ice-cold 25mM potassium phosphate buffer (pH 8.6) for 15 seconds repeated 3 times at 1.75 voltage. The mixture was centrifuged at 12000x g for 45 min at 4^{o}C and the supernatant was assayed into a new tube to form the soluble protein extract. In the non-human primate study, animals were chemically restrained with intramuscular (IM) ketamine HCl (Ketaved®, Saint Joseph, MO) dosed at 10 mg/kg body weight. About 3 ml of blood was obtained from the femoral vein by venipuncture using heparinized Vacutainer® tubes and blood collection sets (Franklin Lakes, NJ). Heparinized blood was centrifuged at 1000 RCF for 15 minutes. Thereafter, plasma was separated and stored at -80^{o} C for subsequent biochemical assays.

3.3.5.3 Assay for cyanide detoxification rates

Cyanide detoxification rates were assessed using the rhodanese assay developed by

Sörbo (Sorbo, 1953). Briefly, a solution of 200mM potassium phosphate buffer (pH 8.6) was mixed with 125 mM sodium thiosulfate and 250mM potassium cyanide. A 20µl of sample to be assayed was added to the solution, mixed, and incubated at 37^{o}C for 20 minutes. The reaction was then stopped by addition of 37% formaldehyde. The end product of the reaction was thiocyanate, which formed a red precipitate on mixing with an equal volume of 410 mM ferric nitrate solution. A standard curve was made from 40mM potassium thiocyanate in deionized water and diluted accordingly. A 200µl of the blank, standard dilutions, plasma, spinal cord and brain samples were assayed in duplicates. The absorbance of the final solution was read at or near 460 nm on a plate reader using the Epoch multivolume spectrophotometer system equipped with a Biotek Gen 5 data analysis software (Biotek instruments, Inc, USA). The concentration of thiocyanate in each sample was calculated from a calibration curve (standard) of potassium thiocyanate solution (0.05 – 40 mM). The enzyme activity was expressed in µmole of thiocyanate formed per minute per mg of protein at pH 8.6 and 37^{o}C. This was later expressed as the number of milliseconds required to produce one µmol of thiocyanate per mg of protein (ms/(µmol/mg) in the tested sample.

3.4 STUDY III: IDENTIFICATION OF MOLECULAR TARGETS OF CYANIDE OR CYANATE TOXICITY UNDER SAA-DEFICIENT DIET IN RATS

3.4.1 Chemicals

Sodium cyanide (NaCN CAS No. 143-33-9, 97.2% purity) and cyanate (NaOCN CAS No. 917-61-3, 96% purity) were bought from Sigma–Aldrich (St. Louis, MO) and stored

at room temperature. All other laboratory reagents were of analytical or molecular biology grades.

3.4.2 Animals

Young adult male heterozygous rats (Crl: NIH-Fox1 rnu/Fox 1+, 6-8 weeks old) (N=52), weighing 140-210g upon arrival were donated by Professor Neuwelt, Department of Neurology, Oregon Health & Science University (OHSU). These rats are known to have a normal phenotype (Charles River technical data sheet 2009). They were caged in an animal room maintained on a 12/12h light/dark cycle in the Center for Research on Occupational and Environmental Toxicology (CROET). Food and water were given *ad libitum*. Experimental protocols were approved by the OHSU institutional animal care and use committee (IACUC).

3.4.3 Diet and dosing regimens

3.4.3.1 Diet

Custom-synthesized isonitrogenous rodent diet with either all amino acids (AAA-diet; code TD09460) or lacking 75% of the SAA-content relative to the control diet (SAA-deficient diet; code TD09463) were purchased from Harlan (Madison, WI) and stored at 4^{o} C until use. We chose a 75%-deficient but not free SAA to allow for animal survival and cyanide detoxification since previous experimentation showed animals fed with SAA-free chow had dramatic weight loss and muscle weakness (Tor-Agbidye et al., 1999a).

3.4.3.2 Dosing regimens

Animals were first acclimated for a 5-day period on a diet consisting of 4:1 portions of normal rodent chow (PMI Nutrition International, NJ) with either AAA-diet (N=28) or 75%-SAA deficient diet (N=24). On the 6th day, they were assigned to experimental groups (N=7-10/group) and treated intraperitoneally (one injection per day) for up to 6 weeks as follows: (1) AAA-diet, 2.5mg/kg body weight (bw) NaCN; (2) AAA-diet, 50 mg/kg bw NaOCN; (3) AAA-diet, equivalent amount of vehicle (1µl/g bw saline); (4) SAA-deficient diet, 2.5mg/kg bw NaCN; (5) SAA-deficient diet, 50mg/kg bw NaOCN; (6) SAA-deficient diet, equivalent amount of vehicle (1 µl/g bw saline). Dose selection for cyanide was informed by previous findings of sublethal and/or lethal cyanide poisoning prior to konzo outbreaks (Banea-Mayambu et al., 1997). Cyanate was given at doses similar to doses known to induce neuropathy in humans (Ohnishi et al., 1975).

3.4.4 Specific protocols

3.4.4.1 Animal observations

Rodents were examined daily in an open field for physical signs including walking difficulties, tremors and hind limb extension reflexes, which were elicited when the animal was gently raised by the tail. Motor function was assessed by animal performance on an accelerating rotating rod. Animals were individually placed on rotating rods in a software-driven rotarod apparatus (AccuScan Instruments, Inc., Columbus, OH) set in an accelerating mode. The rotation speed was gradually increased from 5 to 25 r.p.m. The apparatus had an automatic system for fall detection *via* photobeams. Rotarod performance was analyzed to estimate how odds of remaining on the rod for at least 60 seconds changed over time. Testing was carried out only in

animals maintained on SAA-deficient diet to determine whether SAA dietary restriction had an impact on the toxicity of cyanide and cyanate, our main experimental paradigm of interest.

3.4.4.2 Tissue preparation for proteomics

Serum samples were obtained on day 14 and 28 of the study period. Blood was collected from the salphenous vein using vacutainer tubes with no anticoagulants from all rats and kept overnight at 4^{o} C. The serum was then collected in sterile tubes and centrifuged at 15,000 rpm for 15 min at 4^{o} C. Serum was aliquoted in cryotubes and stored at -80^{o} C till later liquid chromatography mass spectrometry (LC-MS/MS) studies. At study termination, rats were deeply anesthetized with 4% isofluorane (1 l oxygen/min) and transcardially perfused with saline through the ascending aorta to remove the remaining blood. The spinal cords were dissected out, flash-frozen in liquid nitrogen, and stored at -80^{o} C. Prior to LC-MS/MS studies, the frozen spinal cord tissue was thawed by adding 500µl of 100mM ammonium bicarbonate, and the tissue was uniformly dispersed by 3X15 sec of sonication (Sonic Dismembranator, Model 60, Fisher Scientific). Samples were then centrifuged at 12,000g for 30 min at 4º C, the supernatant removed, the pellet re-suspended and centrifugated, and the two supernatants pooled. The pellet was then re-suspended in 250µl 100mM ammonium bicarbonate by 5 sec of sonication as before. The protein content of the spinal cord soluble fraction, suspended pellet, and serum samples were then determined using the BCA assay (Pierce Chemical, Rockford, IL). The equivalent of 50µg portions of the two spinal cord fractions and serum were then dried by vacuum centrifugation in preparation for trypsin digestion.

3.4.4.3 Protein digestion

Fifty µg portions of dried serum, soluble and insoluble spinal cord fractions were dissolved in 50µl of 100 mM ammonium bicarbonate containing 1mg/ml of Rapigest detergent (Waters Corp., Milford, MA). Eight µl of 50mM dithioerythritol (DTT) solution was then added and the samples incubated for 60 min at 60º C, followed by addition of 8µl of 150mM iodoacetamide solution and incubation at room temperature for 30 min. An additional 16µl of 50mM DTT was then added, samples incubated at room temperature for an additional 15 min, and 20µl of 0.1µg/µl proteomics grade trypsin added (Sigma Chemical Co., St Louis, MO). Following an overnight incubation at 37ºC, samples were acidified by addition of 0.5% trifluoroacetic acid (final concentration), incubation at 37°C for 45 min, centrifugation at 13,000 g for 10 min, and the supernatant frozen prior to analysis of peptides by mass spectrometry.

3.4.4.4. Mass spectrometric analysis equipment

Liquid based proteomics involved the identification and quantification of peptides and proteins, as well as identification of post-translational modifications. The equipment (appendix III) used was a hybrid triple quadrupole/linear ion trap (QTRAP 4000) mass spectrometer (ABsciex). For identification of proteins, proteins in solution were digested (normally, with trypsin) and their peptides were injected into the system where they were separated according to their hydrophobicity until they reach the mass spectrometer. Peptides resulted from digestion of complex mixtures of proteins were separated by two-dimensional liquid chromatography. They were separated in the first

dimension by strong cation exchange, resulting in different fractions from different salt concentration. The content of each fraction was then separated in the second dimension by reverse phase chromatography. Proteins were identified by submitting all MS/MS spectra to a database, as SwissProt, with different softwares used for the search: Mascot, Protein Pilot or Peaks, based in different search algorithms.

For identification and location of post-translational modifications (phosphorylations, glycosylations) a proteolytic digestion was performed and modifications were detected by the mass spectrometer using different acquisition modes (Scan or Multiple Reaction Monitoring (S/MRM). Protein Pilot software enabled the searching for hundreds of peptide modifications and was normally used for this type of identification. Quantification of metabolites was achieved by using the mass spectrometer in MRM mode, where the target ion was selected in the first quadrupole, fragmented in the second quadrupole, and just one fragment was selected in the last quadrupole to reach the detector. The MRM mode permitted an increase in sensitivity as well as specificity. In the same way, quantification of peptides and proteins was achieved using the Multiquant software.

The hybrid triple quadrupole/linear ion trap (QTRAP 4000) mass spectrometer was handled as an OHSU Proteomics Shared Resource. The equipment had Eksigent nanoflow 2DLC system and Autosampler. It also had a video monitor capable of magnification view of the 400 nl/min nanoflow spray tip with a 10 micron spray opening. Additionally, up to 1 µg of sample was loaded during each injection.

The QTRAP4000 was equipped with a MicroIon II spray source and was used to generate a set of scan reaction monitoring (SRM) response curves for the unmodified and carbamoylated peptides. To imitate the conditions required for detection of these

peptides, desalted peptides were injected onto a reversed phase C18 home-built 150 µm x 0.5 cm trap column of Magic C18 AQ 5 µm, 200 angstrom column material and separated on a home-built 75 µm x 12 cm column of the same material using an Eksigent 2D nano LC equipped with an auto-sampler. The flow rate was 400 nl/min with QTRAP4000 settings of 2000-2200 ion spray voltage, curtain gas 20, nebulizer gas (GS1) 8 and interface heater temperature (IHT) 150 deg C. The column was interfaced to a 75 µm i.d. coated spray tip with a 10 µm opening (New Objective). SRM pairs were entered into Skyline (publicly available quantitation software from the MacCoss Laboratory, University of Washington) for integration of areas of the SRM responses.

3.4.4.5. Mass spectrometric analysis

Ten µg samples of peptide digests were injected onto a trap cartridge (Michrom Bioresources, Auburn, CA) at 20µl/min, samples were washed for 5 min with 2% acetonitrile, 0.1% formic acid, and the trap cartridge placed in-line with a 0.5 x 250 mm SB-C18 reverse phase column (Agilent Technologies, Santa Clara, CA). Peptides were then eluted using a 200 min gradient of 7-30% acetonitrile containing 0.1% formic acid at a flow rate of 10µl/min. Peptide m/z values (MS spectra) and fragment ions (MS/MS spectra) were collected using an LTQ linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA) with an ion max electrospray source fitted with a 34Ga metal needle. Each survey MS spectrum from 400-2000 m/z was followed by 3 data-dependant MS/MS spectra on the 3 most intense ions found in each survey MS spectrum. The instrument used the dynamic exclusion feature of its control software to ignore previously analyzed ions (repeat count of 1, maximum exclusion list of 50, and

30 sec exclusion time), a tune file configured with one μscan, a maximum ion accumulation time of 200m/sec, and AGC targets of 30,000 and 10,000, respectively, for MS and MS/MS spectra.

MS/MS spectra from each analysis were converted to dta files using Bioworks 3.3 (Thermo Scientific) and each MS/MS spectrum matched to peptide sequences in a database using the program Sequest (Version 27, rev. 12, Thermo Scientific). Searches were performed with a static modification of +57 on cysteines for carboxyimidomethylation, and differential modifications of +16 for oxidation of methionines, and +43 for carbamoylation of lysines. The search also used trypsin specificity. A database of rat sequences was created from the Uniprot database (Swiss Bioinformatics Institute, Geneva, Switzerland) downloaded in Feb. 2011 (15,524 entries), the sequences of common contaminants added, and the database amended with sequence reversed entries to estimate peptide false discovery rates. Sequest results were then processed using the program Scaffold (version 4.0.5, Proteome Software, Portland, OR) to produce a list of identified peptides and their carbamoylation sites. Peptide and protein confidence levels of 95% and 99% were used, and a minimum of 2 unique peptides matching each protein entry was required.

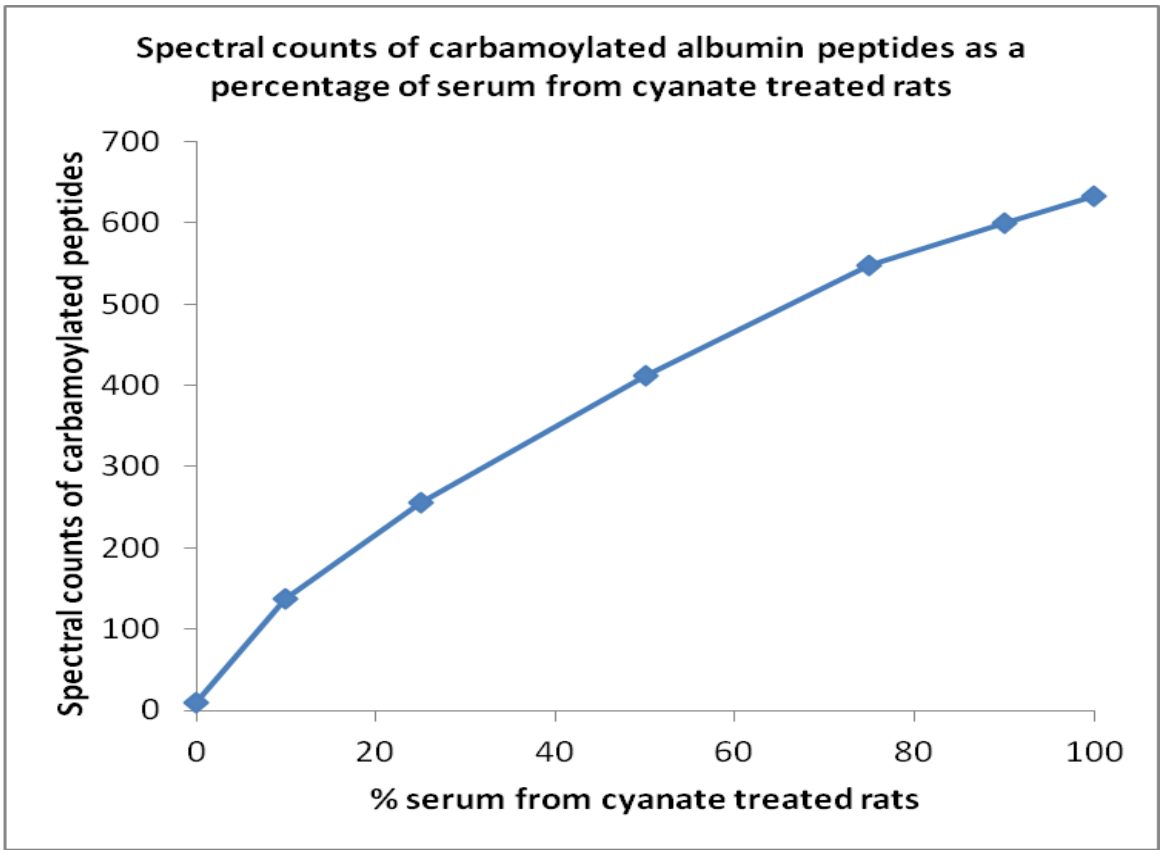
Since few carbamoylated peptides were detected in the soluble fraction of spinal cord, further analysis to test for differences in the level of carbamoylation between different treatment groups was only performed for serum and spinal cord insoluble fractions. Differences in levels of carbamoylation in various treatment groups was determined by

tabulating the numbers of MS/MS spectra assigned to carbamoylated peptides from albumin in serum digests, and the proteins myelin basic protein, neurofilament light polypeptide, 2'3'-cyclic-nucleotide 3'-phosphodiesterase, myelin proteolipid protein, and glial fibrillary acidic protein in digests of water-insoluble proteins from spinal cord. Inclusion lists for each of the identified carbamolyated peptides was created by specifying the m/z value for each in the instrument's control software. This maximized the collection of MS/MS spectra for carbamoylated peptides during the quantitative analysis so that a shorter data collection period was required. This mass spectrometric analysis was identical to those described above. However, a 60 min gradient, inclusion lists specifying the m/z values of the targeted carbamoylated peptides was used, and the dynamic exclusion feature of the instrument's control software was disabled. Differences in the level of carbamoylation in various sample groups was then calculated by summing the numbers of MS/MS scans (spectral counts) assigned to carbamoylated peptides from the target proteins in each sample. The MS/MS scans collected during the analysis were again searched using Sequest and the same database and differential searches for modified peptides as before. However, due to the size of the result files, the Sequest results were filtered using PAW software (Wilmarth, et al 2009), which controlled peptide false discovery using numbers of matches to the sequence reversed entries. Peptides identified as being carbamoylated were separately filtered from unmodified peptides to maintain their false discovery rate below 5%. The linearity of the carbamoylation detection was tested by mixing protein digests from cyanate and vehicle treated animals from the sulfur deficient diet group (**Figure 3.4.4.4-1**). The numbers of identified carbamoylated peptides as a function of percent cyanate treated

serum or spinal cord protein was determined following Sequest searches and filtering of MS/MS data using Scaffold as described above.

