Glutaric Acidemia Type 1: Human pathology and attempted development of an animal model

A thesis presented to the University of Manitoba in partial fulfillment of requirements for the degree of Masters in Science in the Department of Pathology

Presented by

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Glutaric Acidemia Type 1:

Human Pathology and Attempted Development of an Animal Model

BY

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

MASTER OF SCIENCE

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Abstract

Glutaric acidemia type 1 (GA-1) is an autosomal recessive disorder with macrocephaly, mental retardation, severe movement disorders, and seizures, that leads to early childhood death in most cases. The onset of brain damage is often sudden, occurring in infancy during a febrile illness. Lack of glutaryl-CoA dehydrogenase (GCDH) activity causes the accumulation of glutaric acid (GA) and 3-hydroxyglutaric acid (3-OH-GA) in the blood, urine, and cerebrospinal fluid (CSF). Neuropathological features of 5 children and one adult with GA-1 from a single genetic North American aboriginal background are presented. All had enlarged ventricles and atrophy of the striatum. In comparison to age matched controls, there is severe loss of medium-sized neurons in the caudate and putamen, with dorsal areas more severely affected. Near complete neuron loss appears to occur within a few months of the first encephalopathic crisis and is not progressive. This cohort displays similar pathologic characteristics to those previously described in children and adults of varied ethnic background. The pathogenesis of selective neuronal loss remains to be determined. To try to determine the pathogenesis of the human disease we attempted to develop a rodent model of GA-1 that would exhibit similar brain damage. We hypothesized that intrastriatal injections of GA or 3-OH-GA could be used to create specific neuron loss. Adult, 3-week, and 2-week-old rats were given "high" and "low" concentration injections. High concentrations caused necrotic lesions in striatum. Low concentration injections showed a dose-dependent effect ranging from negligible damage, to white matter axonal damage, to small areas of neuron loss in the vicinity of the needle entry site. A separate group of animals was given a intraperitoneal injection of

lipopolysaccharide (LPS) prior to 3-OH-GA administration to create an inflammatory state that mimics the usual human onset during a febrile illness. In no case were the injections associated with widespread selective neuronal loss. We conclude that the simple model of a single GA or 3-OH-GA injection into rat brain does not replicate the neuropathological findings in humans. It therefore cannot be depended upon as a model in which to test pharmacologic interventions.

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List of Abbreviations

3-OH-GA	3-hydroxyglutaric acid
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
GA	Glutaric acid
GA-1	Glutaric acidemia type 1
GCDH	Glutaryl-CoA dehydrogenase
Ca^{2+}	Calcium
cm	Centimeter
CO_2	Carbon dioxide
CP	Creatine phosphate
CSF	Cerebrospinal fluid
CT	Computed tomography
DNA	Deoxyribonucleic acid
GABA	γ-amino butyric acid
GAD	Glutamate decarboxylase
GC	Gas chromatography
GCDH	Glutaryl-CoA dehydrogenase
Gcdh	Mouse glutaryl-CoA dehydrogenase
GFAP	Glial fibrillary acidic protein
H&E	Hematoxylin and Eosin

IBSN	Infantile bilateral striatal necrosis
i.c.	Intracerebral
i.p.	Intraperitoneal
kg	Kilogram
LPS	Lipopolysaccharide
M	Molarity
Mol	Mole
mg	Milligram
ml	Milliliter
mm	Millimeter
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NMDA	N-methyl D-aspartate
PBS	Phosphate buffered saline
RNA	Ribonucleic acid
ROS	Reactive oxygen species
US	Ultrasound
μg	Microgram
μl	Microliter
μm	Micrometer

1.0 Review of literature

1.1 Introduction

Glutaric acidemia type 1 (GA-1) is a rare autosomal recessive disorder of amino acid metabolism caused by the absence of functional glutaryl-CoA dehydrogenase (GCDH) [1], an essential enzyme in the catabolic pathways of L-tryptophan, L-lysine, and L-hydroxylysine [2-4]. GCDH is a nuclear-encoded, mitochondrial matrix enzyme, lack of functional enzyme activity leads to the accumulation of glutaric acid (GA), 3-hydroxyglutaric acid (3-OH-GA), and glutaconic acid in the blood, urine, and to a lesser extent in the cerebrospinal fluid (CSF) [2, 3, 5].

GA-1 affected children appear fairly normal at birth, although they may have large heads (macrocephaly). However, progressive dystonia and choreoathetosis, seizures, developmental delay, and mental retardation [6, 7] appear abruptly at around 6 to 18 months of age, often in conjunction with a febrile illness. Brain damage appears to be localized to the striatum (the caudate nucleus and putamen). In most circumstances the damage leads to death in early childhood but some individuals survive for years despite severe neurologic impairment.

1.2 Human Inheritance, Genetics and Clinical Manifestations

Numerous studies have addressed the genetic background in humans as well as the clinical manifestations of GA-1. The GCDH gene is on chromosome 19p13.2, spans about 7kb, comprises 11 exons and 10 introns and has been fully sequenced [8]. There are close to 100 pathogenic mutations in the gene coding for GCDH which have been identified by many laboratories around the world and although some mutations correspond to excretion levels of GA and 3-OH-GA there seems to be no correlation between genotype and clinical phenotype [9, 10].

Haworth et al. [11] observed that there was an overrepresentation of hereditary metabolic diseases including GA-1 among aboriginals (Ojibway-Cree) in of northern Manitoba and northwestern Ontario [11], Canada, where more than 26 affected children have been identified since 1970. In this particular population the estimated GA-1 carrier frequency is 1 in 10. This mutation is a G-to-T transversion at the +5 position of intron 1 of the GCDH gene that affects the Island Lake aboriginals on northern Manitoba and northwestern Ontario [9, 12]. Although the majority of cases are not the results of overt consanguineous matings, inbreeding is a significant contributing factor to the higher incidence in this population. The clinical phenotype is widely variable [6, 9] with regards to severity movement disorders and levels of GA and 3-OH-GA excretion. Interestingly, this cohort differs markedly from other cases reported in that the amount of GA excreted in their urine is quite small, less than 700μmol/mmol creatinine (average GA-1 patient, 700 to 21,500 μmol/mmol creatinine) [6]. Nevertheless one cannot any assumptions about the severity of the phenotype from the specific mutation.

For the majority of patient affected by GA-1 between 6 and 18 months of age, mild neurological symptoms may become exacerbated by a variety of events such as, fever, infections, immunizations, fasts (required for surgery), or minor head injuries [10].

Hoffmann et al. [13] conducted a retrospective analysis of 57 patients of varying genetic background with GA-1. Patients were identified after the onset of the neurological disease (n=36) or prior to onset (n=21). Carnitine levels were found to be reduced in all patients at diagnosis. Macrocephaly was exhibited in 70% of cases.

Strauss and colleagues [14] present a natural history of 77 patients that they categorize as Amish and non-Amish. The Amish cohort was affected by one specific GCDH mutation while the non-Amish group demonstrated a wide array of mutations. In addition, both groups demonstrated a wide variety of disease severity and they concluded that one cannot judge clinical outcome based on a specific mutation.

1.3 Human biochemical