Fig 3.4.4.4-1

A)



B)

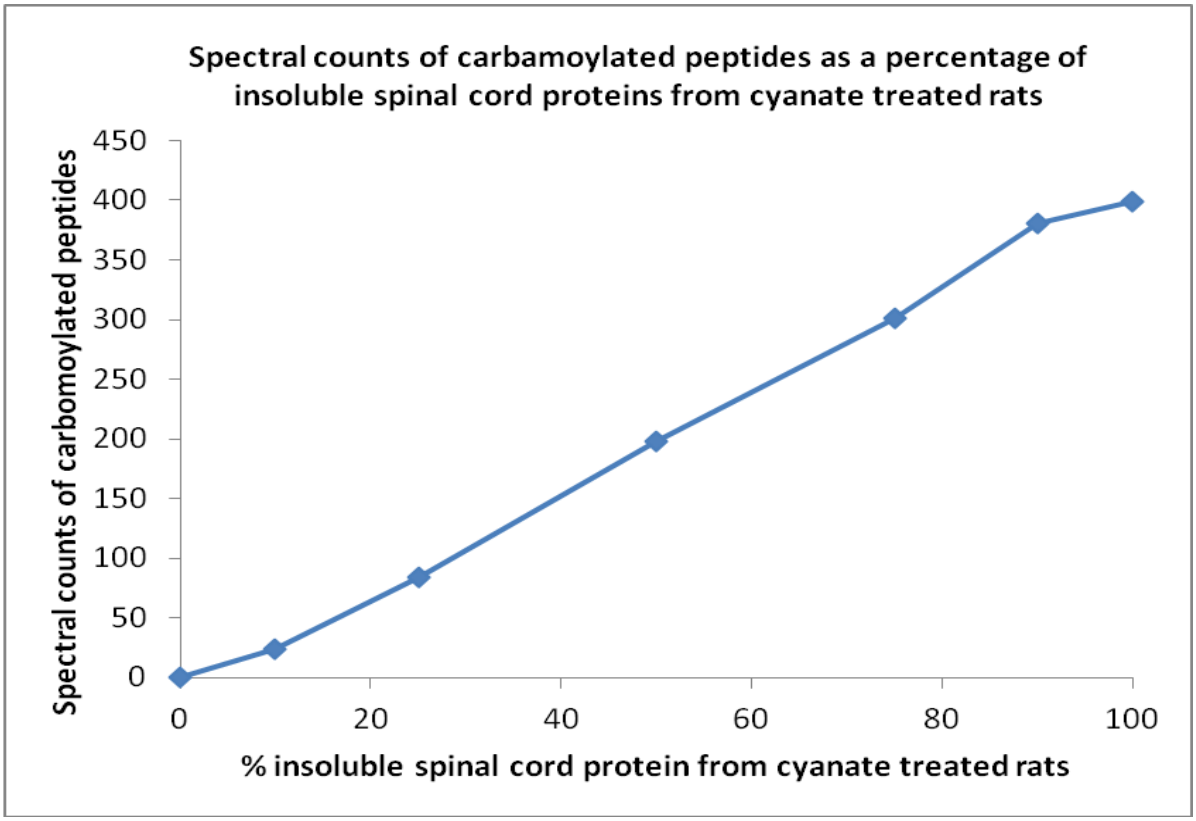


Figure 3.4.4.4-1. Tryptic digests from serum (A) and insoluble spinal cord proteins (B) from cyanate-treated and vehicle-treated rats were mixed and analyzed by LC-MS/MS using inclusion list for the carbamoylated peptides list in supplementary table 2 (**appendix vi & vii**). The resulting numbers of MS/MS scans assigned to carbamoylated peptides (spectral counts) were plotted as a function of the percentage serum and insoluble spinal cord protein from cyanate- treated rats to determine the linearity of carbamoylation assay for serum albumin (R^2=0.97432 and insoluble spinal cord proteins (R^2=0.99658).

3.5 Ethical consideration

Humane handling and prudent use of experimental animals was observed in this study. As regard the rodents, the experimental protocols were approved by the OHSU Institutional Animal Care and Use Committee (IACUC). The non-human primates, the protocols were approved by the Mannheimer Foundation ethical committee on animal care and use (The Mannheimer Foundation, Inc., LaBelle, FL). Animal were feed,

observed and changed for beddings daily to minimize suffering and stress of experimentation. The protocol and good laboratory practice was highly followed.

3.6 Statistical analysis

In study I, rats' body weights were analyzed by ANOVA to determine the effect of treatment on the different parameters of weight. The mean bodyweight changes across the three treatments were analyzed in two stages. A simple linear regression to estimate the initial body weight and mean daily weight change for each animal over the 6 weeks. An ANOVA followed by Bonferroni multiple comparison post hoc tests were used to determine whether the two parameters were affected by treatment. The RAM parameters namely; correct arm entry counts, re-entry counts (WME), entry counts to the unbaited arm (RME), RAM navigation time during working and reference memory were analyzed by ANOVA followed by Bonferroni post hoc tests to establish between and within group differences in terms of the mean and changes of the parameters with each trial over the testing period. All statistical analyses we conducted at the significance level of $p < 0.05$.

In study II, Changes in body weight across the three treatments and two diets were analyzed in a two-stage manner. In the first stage, simple linear regression was used to estimate an intercept and slope coefficient separately for each animal based on the collection of daily weight measurements collected over the course of the study. Thus, the collection of repeated measures per animal was summarized in terms of each animal's initial bodyweight (intercept) and each animal's change in weight per day

(slope). In the second stage, the intercept and slope coefficients were analyzed by ANOVA to determine whether the parameter of interest (intercept or slope) was systematically affected by treatment or diet (or the interaction between the two factors). Rotarod performance was analyzed by dichotomizing performance according to whether the animal remained on the rod for at least 60 seconds. Since each animal contributed exactly two trials, each dichotomized as described above, the final outcome (y) was the number of instances, between the two trials, that the animal lasted for at least 60 seconds (y={0,1,2}). The collection of counted proportions over the study period was analyzed using generalized estimating equations. A secondary analysis of rotarod performance on the termination day used survival analysis to examine time until the animal fell off the rod. A regression analysis was used to determine whether rat CDC were affected by diet, treatment, or their interaction. In non-human primates, total proteins (mg/ml) and CDC were fitted using a mixed-effect model, with treatment and week as the fixed factors and subject (animal) as the random effect. All statistical analyses we conducted at the significance level of $p < 0.05$.

In study III, the rotarod performance was analyzed using generalized estimating equations (GEE) to estimate the odds of remaining on the accelerating rotating rod for at least 60s changed over time (per each additional day of treatment). At study termination, a secondary analysis of the performance on the rotarod was conducted. An overall (log-rank) test on the time needed to fall off the rotarod among the three groups was carried out. Numbers of carbamoylated sites per protein were assumed to follow a Poisson distribution with the mean number of sites dependent upon diet, treatment, or

the interaction between the two. GEE was again used to account for the duplicate samples collected from each animal. Statistical significance was set at 0.05 (0.10 for interaction effects); all p-values are two-sided and confidence intervals set to 95% coverage.

CHAPTER FOUR: RESULTS

4.1 STUDY I: DETERMINATION OF DIFFERENTIAL PATTERNS OF NEUROLOGICAL DEFICITS ASSOCIATED WITH CYANIDE AND CYANATE INTOXICATION

4.1.1 Physical and behavioral observations

The experimental animals did not show any overt physical signs of motor deficit. Except for mild restlessness among the rats treated with cyanide, other signs of acute toxicity namely seizures, breathing problems and restlessness within the initial and subsequent phases of experimentation were not observed with cyanide or cyanate.

4.1.2 Body weight changes

Rats in all the treatment groups gained weight over days albeit differentially (Figure 4.1.2-1). The change in weight was significantly ($F_{2, 19} = 4.11$, $p < 0.05$) influenced by treatment. The mean weight change per day over the experimental period was, NaCN (2.73±0.6), NaOCN (2.28±0.5) relative to controls (3.11±0.5) g. Post hoc test revealed that treatment with NaOCN resulted in significantly ($p < 0.05$) slower rate in weight gain relative to control rats. The change in weight for NaOCN compared to NaCN treated rats was not statistically different ($p=0.59$).

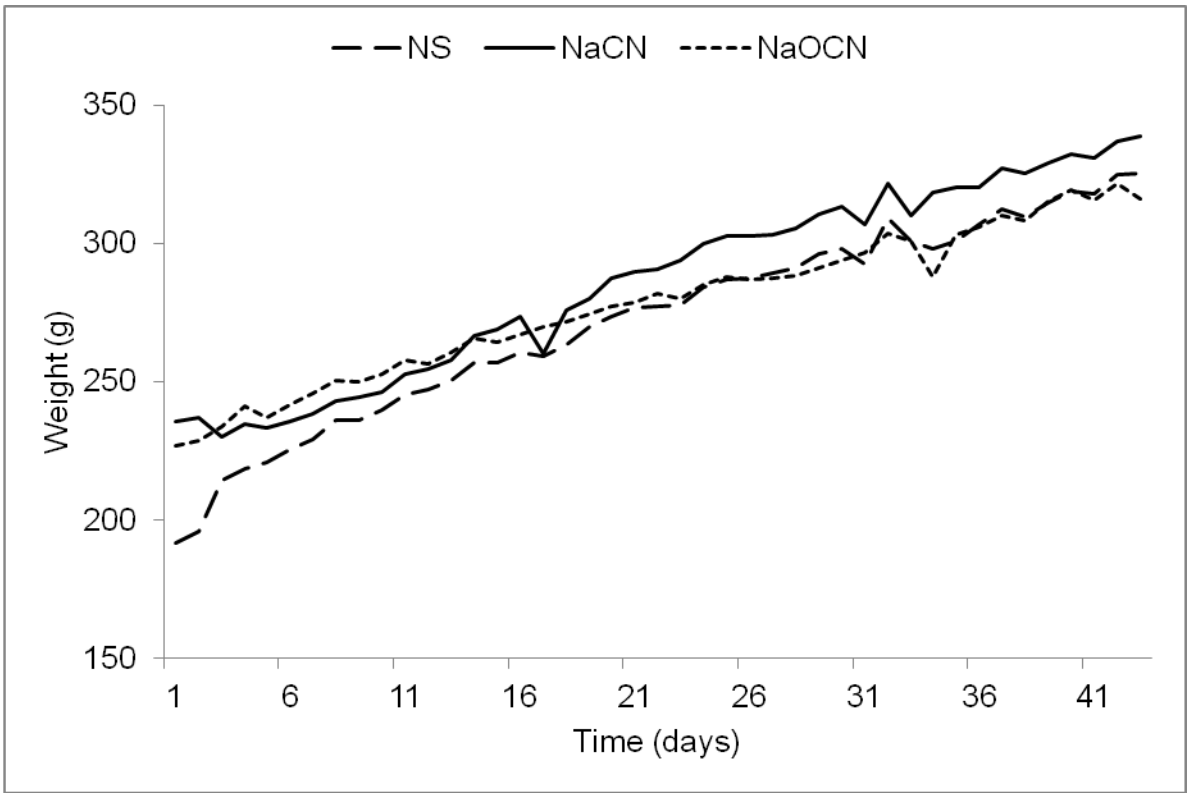


Figure 4.1.2-1. Weight (g) in rodents treated with normal saline, NaCN or NaOCN. Animals treated with NaOCN gained weight slowly ($p < 0.05$) relative to controls but compared to NaCN treated rats, the change was not statistically different.

4.1.3 Short-term memory

The short-term memory results in rats were summarized in terms of number of correct arm entry counts made into all the 8 baited arms, re-entry counts into already visited arms (WME) and the RAM navigation time. There was significant effect ($F_{2, 19} = 4.57$ $p < 0.05$) of treatment on correct arm entry counts into unvisited arm by rats navigating the RAM (**Figure 4.1.3-1a**). The mean correct arm entry counts across the trials among rats treated with NaOCN (3.6±0.7) were fewer relative to NaCN (4.1±0.4) and controls (6.2±0.6). The correct arm entry counts decreased significantly ($p < 0.05$) with each trial in NaOCN (-0.19±0.2), relative to NaCN (0.13±0.2) and saline (0.16±0.3) treated rats.

However, no statistical difference (p=0.8) in correct arm entry counts was found with each trial between NaCN compared to the saline treated rats over the testing period.

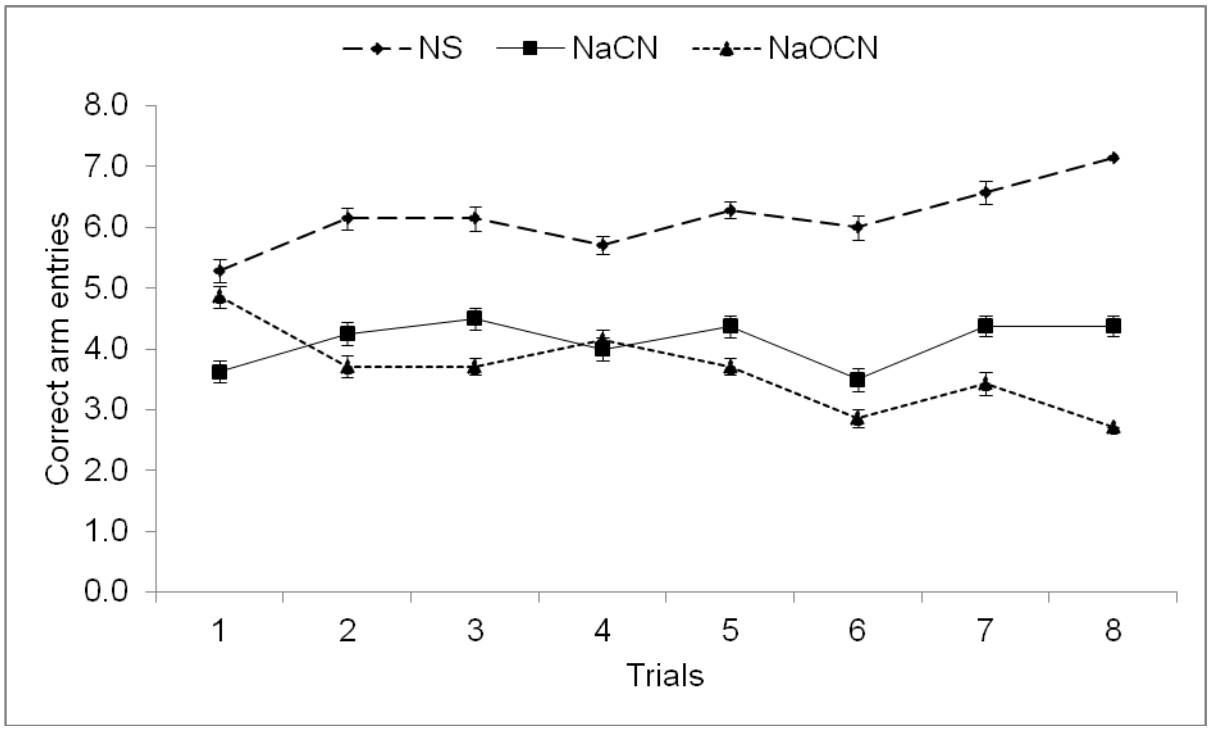


Figure 4.1.3-1a. Correct arm entry counts into 8 baited arms over the 8 trials among rats treated with NS, NaCN or NaOCN. The mean correct arm entry counts over the 8 trials in NaOCN (3.6±0.7) were fewer compared to NaCN (4.1±0.4) and control (6.2±0.6) treated rats, respectively. The entry counts decreased significantly ($p < 0.05$) with each trial in NaOCN (-0.19±0.2) compared to an increase in both NaCN (0.13±0.2) and control (0.16±0.3) groups. However, no statistical (p=0.8) difference between NaCN and controls with each trial was demonstrated.

The re-entry counts into already visited arms (WME) were significantly influenced ($F_{2, 19} = 5.09$, $p < 0.05$) by treatment during RAM testing (**Figure 4.1.3-1b**). The mean re-entry counts across the trials in rats treated with NaOCN (85.05±7.7) were higher ($p < 0.05$) relative to NaCN (77.98±4.4) and saline (26.11±2.6) %, respectively. The re-entries increased with each trial in NaOCN (8.40±2.2) compared to a decrease in NaCN (-1.18±0.4) (p=0.05) and control (-3.21±0.9) (p=0.01) groups. However, no statistical

(p=0.5) difference was found with each trial between NaCN and saline treated rats on re-entry counts over the testing period.

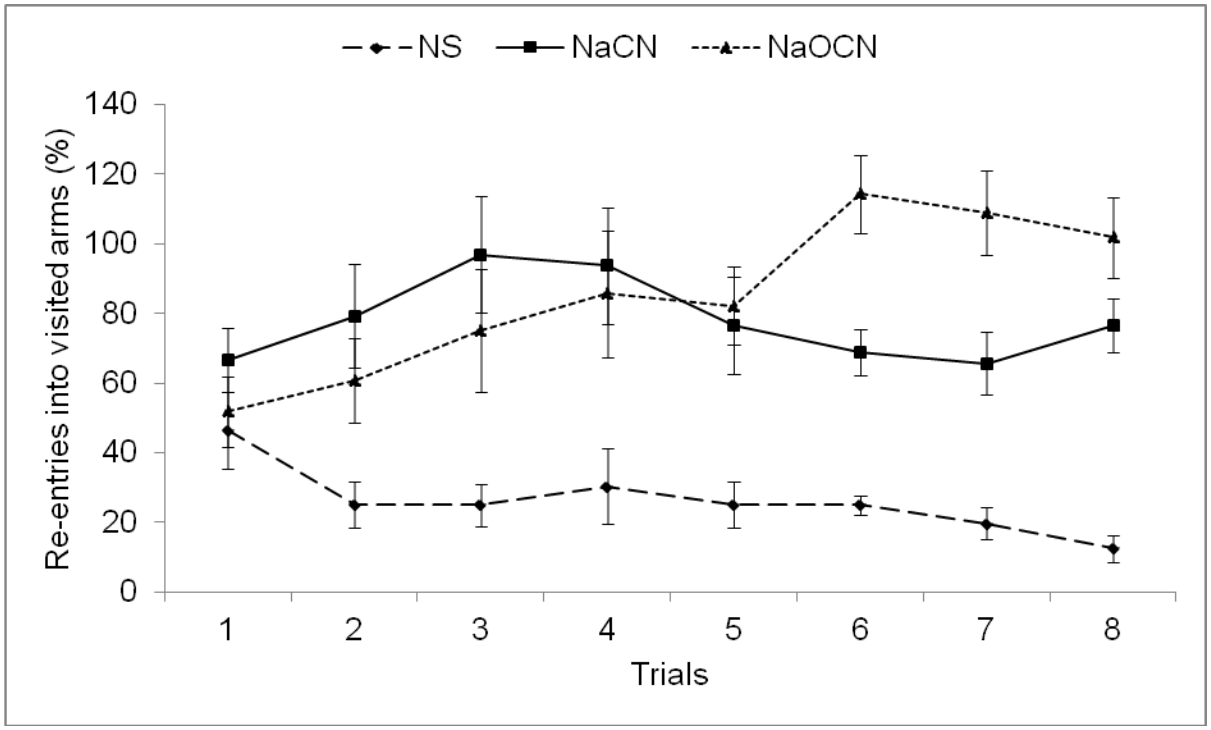


Figure 4.1.3-1b. Re-entry counts into already visited arms (WME) among rats treated with NS, NaCN or NaOCN. The mean re-entry counts into visited arms over the 8 trials in NaOCN (85.05±7.7) were higher compared to NaCN (77.98±4.4) and control (26.11±2.6) treated rats. The re-entry counts increased with each trial in NaOCN (8.40±2.2) compared to NaCN (-1.18 ±0.4) ($p < 0.05$) and control (-3.21± 0.9) ($p < 0.01$) treated rats, respectively. However, no statistical (p=0.5) difference was realized with each trial between NaCN and control treated rats.

The RAM navigation time was significantly ($F_{2, 19} = 3.91$, $p < 0.05$) influenced by treatment among experimental rats (**Figure 4.1.3-1c**). The mean RAM navigation time across the trials in rats treated with NaOCN, NaCN relative to controls during short-term memory testing were (6.26±0.3), (4.72±0.1) and (2.82±0.3) minutes. The navigation time changed with each trial, it increased in NaOCN (0.25±0.03) and NaCN (0.02±0.01) relative to saline (-0.01±0.02) treated rats. The change in navigation time with each trial

was also significantly ($p < 0.05$) higher in the rats treated with NaOCN compared to controls. However, comparing the change between NaOCN relative to NaCN treated rats with each trial, no statistical difference ($p = 0.23$) was yielded over the testing period.

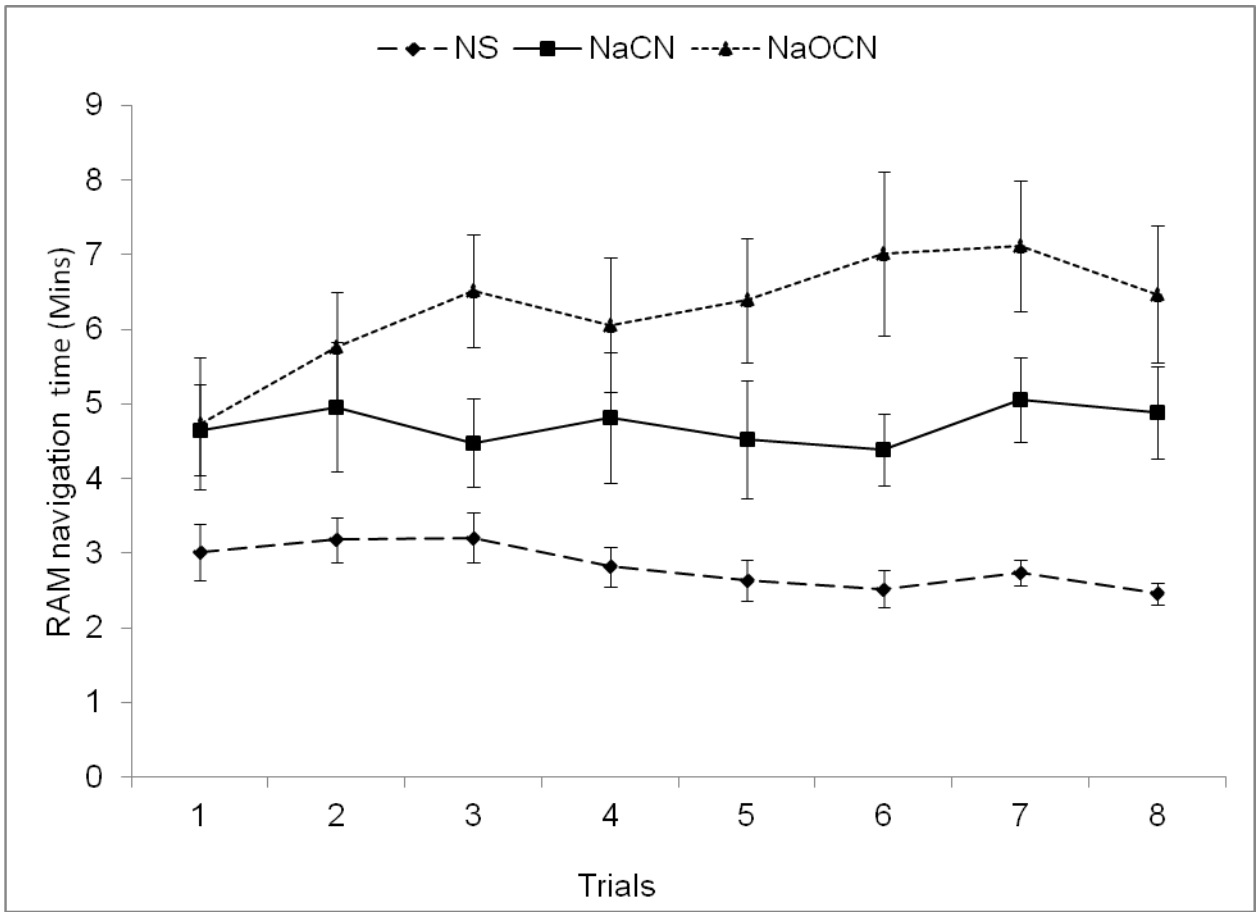


Figure 4.1.3-1c. RAM navigation time into 8 baited arms among rats treated with NS, NaCN or NaOCN. The mean RAM navigation time over the 8 trials among rats treated with NaOCN (6.26±0.3) was longer compared to NaCN (4.72±0.1) and controls (2.82±0.3) minutes, respectively. The navigation time increased with each trial in NaOCN (0.25±0.03) relative to NaCN (0.02±0.01) and controls (-0.01±0.02) ($p < 0.05$), respectively. However, there was no statistical ($p = 0.13$) difference with each trial between NaOCN and NaCN treated animals on RAM navigation time.

4.1.4 Long-term memory

The long-term memory performance results were summarized in terms of number of correct arm entry counts into baited arms, re-entry counts into already visited baited arms (WME), entry counts into un-baited arms (RME) and the RAM navigation time with only 4 out of 8 baited arms. There was significant effect ($F_{2, 19} = 7.45$ $p < 0.01$) of treatment on the correct arm entry counts into the baited arms (**Figure 4.1.4-1a**). The mean arm entry counts across the 10 trials among rats treated with NaOCN, NaCN relative to saline were (3.1±0.6), (3.0±0.3) and (3.8±0.2). The correct arm entry counts decreased with each trial in NaCN (-0.08±0.1), but increased in NaOCN (0.14±0.2) and saline (0.02±0.03) treated rats. The change in the entry counts with each trial was significantly higher among NaCN compared to saline ($p < 0.01$) and NaOCN ($p < 0.05$) treated rats. However, no statistical difference ($p=0.15$) was realized on entry counts with each trial between NaOCN relative to saline treated rats.

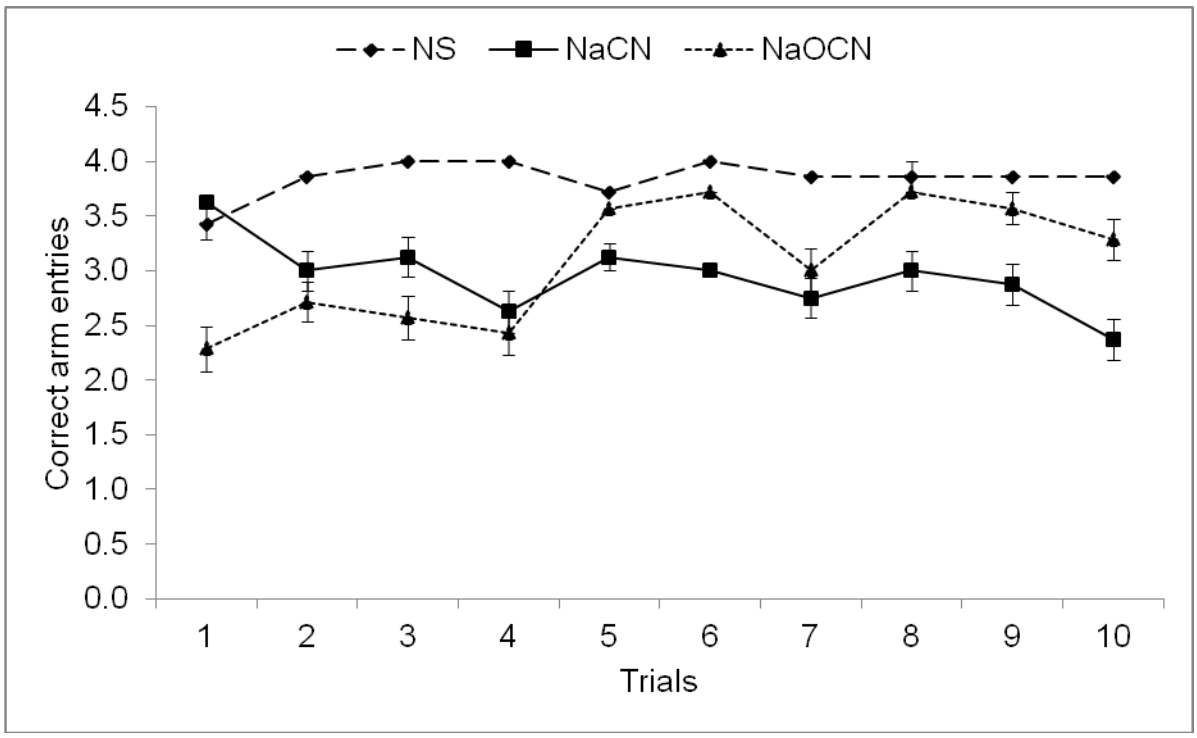


Figure 4.1.4-1a. Correct entry counts into 4 baited arms of RAM in rats treated with NS, NaCN or NaOCN during long-term memory assessment. The mean arm entry counts over the 10 trials among rats treated with NaOCN (3.1±0.6) and NaCN (3.0±0.3) were fewer relative to controls (3.8±0.2), respectively. The entry counts however, decreased with each trial in NaCN (-0.08±0.09) compared to NaOCN (0.14±0.16) ($p < 0.05$) and control (0.02±0.03) treated rats, respectively. However, no statistical ($p = 0.08$) difference was realized with each trial between NaOCN and controls.

There was significant influence ($F_{2, 19} = 9.35$ $p < 0.05$) of treatment on re-entry counts (WME) into the baited arms (**Figure 4.1.4-1b**). The mean re-entry counts into already visited baited arms across the 10 trials in rats treated with NaOCN, NaCN and saline were (25.50±7.5), (30.8±5.7) and (3.8±2.1), respectively. The re-entries changed in each trial, with an overall decrease among animals treated with NaOCN (-8.20±3.4), NaCN (-3.2±2.4) and saline (-4.7±1.3). The decrease in re-entry counts was significantly higher with each trial among NaOCN treated rats compared to NaCN ($p < 0.001$) and

saline (p <0.05) groups. However, compared to the controls, rats treated with NaCN had marginal (p=0.07) decrease in re-entries with each trial into already visited baited arms.

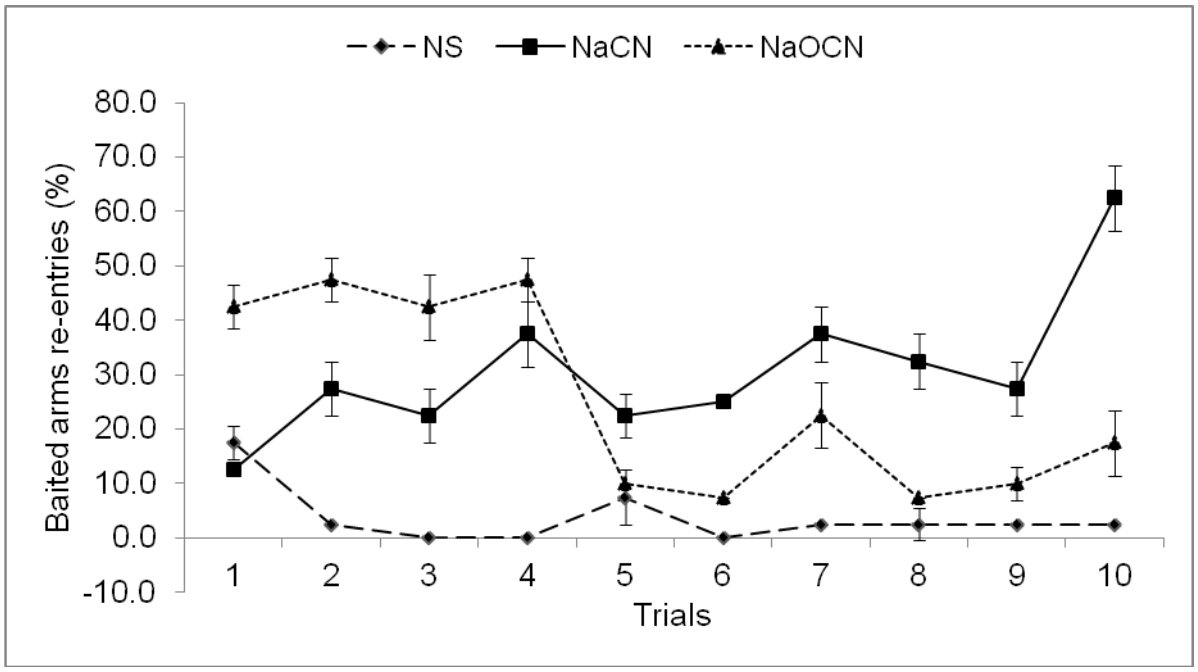


Figure 4.1.4-1b. Re-entry counts into visited baited arms (WME) by rats treated with NS, NaCN or NaOCN during long-term memory assessment. The mean re-entry counts over the 10 trials among rats treated with NaCN (30.8±5.7) were higher compared to NaOCN (25.50±7.5) and controls (3.8±2.1), respectively. The re-entry counts decreased with each trial in NaOCN (-8.20±3.4), compared to NaCN (-3.2±2.4) (p <0.05) and control (-4.7±1.3) (p <0.05), treated rats respectively. A marginal statistical (p=0.07) difference was realized with each trial between NaCN relative to controls.

There was no significant influence ($F_{2, 19} = 1.70$ p =0.20) of treatment on entries into un-baited arms (RME) among rats navigating RAM (**Figure 4.1.4-1c**). The mean entry counts into the un-baited arms across the 10 trials among rats treated with NaOCN, NaCN relative to saline were (4.3±0.8), (5.1±0.6) and (2.2±0.7), respectively. The counts changed with each trial, with an overall decrease in rats treated with NaOCN (-0.35±0.3), NaCN (-0.1±0.1) and saline (-0.29±0.2). The entries decreased more with

each trial among NaOCN treated rats compared to NaCN and control groups, though the difference was not statistically significant.

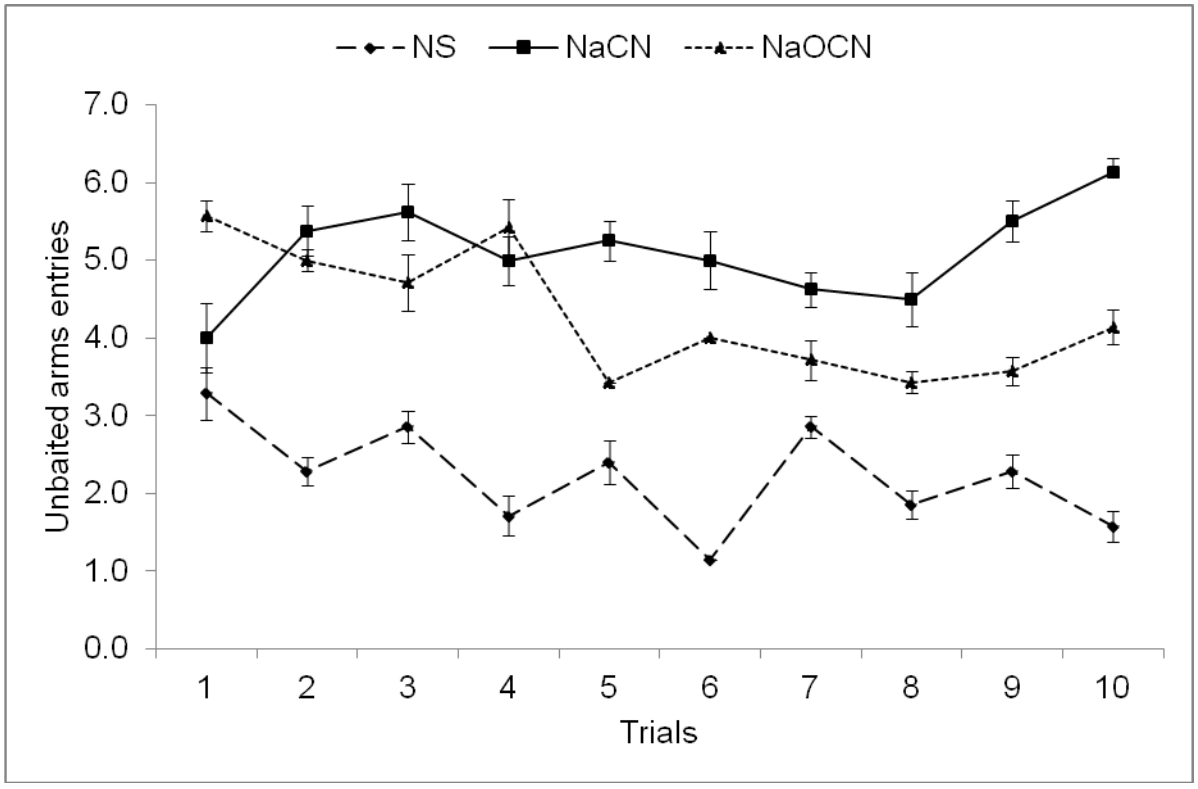


Figure 4.1.4-1c. Entry counts into un-baited arms (RME) by rats treated with NS, NaCN or NaOCN during long-term memory assessment. The mean entry counts into the un-baited arms over the 10 trials among rats treated with NaCN, (5.1±0.6) was higher compared to NaOCN (4.3±0.8), and control (2.2±0.7), respectively. The entry counts decreased with each trial more in NaOCN (-0.35±0.3), compared to NaCN (-0.1±0.1) and controls (-0.29±0.2), respectively, but no statistical difference was yielded.

There was no significant effect ($F_{2, 19} = 2.49$ $p = 0.12$) of treatment on RAM navigation time among experimental rats (**Figure 4.1.4-1d**). The mean RAM navigation time across the 10 trials in rats treated with NaOCN, NaCN relative to controls during long-term memory testing were (3.73±0.9), (3.64±0.4) and (1.90±0.1) minutes, respectively. The navigation time decreased with each trial among rats treated with NaOCN (-0.25±0.03), NaCN (-0.05±0.02) relative to saline (-0.0004±0.1), respectively. The change in RAM

navigation time was significantly ($p < 0.05$) higher with each trial in the rats treated with NaOCN compared to controls. However, a comparison between NaOCN and NaCN treated rats did not demonstrate any statistical difference ($p = 0.10$).

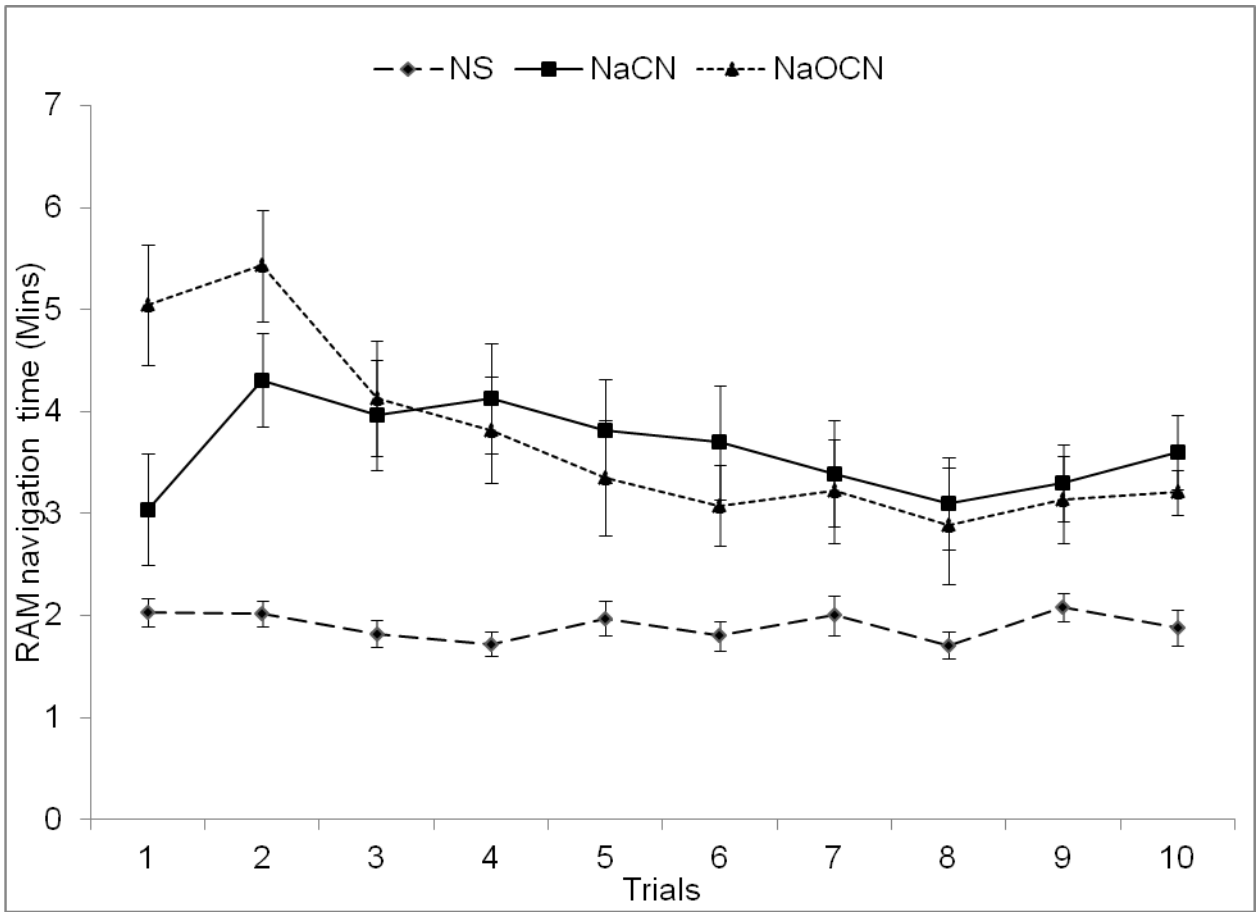


Figure 4.1.4-1d. RAM navigation time among rats treated with NS, NaCN or NaOCN during long-term memory assessment. The mean RAM navigation time over the 10 trials in rats treated with NaOCN (3.73±0.9) and NaCN (3.64±0.4) were longer compared to controls (1.90±0.1) minutes, respectively. The time decreased significantly ($p < 0.05$) with each trial among NaOCN (-0.25±0.03) relative to control (-0.0004±0.1) treated rats. However, there was no statistical ($p = 0.10$) difference realized with each trial between NaOCN and NaCN (-0.05±0.02) treated rats.

4.2 STUDY II: DETERMINATION OF THE IMPACT OF PROTEIN-RESTRICTED DIET ON CYANIDE AND CYANATE TOXICITY IN RODENTS AND NON-HUMAN PRIMATES.

4.2.1 Physical and behavioral changes in rodents and non-human primates

Overt physical manifestations of seizures were observed in rats treated with NaCN and maintained on the SAA-restricted diet. The event seizures occurred after the first week of the study specifically on days 8 (2 rats), 9 (3 rats), 10 (2 rats), 12 (4 rats), 13 (5 rats), 17 (4 rats), 19 (3 rats) and 1 animal per day for days 26, 27, 31, and 32, respectively. All these animals developed seizures within 45 minutes following the injection with NaCN. No Seizures were observed in animals treated with NaCN, NaOCN or vehicle under AAA diet, or NaOCN or vehicle under SAA-restricted diet.

Animals treated with NaOCN/SAA-restricted diet developed motor deficits relative to their NaCN and vehicle-treated counterparts. Odds of remaining on the rotarod for at least 60 seconds (1 minute; a convenient and interpretable choice) did not significantly change over time for NS- and NaCN-treated rats, with the odds decreasing by 4% for each additional day of testing ($p \sim 0.10$ for each group). By contrast, the odds of remaining on the rod decreased 14% (95% CI: 6--21% decrease) per day of testing ($p<0.001$) for animals on NaOCN. A secondary analysis examined motor performance at study termination. An overall (log-rank) test showed the time needed to fall off the rotarod significantly differed across the three groups ($p=0.040$), but the same comparison restricted to NaCN and NS animals showed no difference ($p=0.16$; log-rank test), suggesting the difference in performance was limited to NaOCN. A description of

how the odds of remaining on the rod changed over time (per each additional day of treatment) is shown in **Table 4.2.1-1 and Figure 4.2.2-1**. Regardless of the type of the diet, *macaca fascicularis* remained alert and responsive. No abnormality in gait, arms or hand coordination, were observed during the course of the study. One animal developed a metabolic derangement as a result of hyperglycemia, which required treatment with insulin.

Table 4.2.1-1. Changes in estimated odds of remaining on rotarod for at least 60 seconds for each additional day (SAA diet, separated by treatment).

Treatment	OR (95% CI)	P value for trends	P value for comparisons
Saline (n =7)	0.96 (0.96 – 1.01)	0.099	0.87*
NaCN (n=9)	0.96 (0.92 – 1.01)	0.096	0.046†
NaOCN (n=8)	0.86 (0.79 – 0.94)	0.001	0.029‡

The odd of remaining on the rod significantly decreased over time in animals treated with NaOCN (p=0.001). The differences were significant when comparing with saline (†) or NaCN (‡). No difference was seen between saline and NaCN-treated groups (*).

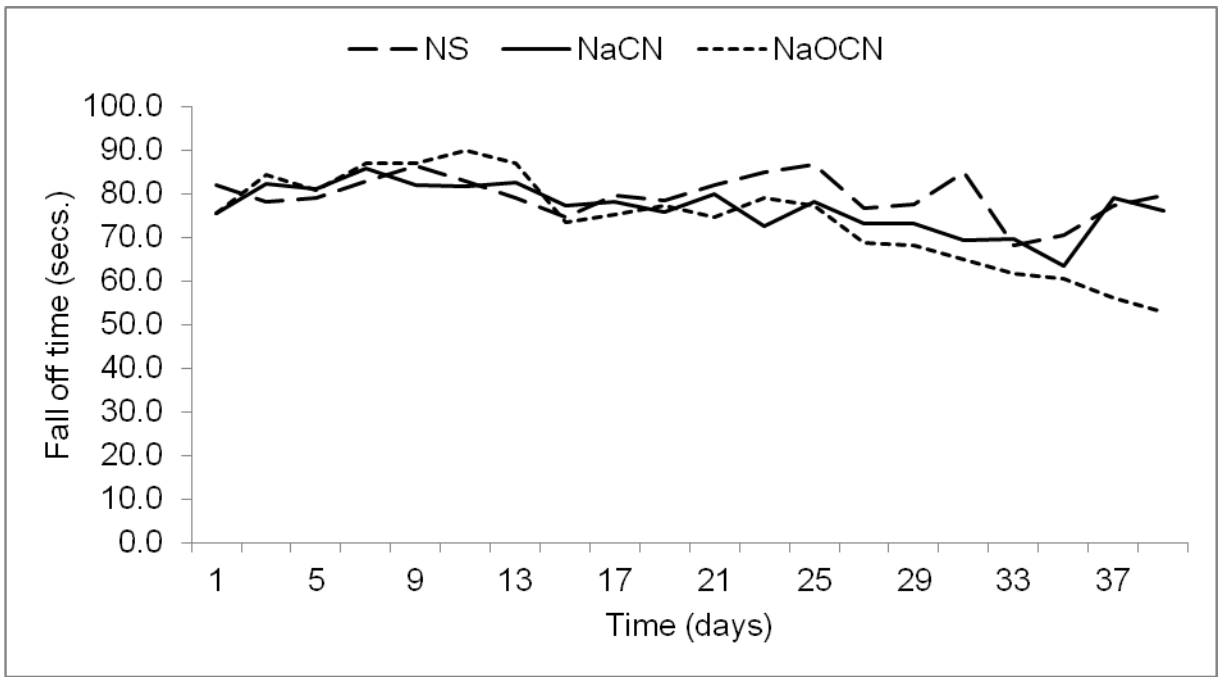


Figure 4.2.1-1. Rotarod performance in rats treated with normal saline (NS), NaCN or NaOCN and fed SAA-restricted diet. Treatment affected rotarod fall off time in rats. The fall time from the rotating rod decreased significantly ($p < 0.001$) among rats treated with NaOCN compared to control and NaCN over time.

4.2.2 Body weight changes

The initial body weight (day 1) of rodents was not significantly different across the three treatment groups for either diet (p=0.47, AAA diet; p=0.19, SAA-restricted diet), although initial weight was, on average, 52.5 (95% CI: 37.3--67.7) grams lower for those animals in AAA diet compared to SAA-restricted diet. Differences across the three treatment groups with respect to daily changes in body weight was influenced by type of diet [$F(2,46)=3.42$, $p=0.001$; test of treatment:diet interaction]; consequently, the effect of treatment was summarized separately within each type of diet. Overall, animals placed on SAA-restricted diet gained less body weight relative to those on the AAA diet, regardless of treatment (**Table 4.2.2-1)**. Follow-up tests revealed that within the AAA

Diet, animals treated with NaOCN gained weight at a significantly reduced rate relative to rates of weight gained by animals in both the NS and NaCN groups, while animals in the NS and NaCN groups gained weight at essentially the same rate (**Figures 4.2.2-1a**). However, within SAA Diet, the average daily change in body weight did not significantly differ among the three treatments [F(2,46)=0.39, p=0.68] (**Figures 4.2.2-1b**).

Table 4.2.2-1. Weight (g) changes in rodents treated with normal saline, NaCN and NaOCN over time under AAA or SAA- restricted diets.

		Weight change per day			
Diet	Treatment	Range	Median (IQR)	Mean	SD
AAA	Vehicle (n=9)	2.90-4.17	3.73 (3.22-3.94)	3.59	(0.46)
AAA	NaCN (n=10)	2.84-4.53	3.32 (3.09-3.57)	3.45	(0.54)
AAA	NaOCN (n=9)	1.67-3.27	2.23* (2.01-2.60)	2.29 *	(0.49)
SAA	Vehicle (n =7)	0.23-1.71	0.84 (0.53-1.21)	0.89	(0.53)
SAA	NaCN (n=9)	0.48-1.59	0.88 (0.72-1.40)	1.00	(0.41)
SAA	NaOCN (n=8)	0.32-1.19	0.81 (0.68-1.04)	0.80	(0.31)

Rodents fed on SAA diet gained weight at a slower rate compare to those under normal AAA diet. Among those on normal AAA diet, animals treated with NaOCN slowly gained weight in comparison to those on saline or NaCN (apteryx) (P<0.05).

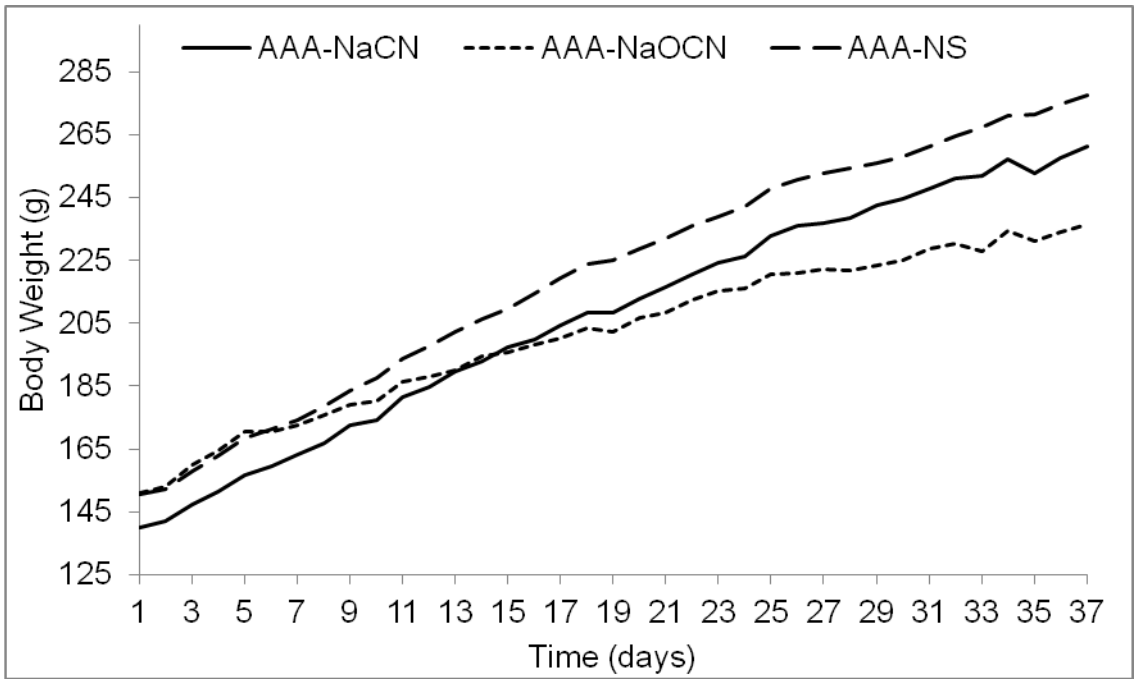


Figure 4.2.2-1a. Weight (g) changes in rodents treated with normal saline, NaCN or NaOCN and fed on AAA diet over time. Rats treated with NaOCN, gained weight slowly compared to control or their NaCN counterparts ($p < 0.05$). Rats treated with NaCN and control gained weight almost at the same rate.

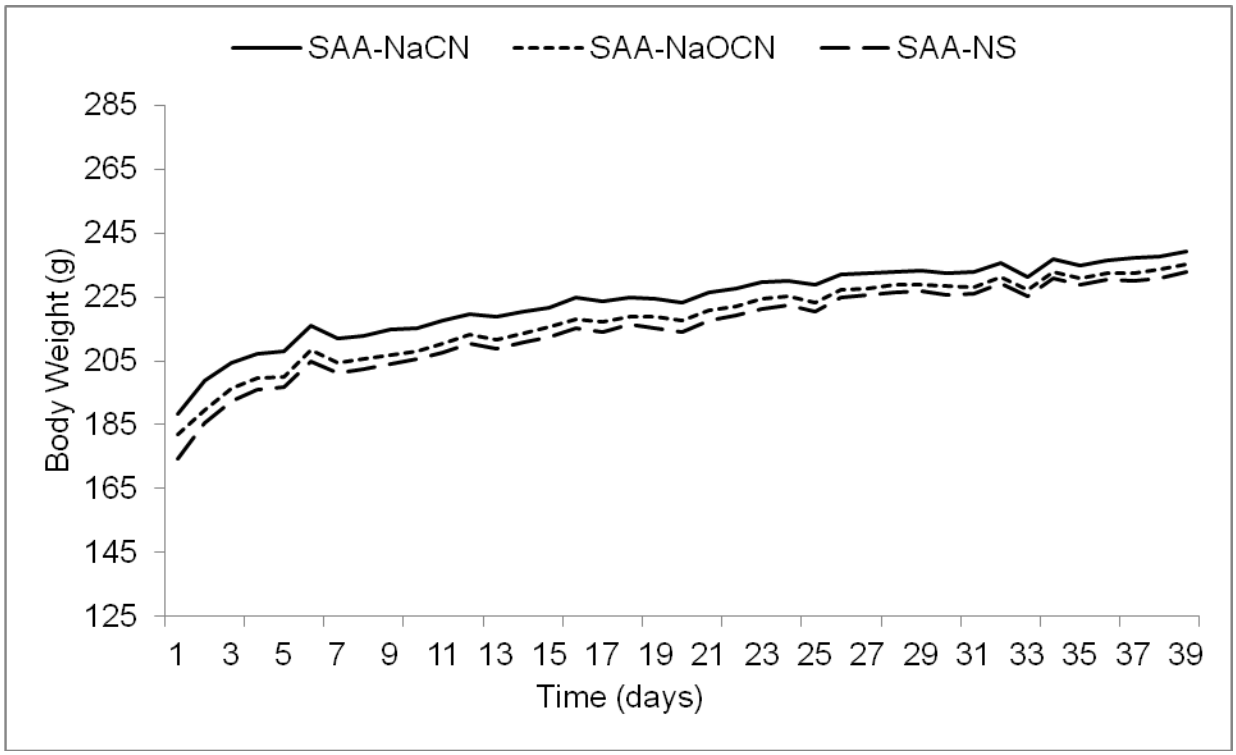


Figure 4.2.2-1b. Weight (g) changes in rodents treated with normal saline, NaCN or NaOCN and fed on SAA diet over time. All animals gained weight essentially at the same rate regardless of treatment. The daily change of weight among rats was approximately 0.9 g regardless of the treatment.

Non-human primates appeared to have similar weight change patterns across the two diets, i.e. cassava versus control primate chow. However, their body weights significantly ($p < 0.05$) decreased over time, regardless of the type of diet. A marginal difference was observed between animals under the cassava-diet, which had lost more weight, and those under the primate chow ($p=0.06$). Relative to the initial body eight, animals under the cassava diet lost more weight (8.22%) compared to those under the primate chow (1.63%) (**Figure 4.2.2-2**).

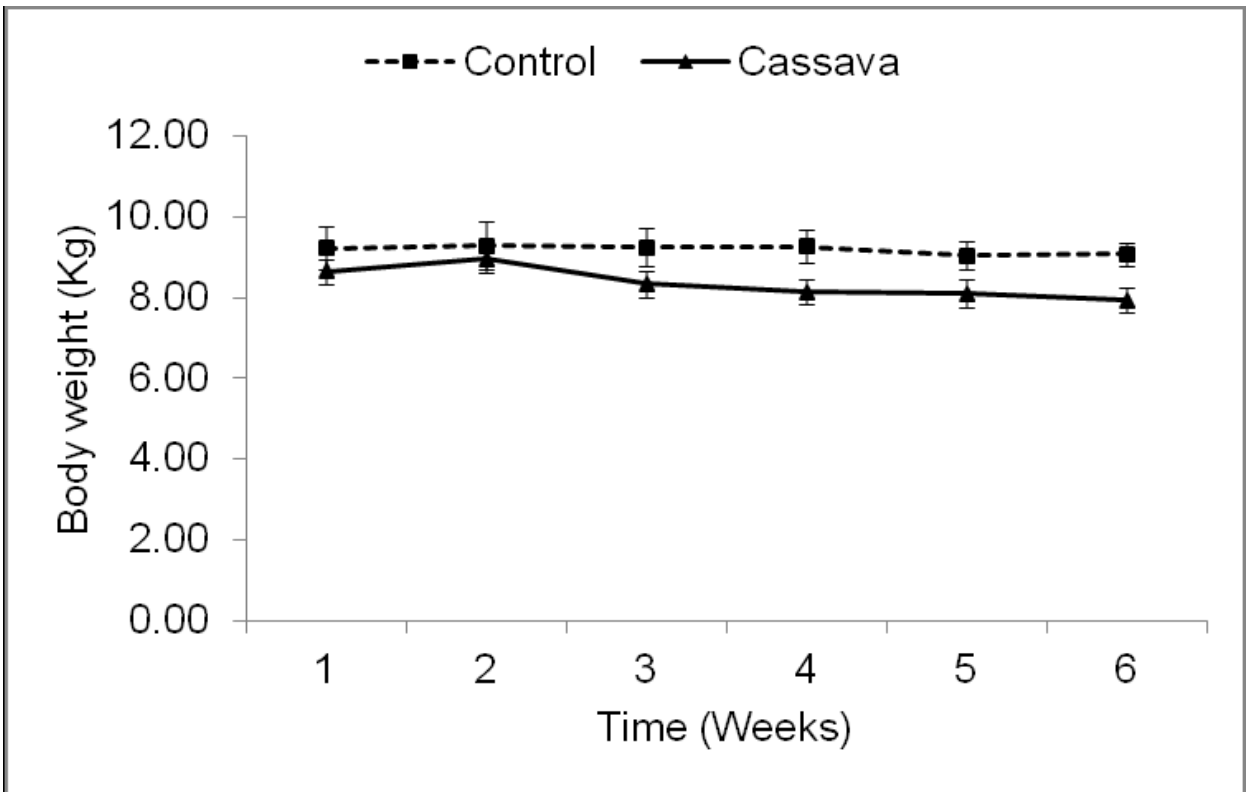


Figure 4.2.2-2. Weight (g) changes in non human primates fed on control or cassava diets over time. Animals fed on cassava lost more weight compared to the group on primate chow control diet which appeared to maintain a stable weight over time.

4.2.3 Total plasma proteins

Neither the diet nor the treatment had a significant effect on total plasma proteins in rodents. In non-human primates, there was no indication that differences between diet changed over the six weeks [$F(5,50)=1.57$, $p=0.18$; test of diet:week interaction]. Consequently, the effects of diet and time were summarized as separate main effects.

Average protein level did not significantly differ between the two diets [$t(10)=0.90$, $p=0.39$]. There were significant changes over time, however [$F(5,55)=9.50$, $p<0.001$],

relative to week 1, with a significant decreased in proteins during the last two weeks (**Table 4.2.3-1**).

Table 4.2.3-1. Plasma proteins (mg/ml) in non human primates across control vs. cassava diet over six-week period.

	Diet	
Week	Control (S.E.M)	Cassava (S.E.M)
1	69.05 (1.98)	71.94 (2.26)
2	68.64 (2.40)	71.53 (2.74)
3	69.89 (1.86)	72.78 (2.79)
4	72.14* (2.80)	75.03* (3.08)
5	65.81* (1.74)	68.69* (2.78)
6	66.47* (2.29)	69.36* (2.32)

Plasma proteins (mg/ml) in non human primates markedly ($p < 0.05$) declined during the last two weeks (5 & 6) of the study period regardless of the diet. *$P<0.05$

4.2.4. Tissue variations in rodents cyanide detoxification rates

Differential pattern of cyanide detoxification capabilities (CDC) across tissues was observed.The highest (faster) detoxification rate was observed in brain, followed by the spinal cord, and lastly, plasma. Overall, the time required to produce one µmol of thiocyanate per mg protein was ~ 80X more in plasma relative to brain and spinal cord regardless of the diet (**Table 4.2.4-1**). Plasma rates were not influenced by diet or treatment (**Figure 4.2.4-1a)**

Table 4.2.4-1. Cyanide detoxification rate time (mean ± S.E.M ms/[µmol/mg] to produce one µmol thiocyanate) in plasma and nervous system tissues of rodents.

		Diet	
Tissue	Treatment	AAA (mean ± S.E.M ms/[µmol/mg)	SAA (mean ± S.E.M ms/[µmol/mg)
Brain	NS	13.97 (1.35)	12.65 (1.98)
	NaCN	19.38 (2.72)	18.05 (1.94)
	NaOCN	20.68 (2.35)	19.35 (1.79)
Spinal cord	NS	18.95 (6.77)	17.63 (5.57)
	NaCN	15.37 (8.30)	60.15 (7.89)
	NaOCN	35.54 (5.89)	28.25 (9.71)
Plasma	NS	1078.51 (125)	1117.92 (38)
	NaCN	1134.89 (26)	1180.89 (55)
	NaOCN	1130.69 (79)	968.14 (46)

Cyanide detoxification rates required to produce one µmol thiocyanate in brain or spinal cord were ~ 80X lower than that required for the same process in rodent plasma i.e. brain and spinal cord detoxify ~ 80X faster than plasma.

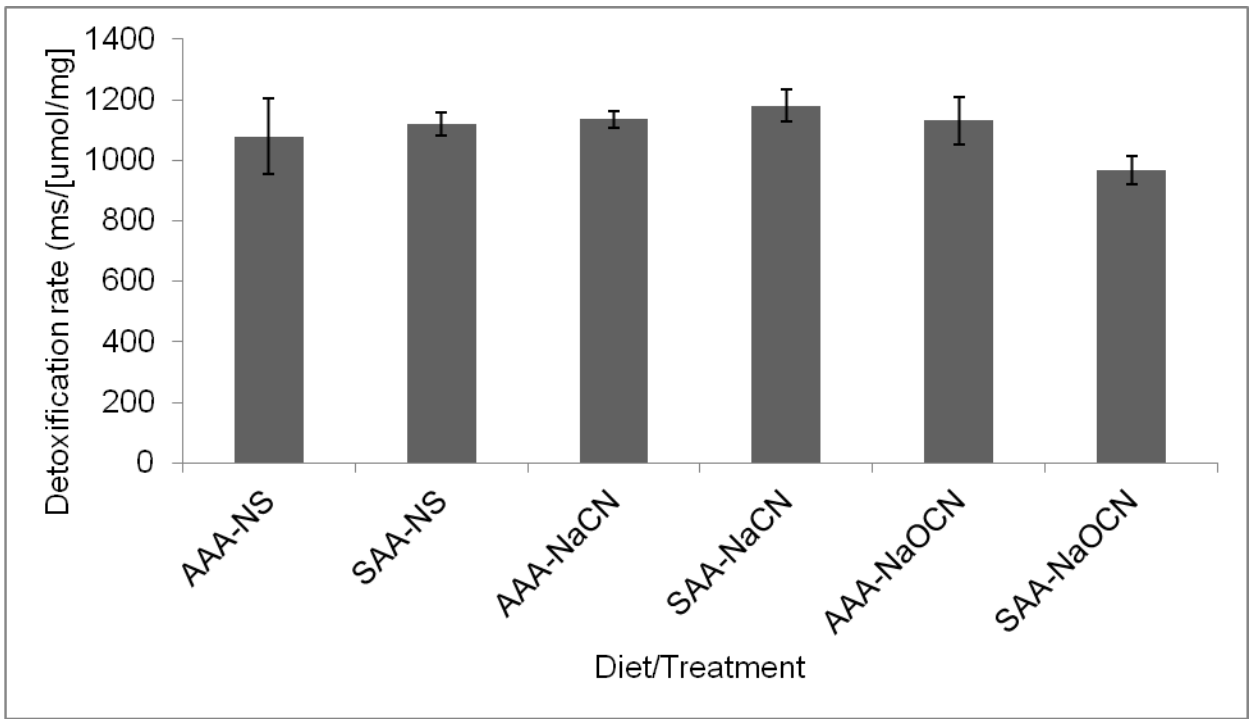


Figure 4.2.4-1a. Cyanide detoxification rates (mean ± S.E.M ms/[µmol/mg] time to produce one µmol thiocyanate) in rat plasma across SAA or AAA diets. The detoxification rates in plasma were not influenced by the diet or treatment with either NaCN or NaOCN. The plasma cyanide detoxification rates among the rodents under the two diets or the three treatments were not statistically different.

With regard to rodent nervous system, CDC was influenced by diet or chemical treatment, in the spinal cord, but only by chemical treatment in the brain. A significant effect of diet-treatment interaction on the detoxification activity in the spinal cord (χ^2=13.2; df =2; p <0.001) was yielded. The detoxification rates in the NaCN group were slower (p<0.001) under SAA-restricted diet relative to the normal AAA diet (up to 60.15 ms vs. 15.37 ms, respectively). Within the SAA-fed group, the detoxification rates in the NaCN-treated animals were slower compared to those in animals treated with saline (p<0.001) or NaOCN (p<0.05), respectively. Within the AAA normal diet, however, the time required to produce one µmol of thiocyanate was not significantly different for either NaCN relative to saline (p=0.74) or between NaOCN compared to saline

(p=0.065). The rates were, however, marginally slower for NaOCN compared to NaCN (p=0.048) (**Figure 4.2.4-1b**).

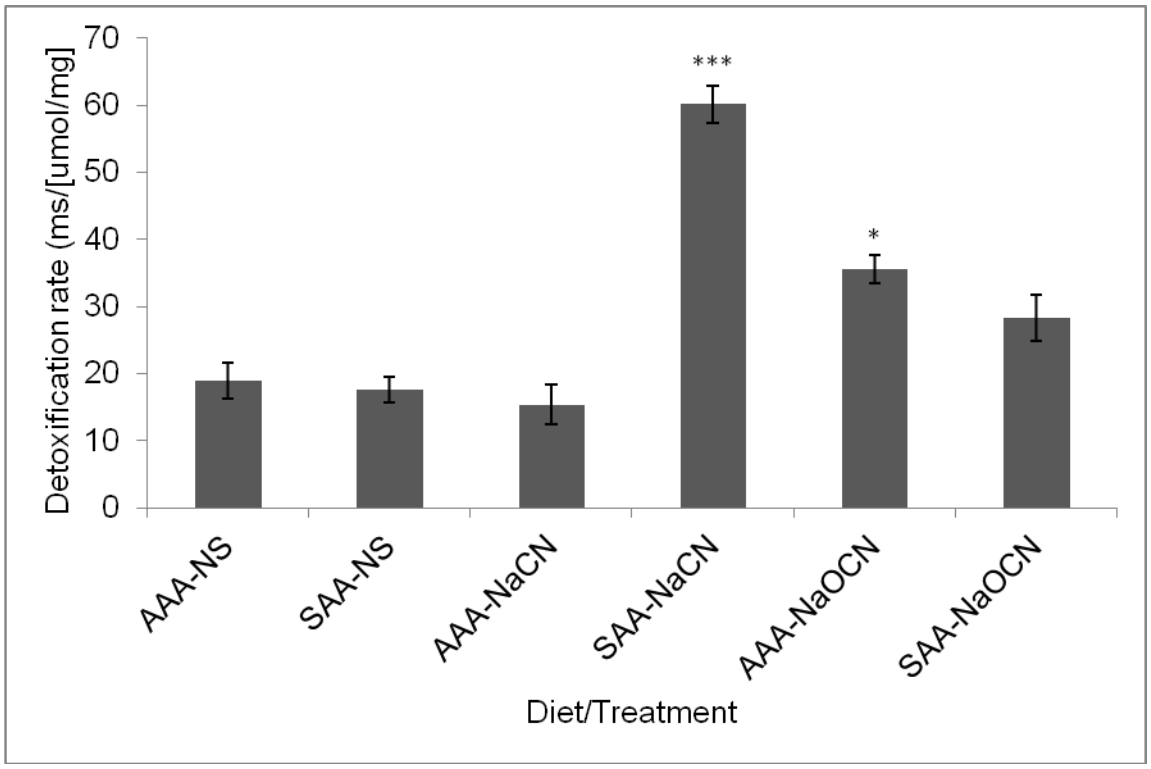


Figure 4.2.4-1b. Rodent cyanide detoxification rates (mean ± S.E.M ms/[µmol/mg] time to produce one µmol thiocyanate) in spinal cord were influenced by diet or treatment. Animals treated with NaCN and fed with SAA-deficient diet needed longer time (***p <0.001) relative to their counterparts under normal AAA diet, or those on SAA diet but under saline or NaOCN. Under the AAA diet, animals treated with NaOCN (*) required more detoxification time relative to those on NaCN.

The CDC of rodent brain was significantly (p<0.05) influenced by chemical treatment only. We found no effect of diet, regardless of treatment (p=0.50). With regards to chemical treatment, rates averaged 5.4 (95% CI: 0.4-10.4) ms slower in animals treated NaCN relative to saline (p<0.05) or 6.7 (95% CI: 2.2-11.2) ms slower in NaOCN treated rats compared to saline (p<0.05) regardless of the diet (**Figure 4.2.4-1c).** The two

NaCN and NaOCN-induced changes were not significantly different from each other (p=0.64).

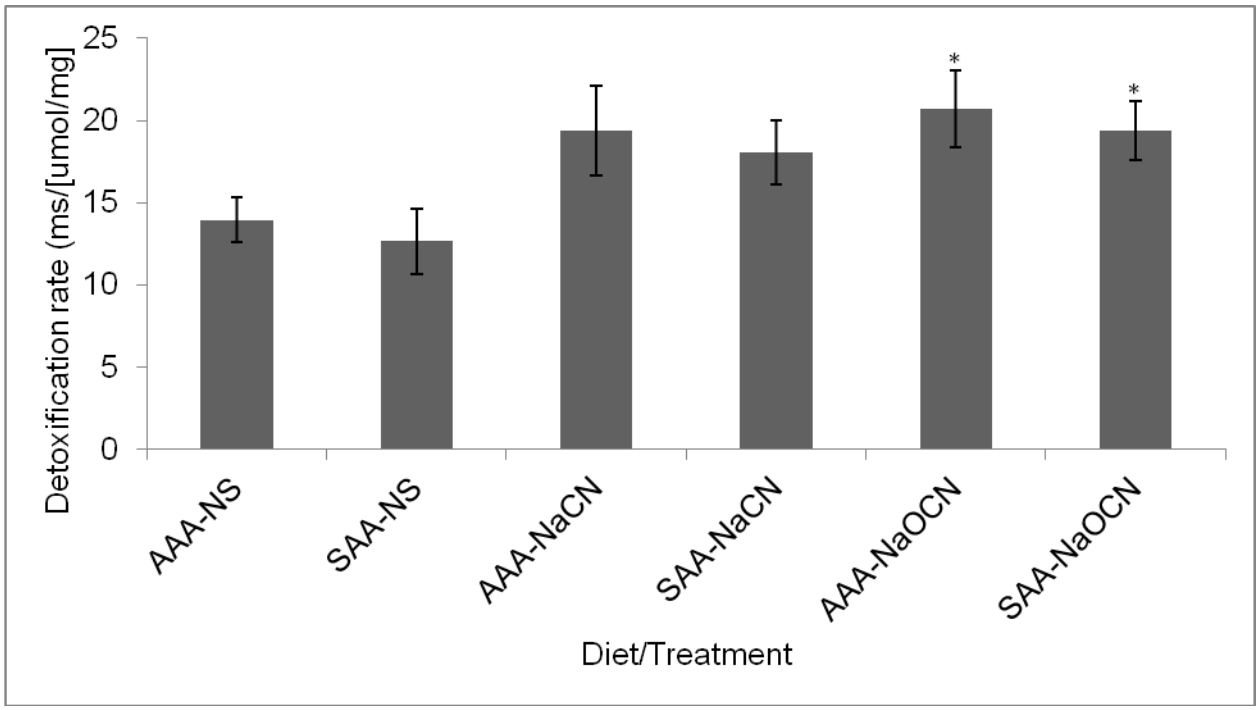


Figure 4.2.4-1c. Rodent cyanide detoxification rate (mean ± S.E.M ms/[µmol/mg] time to produce one µmol thiocyanate) in the brain was significantly ($p < 0.05$) affected by treatment. Rates were ~ 5.4 and 7.4 ms slower ($p < 0.05$) in rats treated with NaCN or NaOCN relative to their respective controls, regardless of the diet.

4.2.5 Plasma cyanide detoxification rates in *macaca fascicularis*

There was no indication that differences between diets changed over the study period [$F(5,50)=0.39$, $p=0.85$; test of diet:week interaction]. Consequently, the effects of diet and time were summarized as separate main effects. Average CDC did not significantly differ between the two diets [$t(10)=1.68$, $p=0.12$]. There were significant changes over time, however [$F(5,55)=6.84$, $p<0.001$], and those differences, relative to Week 1, are shown in (**Table 4.2.5-1**). A positive correlation between changes in total proteins and

CDC in both the control (Spearman r=0.35, p=0.04) and cassava (Spearman =0.36, p=0.03) groups (overall Spearman r=0.43, p<0.01) was found.

Table 4.2.5-1. Cyanide detoxification rates (mean ± S.E.M ms/[µmol/mg] time to produce one µmol thiocyanate) in plasma of non-human primates across diets and weeks.

	Diet	
Week	Control (S.E.M)	Cassava (S.E.M)
1	563.54 (7.45)	629.66 (4.40)
2	459.27* (10.80)	491.89* (8.49)
3	553.35 (6.37)	689.89 (9.29)
4	573.92 (6.77)	584.01(7.11)
5	688.38 (9.82)	701.26 (5.41)
6	669.34 (6.63)	736.11 (7.26)

Cyanide detoxification rates (time) required to produce one µmol thiocyanate in plasma of non human primates inconsistently changed over time. However, time significantly increased during the last two weeks (5 & 6) of the study period regardless of the diet.

4.2.6. Metabolic panel in *macaca fascicluaris*

The metabolic panel (complete blood count (CBC), creatinine phosphokinase (CPK), electrolytes, liver, kidney, and pancreas functions) appeared normal and was not influenced by the type of diet except for an increase in blood glucose in one of the animal, and changes in blood urea nitrogen (BUN) and BUN/creatinine ratio over time. A significant decrease in BUN and BUN/creatinine ratio (**Figure 4.2.6-1**), with a

significant increase in creatinine levels, were observed in animals fed cassava relative to those under the normal diet ($p<0.05$).

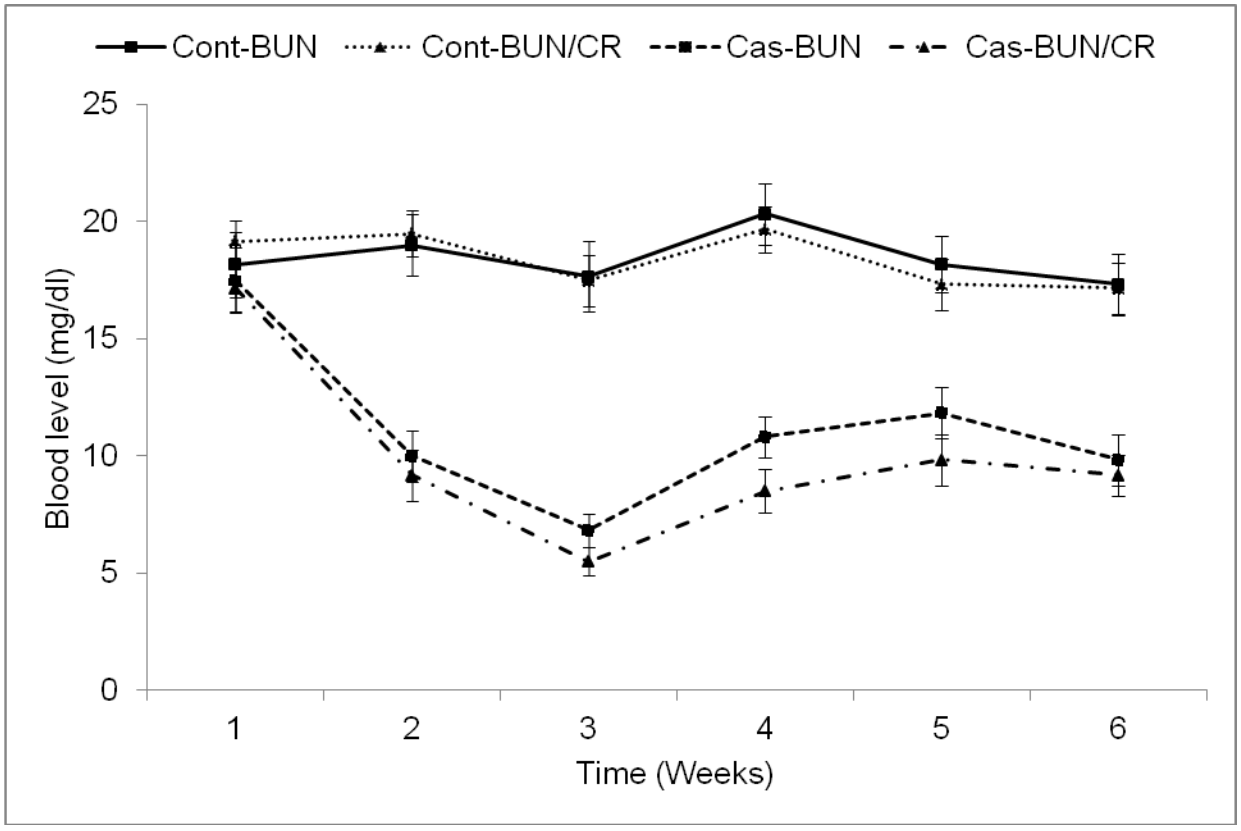


Figure 4.2.6-1. Blood urea nitrogen (BUN) (mean ± S.E.M mg/dl) and BUN/Creatinine (CR) levels in non-human primates fed on cassava were significantly ($p <0.05$) lower compared to control group. Additionally, these parameters significantly ($p <0.05$) decreased over time in animals fed on cassava relative to those on the control diet.

4.3. STUDY III: IDENTIFICATION OF MOLECULAR TARGETS OF CYANIDE AND CYANATE UNDER SAA-DEFICIENT DIET IN RATS

4.3.1 Motor deficits

Over the course of the study, the odds of remaining on the rotarod for at least 60s was significantly ($p < 0.05$) lower for the SAA-deficient fed rats treated with NaOCN compared to those treated with NaCN or vehicle. Treatment affected the rotarod fall off time. The fall time was also significantly ($p=0.040$) shorter on the last testing day in animals treated with NaOCN compared to control and NaCN groups. No statistical difference was found between NaCN relative to the control treated groups ($p=0.16$) (**Figure 4.3.1-1**).

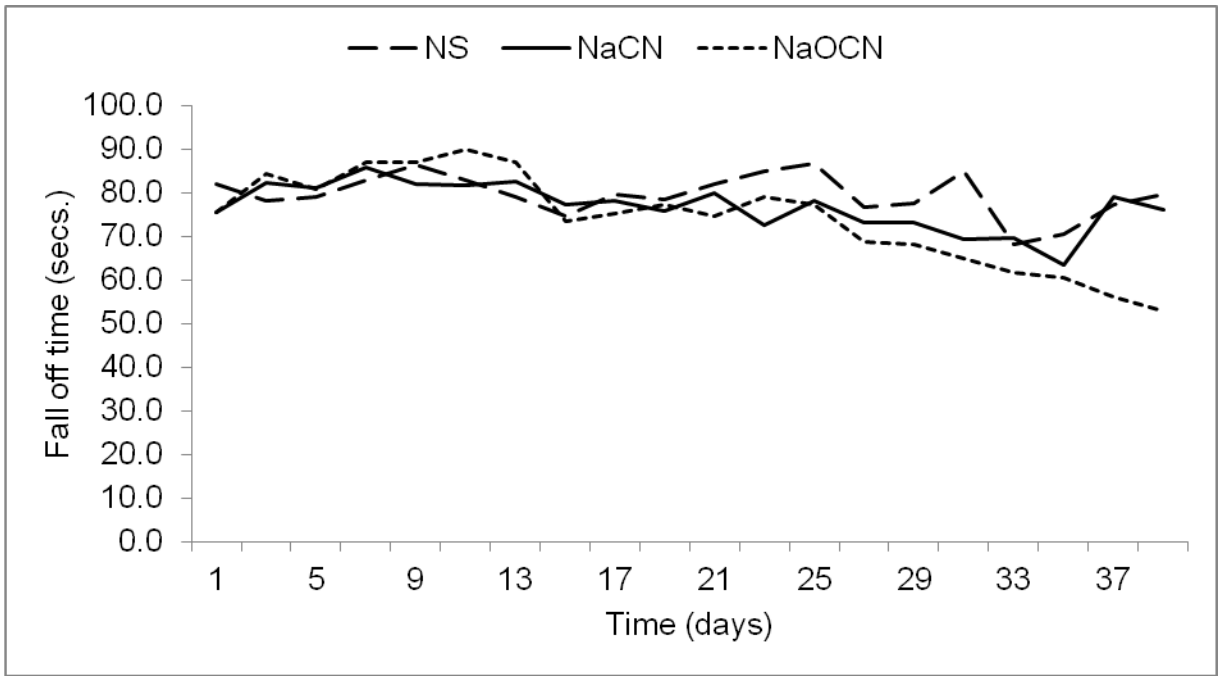


Figure 4.3.1-1. Rotarod performance in rats treated with normal saline (NS), NaCN or NaOCN and fed SAA-restricted diet. Treatment affected rotarod fall off time in rats. The fall time from the rotating rod decreased significantly ($p <0.001$) among rats treated with NaOCN compared to control and NaCN overtime.

4.3.2. Albumin carbamoylation

There was a significant interaction between diet and treatment with respect to mean number of assigned MS/MS spectra to carbamoylated albumin peptides (spectral counts) at 14-day time point (X^2(2df)=5.24, p=0.073). NaOCN overtly induced high levels of carbamoylation of albumin relative to vehicle or NaCN regardless of the diet (P<0.001) (**Table 4.3.2-1**).

Table 4.3.2-1. Estimated mean number of spectral counts (95% CI for the mean response) for carbamoylated albumin peptides and comparisons within AAA or 75%-SAA-deficient diet on day 14 **in rats.**

	Diet	
Treatment	**AAA estimates (spectral counts) (95%CI)**	**SAA estimates (spectral counts) (95%CI)**
Vehicle	22.5 (16.5 - 30.7)	15.8 (11.2 - 22.1)
NaCN	18.0 (13.0 - 25.0)	21.5 (15.7 - 29.4)
NaOCN	521.0 (411.0 - 660.0)	702.0 (554.0 - 888.0)
Comparisons	**Estimated ratios (95% CI)**	**Estimated ratios (95% CI)**
NaCN/Vehicle	0.80 (0.51 - 1.26)	1.37 (0.86 - 2.17)]
NaOCN/Vehicle	23.2 (15.7 - 34.2)*	44.5 (29.5 - 67.3)*
NaOCN/NaCN	28.9 (19.3 - 43.4)*	32.6 (22.0 - 48.3)*

On day 14, NaOCN overtly (*) induced high levels of carbamoylation of albumin relative to vehicle or NaCN regardless of the diet.

At the 28-day time point, there was no evidence of interaction between diet and treatment with respect to mean number of spectral counts for carbamoylated albumin peptides (X^2(2df)=3.83, p=0.15), thereby simplifying comparisons across treatments and diets. The mean number of spectral counts for albumin carbamoylated peptides was 47.4% (95% CI: 16.7 - 86.4%) greater in NaCN-treated animals relative to their vehicle-treated counterparts (p=0.001), regardless of diet. The carbamoylation effect was again amplified in animals treated with NaOCN compared to vehicle, with the mean response for NaOCN animals being 33.9 (95% CI: 28.0 - 41.1) times as much as the mean response for vehicle (p<0.001) or 23 (95% CI: 19.5 - 27.2) times the mean response for NaCN (p<0.001) (**Table 4.3.2-2**). After controlling for treatment, we found no significant differences in carbamoylation that could be attributed to diet (p=0.71).

Table 4.3.2-2. Estimated mean number of spectral counts (95% CI for the mean response) for carbamoylated albumin peptides on day 28 (Based on a purely additive model with diet and treatment as main effects).

	Diet	
Treatment	**AAA estimates (95% CI)**	**SAA estimates (95% CI)**
Vehicle	17.3 (14.3 - 20.9)	16.9 (14.0 - 20.5)
NaCN	25.5 (21.7 - 30.0)	25.0 (21.2 - 29.4)
NaOCN	587.0 (535.0 - 644.0)	574.0 (525.0 - 629.0)

On day 28, carbamoylation effects were significantly seen in NaCN and NaOCN-treated rats relative to their vehicle-treated counterparts, with a pronounced effect of NaOCN regardless of the diet.

When comparing the levels of albumin carbamoylation at Day 14 vs. Day 28, we found no evidence of an interaction between diet and treatment (X2(2df)=2.27, p=0.32). With

the exception of animals treated with NaCN under the normal AAA diet, the relative change in levels of carbamoylation was not significant. In the NaCN/AAA group, the mean number of spectral counts for carbamoylated albumin peptides was estimated to increase 55% (95% CI: 20 - 101%; p=0.001) over the 14-day span. All other entries, however, showed relative changes not significantly different (p≥0.15) from unity (**Table 4.3.2-3**).

Table 4.3.2-3. Estimated ratios (95% CI) of the mean number of spectral counts for carbamoylated albumin peptides on day 28 relative to day14.

	Diet	
Treatment	**AAA ratios (95% CI)**	**SAA ratios (95% CI)**
Vehicle	1.06 (0.80 - 1.40)	0.80 (0.61 - 1.07)
NaCN	1.55 (1.20 - 2.01)*	1.18 (0.91 - 1.53)
NaOCN	1.17 (0.94 - 1.46)	0.89 (0.71 - 1.11)

Comparison of the levels of albumin carbamoylation at day 28 vs. day 14 revealed increased carbamoylation (*) in the group of animals treated with NaCN and fed with a normal diet.

4.3.3. Spinal Cord proteomics

On the last experimental day, a differential pattern of susceptibility to carbamoylation in spinal cord proteins was demonstrated. Carbamoylation was almost exclusively induced by NaOCN so that comparisons between diets (AAA/SAA) were limited to this one neurotoxicant. NaOCN-targeted proteins included myelin basic protein (MBP), myelin proteolipid protein (MPP), neurofilament light polypeptide (NFLP), glial fibrillary acidic protein (GFAP), and 2', 3' cyclic-nucleotide 3'-phosphodiesterase. MBP was 18.8%

(95% CI: 2.3 - 38.1%; p=0.024) more carbamoylated in animals under the SAA-restricted diet relative their AAA counterparts. With regard to MPP, animals in the SAA-restricted diet had 23.3% (95% CI: 5.8 - 43.7%; p=0.007) more carbamoylation sites compared to their AAA counterparts. Similarly for NFLP, the SAA-restricted diet had an impact on carbamoylation. Under the SAA-restricted diet, the animals had 39% (95% CI: 2.6 - 88.2%; p=0.03) more carbamoylation sites relative to their AAA counterparts. For GFAP, the SAA-deficient group had 34.2% (95% CI: 5.9 - 69.9%; p=0.02) more carbamoylation sites relative to the AAA-group. With regard to 2', 3' cyclic-nucleotide 3'-phosphodiesterase, the SAA-restricted diet produced an average number of carbamoylation sites that was 2.85 (95% CI: 1.8 - 4.5; p<0.001) times the corresponding average response of animals fed the normal AAA diet (**Table 4.3.3-1**).

Table 4.3.3-1. Estimated mean numbers of carbamoylated sites (95% CI for the mean response) on spinal cord proteins in animals treated with NaOCN.

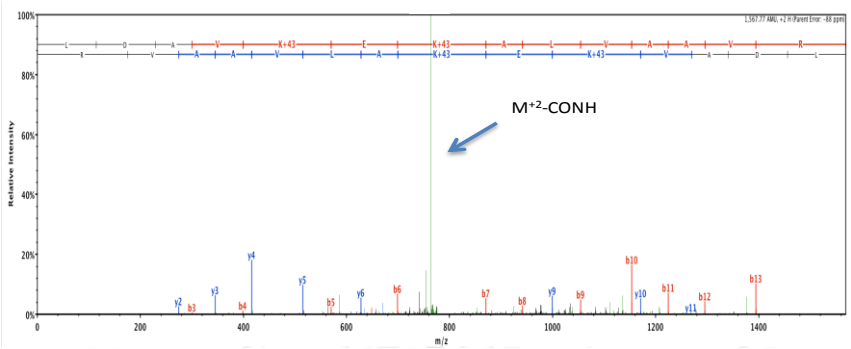
Protein	**AAA Diet**	**75% SAA-Deficient Diet**
Myelin basic protein	78.25 (70.0 - 87.4)	93 (84.0 – 102.0)
Myelin proteolipid protein	74 (66.0 – 82.9)	91.25 (82.4 – 101.0)
Neurofilament light polypeptide	48.75 (39.0 – 60.8)	67.75 (55.0 - 83.4)
Glial fibrillary acidic protein	30 (25.1 - 35.9)	40.25 (34.5 - 47.0)
2', 3' cyclic-nucleotide 3'-phosphodiesterase	6.5 (4.4 – 9.6)	18.5 (14.6 – 23.5)

Day-28 carbamoylation pattern in spinal cord proteins indicates an exacerbating effect of the SAA-deficient diet, mostly for 2', 3' cyclic-nucleotide 3'-phosphodiesterase.

None of the animals in the Vehicle- or NaCN-treated groups (either diet) had any

carbamoylated sites for MBP or GFAP. For MPP, only one animal had 1 carbamoylated site in the Vehicle/AAA group; all other Vehicle and NaCN treatments (both diets) were otherwise uniformly silent. For the NFLP, only one animal had 1 carbamoylated site in the NaCN/SAA group; all other Vehicle and NaCN treatments (both diets) were otherwise uniformly silent. A representative MS/MS spectra of carboylated peptides for both albumin and spinal cord protein residues are shown (**Figure 4.3.3-1**).

A) Albumin 206-219



B) Neurofilament Light Polypeptide 148-164

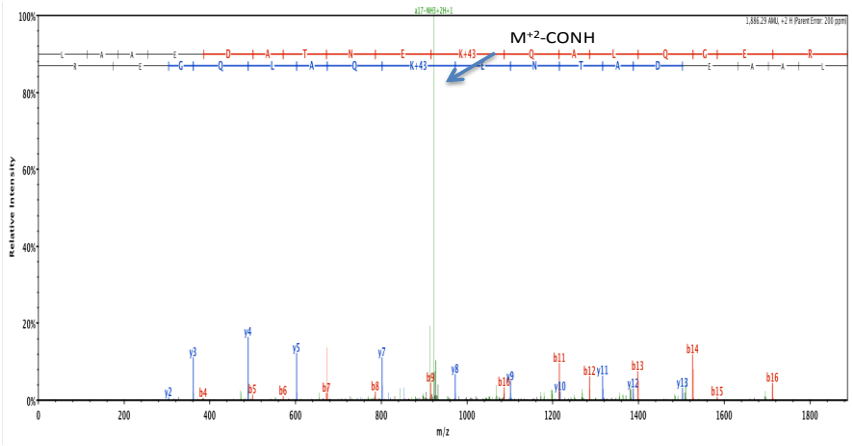


Figure 4.3.3-1. Representative MS/MS spectra of carbamoylated peptides from albumin, residues 206-219 (A) and neurofilament light polypeptide, residues 148-164 (B) prepared from the serum and spinal cord insoluble protein of cyanate treated rats, respectively. Sites of carbamoylation were detected by a +43 mass shift localized to lysine residues. Note that peptide 206-219 of albumin was doubly carbamolyated. Each doubly charged peptide also underwent a neutral loss of 43 mass units due to a partial loss of a carbamoyl group during fragmentation, designated by $-M^{+2}$-CoNH.

CHAPTER FIVE: DISCUSSION

5.1 The biomarkers and mechanisms of konzo: The "multiple hit" hypothesis

Cyanogenic toxic exposures impaired short-term working memory as evidenced by fewer correct arm entries, higher working memory errors, and longer RAM navigation time in rodents treated with NaOCN relative to NaCN and saline. However, treatment with NaCN resulted in long-term working memory impairment as signified by fewer correct arm entries and increased working memory errors relative to NaOCN or control animals. Reference memory was neither affected by cyanate nor cyanide treatments.

The findings demonstrate that cassava cyanogenic analogs notably cyanide and its metabolite cyanate induced differential pattern of cognitive deficits in rodents reflecting dual toxicity mechanism. Whether these patterns are attributable to mechanism commonly associated with both cyanide (cassava linamarin metabolite) and cyanate intoxications is yet to be determined. The observed pattern of cognitive deficits in animals treated with NaOCN vs. NaCN under normal diet could be possibly explained by the fact that cyanate may be capable of instantaneously adducting proteins thus impairing cognitive function (Crist et al. 1973). Modifications by cyanate such as carbamoylation of brain proteins and other yet to be explored mechanisms, may affect signal transduction, synaptic plasticity and memory formation processes critical in learnig and memory (Crist et al. 1973; Fando and Grisolia 1974).

Cyanide on the other hand appeared to induce cognitive deficit following substantial time lapse as aforementioned. There is possibility that cyanide is effectively detoxified

without cumulative effects despite higher doses (Hayes 1967). Thus neurological damage after cyanide exposure is attributed primarily to its hypoxic action, with subsequent reduction in cellular metabolism and energy through inhibition of cytochrome c oxidase (Way 1984). However, our findings showed a delayed effect of cyanide on cognitive processes. The cognitive deficit pattern observed in the rodents treated with NaCN may be attributed to build up of cyanate overtime and its cumulative effects following NaCN metabolism (Swenne et al. 1996; Tor-Agbidye et al. 1999) and subsequent carbamoylation thereof.

Chronic cassava as evidenced by high serum levels of thiocyanate the main CN metabolite has been associated with thyroid toxicity, Thiocyanate is a pseudohalide that interferes with iodine uptake in the thyroid gland and may aggravate iodine-deficiency disorders (Spencer, 1999; Rosling and Tylleskar, 1999). The effect of cyanide is proportional to the body weight and therefore children tend to be more susceptible to its poisoning than adults. In iodine deficiency endemic areas, cases of goiter and cretinism are high, and thus cyanide intake from cassava exacerbates these conditions (Delange et al, 1994). The resultant effects are arrested growth and stunting among children. Therefore, cassava dependency has been asssociated with thyroid dysfunction and growth stunting (Ihedioha and Chineme, 1999, Banea Mayambu et al, 2000). Thyroid dysfunction especially hypothyroidism has been associated with cognitive deficit including working memory (Samuel 2014). This is unlikely to have been the mechanism by which cyanide and/or cyanate impaired learning and memory especially because rats gained weight normally, while the activity levels were normal. In any case, were we to

speculate on the thyroid pathway then one should expected cyanate treatment to have no effect on cognition, which was not the case. Indeed, cyanate was explicit in impairing the short-term memory as opposed to the cyanide. However, high serum levels of thiocyanate the main CN metabolite, TSH, T3, T4 assements would have been critical in confirming our hypothesis.

Furthermore, the differential pattern observed with cassava cyanogens on cognition in rodents is reminiscence of the complexity of effect of toxic cassava. The complexity is also influenced by the cyanogens and/or the nutritional status of the animal and/or their combinations underscoring their role in metabolism and detoxification capability (Howlett et al 1990, Tylleskär et al 1991, Banea-Mayambu et al 1997, Cliff et al 1997). In the recent past, children with or without konzo hailing from cassava eating areas demonstrated poor cognitive performance compared to the controls (Boivin et al. 2013). The findings, together with this report corroborate the role of cyanogenic cassava and analogs in cognitive impairment. Whereas it is difficult to single out a specific agent culpable for cognitive deficit and/or konzo, it appears multiple causation theory holds the most plausible explanation. This is accounted for by intoxication by cyanide, cyanate, sulfur amino acid deficiency or their combinations. These findings together with others (Tshala-Katumbay et al. 2000; Boivin et al. 2013) add credence to the growing public health burden of cyanogenic cassava related toxicity. These findings still retain some level of public health relevance as peak in cyanide exposures, together with acute signs of cyanide poisoning and lethality do precede outbreaks of konzo (Banea-Mayambu et al. 2000).

From the aforementioned, adequate processing of cassava prior to its human consumption is critical to minimize the levels of cyanogenic toxins. Additionally, dietary supplementation with proteins (animal or plant) may confer the needed cyanide detoxification capabilities thus providing neuronal protection and/or mitigating neurodegeneration. However, more studies are needed to unravel the critical time of the onset of the possible deleterious effects on cognition especially in the light of most vulnerable groups (children and women). Studies are also needed to elucidate the biological markers that best correlate with levels of cognition deficits among cassava-reliant children. Such studies will inform choice for interventions to reduce the burden of cassava associated neurological disease.

The findings showed that under normal diet (AAA), the CDC were up to ~ 80X faster in the nervous system (14 milliseconds to produce one µmol of thiocyanate from the detoxification of cyanide) relative to plasma. In addition, the spinal cord CDC was impaired by NaCN, NaOCN, or SAA-deficient diet. In non-human primates, the plasma CDC changed proportionally to total proteins. However, the plasma CDC in non-human primates was ~ twice faster compared to the rodent capabilities. Generally, the metabolic panel in non-human primates showed a time-dependent decrease in BUN/creatinine ratio under the cassava diet.

The findings showed that chronic dietary reliance on cyanogenic cassava may cause metabolic derangement including poor cyanide detoxification capabilities (CDC). The CDC in mammals depicted a differential pattern across species (rodents vs *Macaca*

fascicularis) and tissues which may be impaired by dietary deficiencies, cyanide or cyanate toxicity. In rodents differential pattern of CDC across tissues with higher detoxification rates in the brain followed by the spinal cord, then plasma, respectively, while *Macaca fascicularis* displayed ~ twice as fast plasma CDC than rodents. This is consistent with findings that CDC may vary across species, tissues, and cellular compartments within the same tissue (Al-Qarawi et al 2001, Aminlari et al 1989, Cipollone et al 2007, Drawbaugh and Marrs 1987, Mimori et al 1984). Higher CDC in the nervous system tissues of rodents, i.e. ~ 80X faster (14 milliseconds to produce one µmol of thiocyanate from the detoxification of cyanide) relative to plasma, may reflect an ontogenetic adaptation of organs that prominently rely on aerobic energy metabolism (Cooper and Brown 2008, Isom et al 1982, Isom and Way 1974).

In the nervous system, particularly the spinal cord CDC were impaired by SAA dietary deficiency and/or chemical treatment with NaCN or NaOCN, but only treatment with the dual (NaOCN and NaCN) affected the brain. Whether this differential pattern of susceptibility reflects the existence of more robust sulfur- buffering system in the brain relative to the spinal cord remains to be determined. The same hypothesis may hold true for the plasma since CDC were not impaired by SAA deficiency possibly arising from a compensatory effect due to existence of abundant sulfur donors such as albumin (Drawbaugh and Marrs 1987, Wrobel et al 2004). Absence of chemical impact on plasma CDC may be explained by the fact that in plasma, CDC mostly depends, conceivably, on the bioavailability of sulfane sulfur whereas in the brain the CDC also depends on the activities of sulfurtransferases; of which the enzyme activities may be

inhibited upon oxidative damage and/or protein carbamoylation associated with treatment with NaCN and/or NaOCN, respectively (Kassa et al 2011, Kraus and Kraus 2001, Nagahara 2013). The variation in primate plasma CDC proportionally to total plasma proteins indicate that total pool of sulfur donors may be critical for the detoxification capabilities of cyanide in plasma.

The time-dependent decrease in BUN, BUN/Cr ratio along with the increase in creatinine in *Macaca fascicularis* fed cassava may be explained by a metabolic adjustment to a protein-restricted (cassava) diet (Kumar et al., 1972). These changes were reminiscent of cyanide metabolism in humans (Mukherjee et al., 2009) suggesting that feeding and toxicological studies using *Macaca fascicularis* may help modeling the biochemical changes associated with cassava cyanogenesis and neurotoxicity.

Regardless of the type of the diet, *macaca fascicularis* remained alert and responsive. No abnormality in gait, arms or hand coordination, were observed during the course of the study. This may be explained by the short duration of experimentation and the low levels of cyanogenic glycoside in our cassava model (*Manhot dulcis*). This is supported by one literature report that, prolonged feeding of a cassava-rich diet (75% cassava, 25% chow) to young male rats induced a slowly developing motor-system disease that is first detectable on the rotarod test after 8 months (Mathangi et al, 1999). Although this are different species of animal, it is clear that for motor deficits to be elicited, one has to feed on cassava chronically.

In sum, the findings have shown that mammals CDC may vary across species and tissues. Both NaCN and NaOCN toxicities, and SAA dietary restrictions, may exert inhibitory effects on CDC maintaining a vicious cycle of increased susceptibility of the nervous system to NaCN and/or NaOCN. This level of complex interactions and tissue variations of CDC may dictate the phenotypic expression and molecular signatures of cyanide-associated neurological diseases.

The study findings show that NaOCN overtly induced motor deficits and high levels of carbamoylation relative to NaCN and vehicle. At two week time point there was, a diet-treatment interaction impacting on albumin carbamoylation. However, at Day 28, there was no effect attributed to diet. The mean number of NaCN-associated carbamoylated sites on albumin became 47.4% significantly higher relative to vehicle. In the nervous system, only NaOCN carbamoylated spinal cord proteins, which was more pronounced under the SAA-restricted diet. The spinal cord carbamoylated proteins included myelin basic protein, myelin proteolipid protein, neurofilament light polypeptide, glial fibrillary acidic protein, and 2', 3' cyclic-nucleotide 3'-phosphodiesterase.

Cyanate (NaOCN) induced motor deficits and significant carbamoylation of albumin and select spinal cord proteins. The findings suggest that cyanate neuropathy may be mediated through carbamoylation. This proposal is consistent with earlier studies that showed a positive correlation between memory deficits and levels of carbamoylation in rodent brain (Crist et al 1973) or the suggestion that carbamoylated albumin may be clinically used to predict risks for neuropathy in uremic patients (urea is broken down

into cyanate) (Berg et al 2013, Kalim et al 2013, Kraus and Kraus 2001). The observed pattern of carbamoylation is consistent with the myelinotoxic effects of cyanate as previously reported (Ohnishi et al 1975, Tellez et al 1979, Tellez-Nagel et al 1977). OCN carbamoylates proteins at amino and/or sulfhydryl sites leading to a modification that may induce changes in protein structure and function (Farías et al 1993, González et al 1998, Kraus and Kraus 2001). NaOCN targets included major myelin proteins and corresponding binding proteins such as neurofilament light polypeptide, glial fibrillary acidic protein, and 2', 3' cyclic-nucleotide 3'-phosphodiesterase; with the latter showing a greater carbamoylation effect. This pattern of susceptibility should inform future studies to elucidate the exact pathways and mechanisms leading to cyanate neuropathy (Han et al 2013).

Neurofilaments are the main cytoskeletal components of neuronal cells (Carden et al 1985) important in axonal and dendritic structural integrity and caliber, thus facilitating conduction of nerve impulses (Kriz et al 2000, Xu et al 1996, Kong et al 1998). Misfolded and/or deformed neurofilaments affect morphological integrity leading to leakage into the extracellular fluid, thus increasing their concentration and neuronal impairment. Abnormal accumulation of neurofilament has become biomarkers of most human neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), Parkinson, Alzheimer, Charcot- Marie-Tooth, dementia with Lewy bodies (Liu et al, 2004, Al-Chalabi and Miller 2003), and toxic neuropathy (Zhu et al 1998). Whether similar mechanism is plausible with pathogenesis of cassava neurotoxicity remains

unclear, but, spinal cord neurofilament light polypeptide were specifically targeted in our study model.

Furthermore, hyperphosphorylation and pathological accumulation of neurofilaments is a key finding in the aforementioned disorders (Al-Chalabi and Miller 2003). Hyperphosphorylation results from misregulation of the kinases and phosphatases that are responsible for neurofilament phosphorylation (Perrot et al 2008, Rudrabhatla and Pant 2010, Shea and Chan 2008). However, many other changes may potentially lead to accumulation of neurofilaments, including mutation, disregulation of neurofilament protein synthesis, defective axonal transport, and abnormal phosphorylation/glycosylation/oxidation (Dong et al 1993, Al-Chalabi et al 1999, Jordanova et al 2003, Mersiyanova et al 2000). This mechanism need to be investigated in patients with konzo.

Glial fibrillary acidic protein (GFAP) on the other hand is a critical intermediate-filament protein of astroglial lineage (Eng et al 1971, Reeves et al 1989, Barclay et al 2010, Messing and Brenner 2003). Its expression is essential for normal white matter architecture and integrity of blood–brain barrier while the absence leads to late-onset CNS dysmyelination (Liedtke et al 1996). GFAP can be a marker for gliosis and has been expressed in hippocampal neurons of patients with Alzheimer's disease a neurodegenerative disorder (Renkawek et al 1994, Hol et al 2003). The severity of the disease has been strongly associated with the density of the activated astrocytes (Muramori et al, 1998). Whether this was the mechanism that led to increased carbamoylation in this study remains unclear.

The CNS myelin and oligodendrocytes have vibrant 2′ 3′- cyclic nucleotide 3′-phosphodiesterase enzyme activity (Braun et al 2004). The enzyme binds to cytoskeletal elements such as F-actin and tubulin promoting outgrowth of processes. Thus, 2′ 3′- cyclic nucleotide 3′-phosphodiesterase enzyme may be involved in regulating cytoskeletal dynamics promoting process outgrowth and differentiation in oligodendrocytes and CNS myelination (Braun et al 2004). Problems of 2′ 3′- cyclic nucleotide 3′-phosphodiesterase sequencing result in phenotype of axonal swelling, neurodegeneration and premature death (Braun et al, 2004) underscoring the importance of the protein. Whether this process is important in carbamoylating the select proteins requires further investigation.

The effect of dietary SAA-deficiency on the carbamoylation of the aforementioned spinal cord proteins remains to be explained. In serum, SAA deficiency had no impact on the carbamoylation of albumin after 28-days of treatment with cyanate. This may possibly be explained by a “sulfur buffering effect” through abundant serum sulfur donors including albumin (Westley et al 1983). Absence of incrimental cyanate carbamoylation at 4 weeks relative to 2 weeks may be due to a metabolic clearance of carbamoylated proteins.

With regard to cyanide, however, the increase in levels of carbamoylation could probably portray a cumulative effect of cyanide detoxification with resultant cyanate production and subsequent carbamoylation of albumin. This proposal would however, need to be confirmed with quantitative measures of tissue contents in cyanide, cyanate,

and SAA; which, along with a small sample size, are among the limitations of this study. NaCN however, did not cause any carbamoylation of spinal proteins. Carbamoylation studies were not conducted in non-human primates plasma and nervous tissue due to high cost of carrying out proteomic analyses. In addition, because of observing the principle of reduction in the prudent use of animal for research, the animals could not be sacrificed to obtain the nervous tissue. This therefore forms a limitation for the study given that non-human primates are phylogenically higer than rodents and also close to humans.

The study failed to confirm the primary hypothesis that under SAA deficiency, cyanide toxicity induces carbamoylation patterns similar to that of cyanate neuropathy. However, in light of the observed response on albumin, chronic exposure to cyanide may conceivably lead to greater carbamoylation effects. This hypothesis should be tested on subjects chronically exposed to cyanide notably those living in konzo-affected areas. In the undernourished subjects, cyanide is hypothesized to undergo oxidative detoxification with subsequent increase in the production of cyanate, a well-known carbamoylating neurotoxin.

The exact biomarkers and mechanisms of konzo remain unclear. Currently, research efforts focus on elucidating the biomarkers and mechanisms of the disease. Candidates for such neurodisability include cyanide, or cyanate, sulfur deficiency, or their respective combinations (Kassa et al 2011). Limited progress has been made partially due to the lack of experimental models. Previously studies have shown that proteomics

methodologies using LC/MS-MS were able to demonstrate carbamoylation patterns induced by NaOCN and cassava glycoside linamarin that may help unveil biomarkers of cyanogenic exposure (Kassa et al 2011). The findings of this study indicate that carbamoylated albumin may be a useful marker of exposure to cyanate and cyanide metabolites of cyanogenic cassava implicated in the pathogenesis of konzo.

Taken together, our findings suggest that the pathogenesis of konzo may involve pathological protein carbamoylation and/or dysregulation of cellular functions involved in thiol-redox mechanisms, and cytoskeleton integrity. The neurotoxic insults seen in konzo subjects could arise from a "multiple hit" process that combines of a direct mitochondrial hit by cyanide, a protein hit through carbamoylation, and a thiol-redox hit due to SAA-deficiency (Berg et al 2013, Kassa et al 2011, Tor-Agbidye et al 1999a, Tor-Agbidye et al 1999b). Since only NaOCN-treated animals developed motor deficit and carbamoylation of spinal cord proteins, the findings are in favor of a recent proposal that suggests that blocking carbamoylation may have neuroprotective effects (Berg et al 2013).

5.2 Nutrition, Cassava and Cyanide poisoning: A self-maintaining cycle of increased susceptibility of cyanogenic toxicity

The rodent growth pattern as evidenced by gain in weight was influenced by both diet and treatment. Overall, animals placed on SAA-deficient diet gained less weight relative to those on the AAA diet, regardless of treatment. Under normal diet, rodents treated with NaOCN gained weight slowly relative to NaCN and controls whereas under SAA

diet the weight gain was essentially the same. Among the non-human primates, the pattern of weight change across cassava compared to control primate diet, a notable declined was observed over time. Animals fed on the cassava-diet lost (8.22%) more weight compared to those under the primate chow (1.63%) taking the terminal relative to their initial body weight.

Nutritional balance is essential for optimal growth pattern and developmental milestones for both young and adult mammals. Cassava is a carbohydrate rich crop with very low protein content, including sulfur amino acids (Diasolua Ngudi et al 2002, Nassar and Sousa 2007, Nunn et al 2011) and therefore may be lacking in some essential amino acids. Cassava main glycoside linamarin is metabolically converted cyanogens namely; cyanohydrins and hydrogen cyanide (HCN) and thiocyanate under normal nutritional conditions. Under SAA-deficiency, oxidative pathways are favored and there is an increased production of cyanate, a well-known motor system toxin (Tellez et al 1979, Diasolua Ngudi et al 2002, Soler-Martin et al 2010, Sreeja et al 2003, Boivin et 2013). Cassava cyanide or cyanate, sulfur deficiency, or their respective combinations (Kassa et al 2011) may play a major role in the initiation and perpetuation of cassava related neurodegeneration and other pathophysiological processes.

Conditions of SAA deficiency together with cassava cyanogenesis increased susceptibility to slow and/or arrested growth in both rodents and non-human primates. This is consistent with the pivotal role essential amino acids play on growth and development in mammals. In the presence of the former, the animals develop some

degree of adaptation. However, it is difficult to achieve full adaption (Sikalidis and Stipanuk, 2010) without long-term slowing of weight gain (Dollet et al, 1985). The adaptation mechanism involves decreased proteolysis, conservation of protein and reduction in oxidation to free amino acids from being burned down, allowing for biosynthesis and other essential metabolic events (Tawa and Goldberg, 1992). The slow weight gain has been observed among children population subsisting mainly on cassava because of its poor protein and cyanogenic content (Banea-Mayambu et al. 2000; Nunn et al. 2011).

The non-human primates also tended to lose weight while exclusively being fed on cassava. It is noteworthy to mention that the control group also lost weight over the experimental period probably as a result of stress of the study. While comparisons across dietary groups require caution (different experimental groups and species), the results do suggest that dietary deficiency in SAA and/or proteins may be accompanied by growth retardation. This is consistent with previous studies that showed dramatic weight loss in rodents fed SAA-deficient diet or evidence of stunting in children who rely on cassava as staple food (Banea-Mayambu et al 2000, Stephenson et al 2010, Tor-Agbidye et al 1998). Poor gain in body weight in animals treated with NaOCN is consistent with previous findings (Alter et al 1974). These findings underscore the role of proteins on weight gain, growth and development and detoxification capabilities.

The physical and behavioral signs associated with cassava cyanogenesis included acute overt convulsion induced by NaCN under conditions of SAA-deficiency diet in

rodents. Cyanide together with SAA deficiency may modulate acute toxicity seen in rodent maintained under dietary deficient conditions. However, it is worth pointing out that cyanide may also induce seizures through its 2-imino-4-thiazolidine-carboxylic acid metabolite (Spencer, 1999). Cassava cyanide detoxification capabilities appear to be dependent on the bioavailability of sulfur donors including the sulfur amino acids (SAA) cystein and methionine suggesting that SAA- or protein-deficient diet may increase the individual susceptibility to cassava-associated neurodegeneration and acute poisoning (Banea-Mayambu et al., 1997, Kassa et al., 2011, Tor-Agbidye et al., 1999a, Tylleskar et al., 1992). SAA deficiency may occur as result of chronic malnutrition or dietary reliance on cassava due to its poor protein content (Diasolua Ngudi et al., 2002, Nassar and Sousa, 2007). There were no seizures seen in animals fed on AAA diet or treated with NaOCN. In addition, no seizures were observed in non human primates fed on cassava. This explains the role of SAA-deficiet diet in predisposing the brain to acute cyanide insults.

In sum, the association of cassava cyanogensis to the pathogensis of konzo, is as a result of viscious cycle of “multiple hit” phenomena notably, acute as well as chronic effect of cyanide, cyanate and SAA-deficiency or their combination on physico-neurodevelopment status of the consumer. Taken together, malnutrition especially protein deficiency, exclusive reliance on cassava as staple diet, and cyanogenic toxicity arising from consumption of unsufficently processed root appear to be the main players underlying pathogenesis of konzo. These factors are typically prevalent in Sub-Saharan Africa where the greatest burden of the konzo is common. The factors appear to be

maintained and perpetuated by ecological process such as drought and famine, political systems such as civil unrest and socio-economic factors notably poverty and land ownership issues. The aforementioned factors compromise the time and steps of processing cassava tuber to reduce the cyanogenic content. The factors serves as a catalyst for food insecurity leaving the communities with no alternative source of food, but only cassava which is torelant to drought, war and other calamities. The most vulnerable in the society namely women and children are hard hit because they have limited capacity to supplement their diet including proteins because of lack of food access and money in case of the already mentioned adverse events. These factors togethers continue to sustain a vicious cycle of public health issue of importance abeit overlooked. The problem will continue to be a major health set back to Africa for many years to come if no infrastructural planning, focused agenda set up to adress nutritional challenges, processing to reduce cyanide in cassava and promotion of socio-economic-political stability.

5.3 Key findings

Treatment of rodents with cyanogenic cyanate impaired short-term working memory while sodium cyanide resulted in long-term working memory impairment. Functionally, NaCN under SAA caused acute seizures whereas NaOCN overtly induced motor deficits under SAA diet in rodents. Weight gain was impaired by SAA-deficient diet and treatment with NaOCN in rodents.

Biochemically, under normal diet, the CDC were up to ~ 80X faster in the nervous system relative to plasma. The spinal cord CDC was impaired by NaCN, NaOCN, or SAA-deficient diet. Additionally, among the non-human primates, the plasma CDC changed proportionally to total proteins. The plasma CDC in non-human primates was ~ twice faster compared to the rodent capabilities. The metabolic panel in non-human primates showed a time-dependent decrease in BUN/creatinine ratio under the cassava diet.

Lastly, NaOCN induced high levels of carbamoylation relative to NaCN and controls. Diet-treatment interaction impacted on albumin carbamoylation at 2 week time point. The mean NaCN-associated carbamoylated sites on albumin became 47.4% significantly higher relative to control at 2 week time point. In the nervous system, only NaOCN carbamoylated spinal cord proteins, which was more pronounced under the SAA-restricted diet. The spinal cord carbamoylated proteins included myelin basic protein, myelin proteolipid protein, neurofilament light polypeptide, glial fibrillary acidic protein, and 2', 3' cyclic-nucleotide 3'-phosphodiesterase.

5.4 Conclusion

The findings provide an experimental evidence for the biological plausibility that cassava cyanogens may induce cognition deficits. The cognitive deficits may result from dual toxicity effect of cyanide and cyanate reflecting their differential toxicity machanisms. Chronic dietary reliance on cassava may cause metabolic derangement including poor cyanide detoxification capabilities (CDC) and acute seizures. The CDC in

mammals demonstrate a differential pattern across species and tissues. Cyanide detoxification capabilities in plasma decreased proportionately to total plasma proteins in non-human primates. Therefore, susceptibility to cyanide poisoning appeared to be modified by diet and endogenous tissue variations in CDC. Cassava cyanate induced motor deficits and significant carbamoylation of albumin and select spinal cord proteins including major myelin proteins and corresponding binding proteins such as neurofilament light polypeptide, glial fibrillary acidic protein, and 2', 3' cyclic-nucleotide 3'-phosphodiesterase. The nervous system susceptibility to food (cassava) cyanogenesis and neurotoxic insults seen in konzo subjects may result from a “multiple hit” process including cyanide toxicity, deficiency in sulfane sulfur, and cyanate-induced toxicity and carbamoylation. The multiple hit processes may combine direct mitochondrial insults, protein carbamoylation and a thiol-redox derangement. This level of pathogenetic complexity should be considered in biomarker studies and efforts to prevent neurotoxicity effects of cassava. Lastly, cyanate protein modification notably carbamoylated albumin may be a useful marker of exposure to chronic cassava cyanogenesis.

5.5 Study limitations

The study utilized pure cyanogenic analogs namely cyanide and cyanate as the chemical articles and not cassava or its main content linamrin, the precursor of cyanide. This was necessary because of cost and time constraints. Whereas, the ideal approach would have been to feed the rodents on cassava, the experiments would require 8 months of to elicit the anticipated endpoints. This is possibly because of low levels of

cyanogens that would have been contained in volume of cassava to be eaten by rodents. In addition, such experiments are difficult to conduct because of challenges in measuring the amount of cassava each rodent eats. Therefore to cut on experimental time and circumvent on the aforesaid problem pure analogs were used.

Additionally, experiments were conducted with cassava fed to non- human primates and the findings on weight gain gave credice to our rodent experimental model. Another, viable experimental approach would have been to use linamarin the main cassava content which is metabolized into cyanide under normal conditions. However, linamarin is extremely expensive and given our resources constraint it would have been difficult to sustain the study to achieve the expected endpoints.

Studies of relevance public implications would require studies on subjects ordinarily affected by the disease konzo. Our studies involved rodents and non-human primates, whereas they form a basis for experimental modeling of cassava neurotoxicity, there may be issues of their extrapolation to humans given that rodents and non-human primates are physiologically different and lower phylogenically. However, for ethical reasons, it is almost impossible to conduct such robust experimental studies on humans and therefore the need for animal use in resaerch.

Carbamoylation studies were not conducted in non-human primate's tissue due to high cost of carrying out proteomic analyses. This may have been a missed opportunity to compare the carbamoylation patterns in both rodent and non human primates under

different dietary and treatment regime. However, this could still be done in future because the samples have been stored under -80 ^{o}C conditions. Additionally, because of the principle of reduction in the prudent use of animal for research, the animals could not be sacrificed to obtain the nervous tissue. This therefore forms a limitation for the study given that non-human primates are phylogenically higher than rodents but also close to humans. The aforementioned reason explains why the CDC in Nervous system in non-human primates was not carried out.

In this study, the seizure events were deemed to have happened based on overt convulsions. This may be challenged because there may be seizure activity without overt signs of convulsion. This stems from the fact that there may be dissociation between overt convulsions and seizure activity. Thus, the presence or absence of motor convulsions may be an unreliable indicator in the assessment seizures (Katalan et al 2013). However, cyanide causes acute toxicity which is manifested as seizures among other signs, therefore it would have been unlikely for the convulsions to be observed beyond one hour. The use more accurate to measure and record seizures eg use of electroencephalography (EEG) should be explored.

Finally, the cognitive studies were conducted by the investigator while observing and recording of the animal activities manually. Though this was a rudemental way of conducting research with the level of technological advancement in neuroscience, all attenpts were made to minimize observer bias as much as possible.

Generally, these studies have established a strong foundation for molecular and mechanistic investigation for konzo, therefore, opening other line of opportunities to carry out translational research with human subjects.

5.6 Recommendations for future work

There is need for experimental modeling of cassava neurotoxity using non-human primates. This is because they are higher phylogenically and they feed on cassava diet in their natural life. This approach will serve to confirm our rodent findings and experimental model. The experimental approach will have to use the same doses of the cyanogenic analogs or feed on cyanogenic cassava and/or feed them on custom-made diet mimicking our rodent/primate studies. The animals should be submitted to a battery of test to test for motor function, behavior and cognition assessment. In addition, biochemical test should be carried out to elicit cyanide detoxification rates and protein carbamoylation in the serum as well as the nervous system.

Studies on humans affected by cassava-associated neurological diseases are needed to determine whether chronic cassava cyanogenic exposure is associated with cyanate-like carbamoylation patterns. The carbamoylation response on albumin suggests that chronic exposure to cyanide may share similarities with cyanate-induced carbamoylation, and possibly, neuropathy. Such hypothesis may be tested in studies on subjects chronically exposed to cyanide such as those living in konzo-affected areas. The study should measure the protein levels and correlate with levels of carbamoylation in subjects with konzo and compare with performance in motor as well as cognition.

More studies are needed to unravel the critical time of the onset of the possible deleterious effects on cognition especially in the light of most vulnerable groups. Studies are also needed to elucidate the biological markers that best correlate with levels of cognition deficits among cassava-reliant children. Such studies will inform choice for interventions to reduce the burden of cassava associated neurological disease.

The CDC in Nervous system of rodents and non-human primates need to be compared because it may have relevance to cassava neurodegeneration in humans.

NaOCN targets included major myelin proteins and corresponding binding proteins such as neurofilament light polypeptide, glial fibrillary acidic protein, and 2', 3' cyclic-nucleotide 3'-phosphodiesterase; with the latter showing a greater carbamoylation effect. This pattern of susceptibility should inform future studies to elucidate the exact pathways and mechanisms leading to cyanate neuropathy (Han et al 2013).

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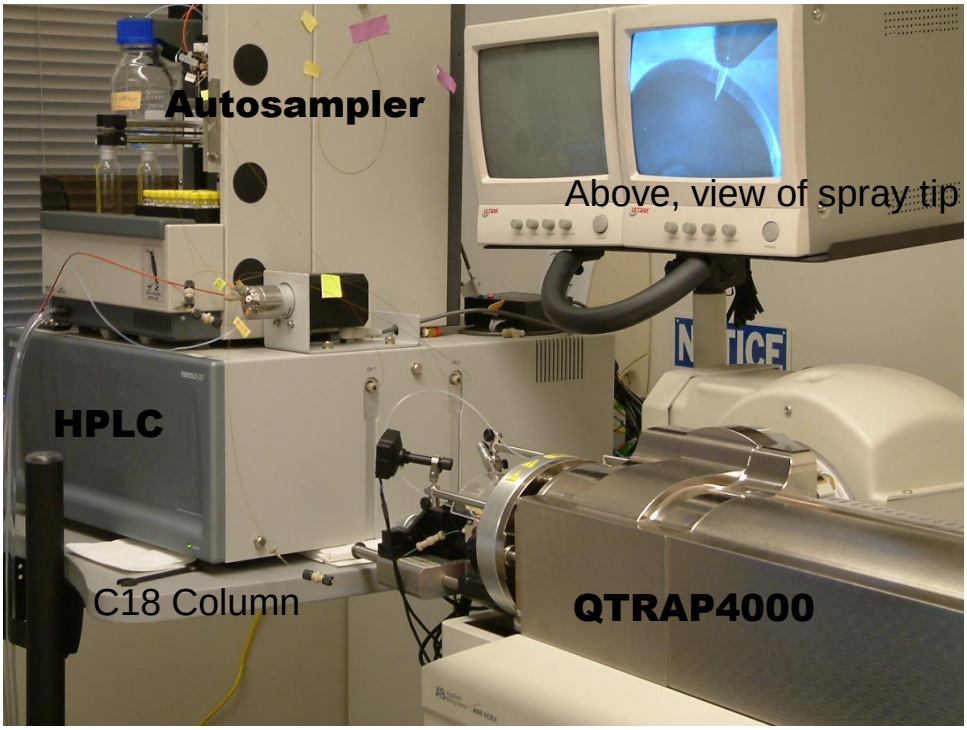
APPENDIX I: Radial arm maze equipment

APPENDIX II: Rotarod equipment

APPENDIX III: Proteomic equipment



Proteomic equipment at OHSU; a shared Resource: Hybrid triple quadrupole/ion trap QTRAP4000 from AB Sciex (right) with Eksigent nanoflow 2DLC system (lower left) and Autosampler (upper left). Top right video monitor shows a magnified view of the 400 nl/min nanoflow spray tip with a 10 micron spray opening. Up to 1 ug of sample can be loaded on each injection.

APPENDIX IV: PUBLISHED PAPERS

1. Kimani S, Sinei K, Bukachi F, Tshala-Katumbay D, Maitai C. (2014) Memory deficits associated with sublethal cyanide poisoning relative to cyanate toxicity in rodents. Metabolic Brain Disease, 29(1):105-112

2. Samuel Kimani, Victor Moterroso, Mike Lasarev, Sinei Kipruto, Fred Bukachi, Charles Maitai, Larry David and Desire Tshala-Katumbay (2013). Carbamoylation correlates of cyanate neuropathy and cyanide poisoning: relevance to the biomarkers of cassava cyanogenesis and motor system toxicity. SpringerPlus, 2013; 2:647.

3. Kimani S, Moterroso V, Morales P, Wagner J, Kipruto S, Bukachi F, Maitai C, Tshala-Katumbay D (2014). Cross-species and tissue variations in cyanide detoxification rates in rodents and non-human primates on protein-restricted diet. Food Chem Toxicol. 2014;66: 203-9.

APPENDIX V: CONFERENCE PRESENTATIONS

1. Kimani S, Monterroso V, Tshala-Katumbay D, Maitai C, Bukachi F. Impact of Sulphur Amino Acid (SAA) dietary deficiency on nervous system response to cyanogenic intoxication. The 1st international scientific conference, CHS-UoN, Nairobi Kenya, 15-17th June 2011.

2. Samuel Kimani, V. Monterroso, K. Sinei, F. Bukachi, C. Maitai, D. Tshala-Katumbay. Probing mechanisms of cyanogen neurotoxicity: relevance to the pathogenesis of konzo, a motor neuron disease highly prevalent in Sub-Saharan Africa, XXth world congress of Neurology 2012, Rabat, Morocco

3. Kimani S, Sinei K, Bukachi F, Tshala-Katumbay D, Maitai C. Cassava cyanogenesis and neurotoxicity: experimental modeling. Part I. Memory deficits associated with sublethal cyanide poisoning relative to cyanate toxicity in rodents. Brain Disorders in the Developing World Tenth Anniversary Symposium Feb 10-13, 2014. Washington DC, USA

4. Kimani S, Moterroso V, Morales P, Wagner J, Kipruto S, Bukachi F, Maitai C, Tshala-Katumbay D. Cassava cyanogenesis and neurotoxicity: experimental modeling. Part II. Cross species and tissue variations in cyanide detoxification rates in rodents and non-human primates on protein-restricted diet. Brain Disorders in the Developing World Tenth Anniversary Symposium Feb 10-13, 2014. Washington DC, USA

APPENDIX VI: Supplementary Table 2; Carbamoylated peptides in albumin used to quantify the relative amount of carbamoylation in plasma digests. The observed m/z value for each modified peptide was used to create an inclusion list in subsequent LC/MS/MS runs to maximize the collection of MS/MS spectra for carbamoylaed peptides, and inclrease the throughput of the analysis.

Carbamoylated albumin peptide*	**residue numbers**	**Observed m/z value**	**Charge State**	**Numbers of assigned MS/MS spectra during longer shot-gun analysis**
AAcLTPkLDAVK	199 - 210	665.5	2	4
AADkDNcFATEGPNLVAR	585 - 602	996.7	2	1
AETFTFHSDIcTLPDkEK	528 - 545	728.3	3	4
AETFTFHSDIcTLPDKEkQIK	528 - 548	851.4	3	1
ATEDQLkTVMGDFAQFVDK	563 - 581	1093.9	2	4
ATEDQLkTVMGDFAQFVDK	563 - 581	729.7	3	5
ccAEGDPPAcYGTVLAEFQPLVEEPkNLVK	384 - 413	1145.5	3	5
cckAADKDNcFATEGPNLVAR	582 -602	814.6	3	3
DNYGELADccAkQEPER	106 - 122	1049.6	2	1
DNYGELADccAkQEPER	106 - 122	700.6	3	2
DNYGELADccAkQEPERNEcFLQHK	106 - 130	1052.3	3	2
EAHkSEIAHR	25 - 34	611.0	2	1
EkALVAAVR	211 - 219	500.3	2	6
FKDLGEQHFkGLVLIAFSQYLQK	35 - 57	918.7	3	1
FPNAEFAEITkLATDVTK	247 - 264	1019.9	2	1
GLVLIAFSQYLQkcPYEEHIK	45 - 65	860.9	3	1
HPYFYAPELLYYAEkYNEVLTQccTESDK	170 - 198	1225.9	3	1
INkEccHGDLLEcADDR	265 - 281	716.9	3	4
INkEccHGDLLEcADDRAELAK	265 - 286	887.7	3	2
kQTALAELVK	549 - 558	382.2	3	1
kQTALAELVK	549 - 558	1143.5	1	1
kQTALAELVK	549 - 558	572.6	2	18
kYEATLEK	376 - 383	513.4	2	1
LcAIPkLR	98 - 105	507.1	2	4
LDAVkEK	206 - 212	846.1	2	3
LDAVkEkALVAAVR	206 - 219	523.8	3	3
LDAVkEkALVAAVR	206 - 219	784.9	2	3
LQAccDkPVLQK	299 - 310	751.7	2	3
LQAccDkPVLQK	299 - 310	501.6	3	2
LRDNYGELADccAkQEPER	104 - 122	790.0	3	3
LVQEVTDFAkTcVADENAENcDK	66 - 88	900.9	3	3
MkcSSMQR	222 - 229	536.1	2	1
NLVkTNcELYEK	410 - 421	777.6	2	3
PVLQkSQcLAETEHDNIPADLPSIAADFVEDK	306 - 337	1199.1	3	1
RHPYFYAPELLYYAEkYNEVLTQccTESDK	169 - 198	1278.2	3	3
RPcFSALTVDETYVPkEFK	509 - 527	777.1	3	5
SIHTLFGDkLcAIPK	89 - 103	582.1	3	3

SIHTLFGDkLcAIPK	89 - 103	871.9	2	2
SQcLAETEHDNIPADLPSIAADFVEDkEVcK	311 - 341	1182.6	3	1
TcVADENAENcDkSIHTLFGDK	76 - 97	856.6	3	2
TVMGDFAQFVDkccK	570 - 584	617.2	3	3
TVMGDFAQFVDkccK	570 - 584	925.1	2	4
VGTkccTLPEAQR	457 - 469	522.2	3	1
VGTkccTLPEAQR	457 - 469	782.1	2	9
VTkccSGSLVER	497 - 508	720.6	2	1
YMcENQATISSkLQAccDKPVLQK	287 - 310	972.5	3	2
YNEVLTQccTESDkAAcLTPK	185 - 205	844.4	3	2
YNEVLTQccTESDkAAcLTPK	185 - 205	1265.9	2	2
YTQkAPQVSTPTLVEAAR	435 - 452	668.4	3	5
YTQkAPQVSTPTLVEAAR	435 - 452	1002.0	2	8

*Lower case k indicates site of carbamoylation

APPENDIX VII: Supplementary Table 2; Carbamoylated peptides in the water-insoluble fraction of rat spinal cord used to quantify the relative amount of carbamoylation. The observed m/z value for each modified peptide was used to create an inclusion list in subsequent LC/MS/MS runs to maximize the collection of MS/MS spectra for carbamoylaed peptides, and increase the throughput of the analysis.

Protein	Carbamoylated peptide*	Residue No	Observed m/z value	Charge State	No of assigned MS/MS spectra during longer shot-gun analysis
Myelin basic protein S OS=Rattus norvegicus GN=Mbp PE=1 SV=3	FSWGAEGQkPGFGYGGR	138-154	616.1	3	1
Myelin basic protein " " " " " "	GFkGAYDAQGTLSK	167-180	770.9	2	2
Myelin basic protein " " " " " "	HGSkYLATASTMDHAR	164-177	743.5	2	2
Myelin basic protein " " " " " "	TQDENPVVHFFkNIVTPR	11_26	597.2	3	5
Myelin basic protein " " " " " "	TQDENPVVHFFkNIVTPR	104-121	1092.8	2	3
Myelin basic protein " " " " " "	TTHYGSLPQk	104-121	728.9	3	4
Myelin basic protein " " " " " "	TTHYGSLPQkSQR	91-100	588.2	2	1
Myelin basic protein " " " " " "	TTHYGSLPQkSQR	91-103	516.0	3	2
Myelin basic protein " " " " " "	TQDENPVVHFFkNIVTPR	91-103	774.1	2	1
Neurofilament light polypeptide OS=Rattus norvegicus GN=Nefl PE=1 SV=3	TTHYGSLPQkSQR	91-103	774.1	2	1
Neurofilament light polypeptide " " " " "	AkTLEIEAcR	315-324	617.6	2	3
Neurofilament light polypeptide " " " " "	LAAEDATNEkQALQGER	148-164	945.2	2	3
Neurofilament light polypeptide " " " " "	LAAEDATNEkQALQGER	148-164	629.8	3	2
Neurofilament light polypeptide " " " " "	TQEkAQLQDLNDR	88-100	801.7	2	1

2',3'-cyclic-nucleotide 3'-phosphodiesterase OS=Rattus norvegicus GN=Cnp PE=1 SV=2	DkPELQFPFLQD EDTVATLHEcK	28-50	935.6	3	2
2',3'-cyclic-nucleotide 3'-phosphodiesterase " " " "	EKNQWQLSLDDL kK	162-175	597.3	3	1
2',3'-cyclic-nucleotide 3'-phosphodiesterase " " " "	LDcAQLkEK	155-163	574.5	2	1
2',3'-cyclic-nucleotide 3'-phosphodiesterase " " " "	LkPGLEKDFLPLY FGWFLTK	176-195	819.7	3	1
2',3'-cyclic-nucleotide 3'-phosphodiesterase " " " "	NQWQLSLDDLkK	164-175	511.1	3	1
2',3'-cyclic-nucleotide 3'-phosphodiesterase " " " "	RPPGVLHcTTkFc DYGK	244-260	694.3	3	1
Myelin proteolipid protein OS=Rattus norvegicus GN=Plp1 PE=1 SV=2	GLSATVTGGQkG R	112-124	426.0	3	3
Myelin proteolipid protein " " " " "	GLSATVTGGQkG R	112-124	637.9	2	4
Myelin proteolipid protein " " " " "	MYGVLPWNAFP GkVcGSNLLSIcK	206-229	1377.8	2	4
Myelin proteolipid protein " " " " "	MYGVLPWNAFP GkVcGSNLLSIcK	206-229	919.2	3	7
Myelin proteolipid protein " " " " "	QIFGDYkTTIcGK	99-111	787.5	2	2
Myelin proteolipid protein " " " " "	TTIcGkGLSATVT GGQK	106-122	575.3	3	1
Myelin proteolipid protein" " " " "	TTIcGkGLSATVT GGQK	106-122	861.6	2	3
Glial fibrillary acidic protein OS=Rattus norvegicus GN=Gfap PE=1 SV=2	AkEPTkLADVYQA ELR	104-199	640.2	3	1
Glial fibrillary acidic protein" " " " "	FLEQQNkALAAE LNQLR	87-103	677.3	3	1
Glial fibrillary acidic protein " " " " "	SkFADLTDVASR	257-268	676.6	2	4

*Lower case k indicates site of carbamoylation

APPENDIX VIII: Ethical approval letter for non-human primate studies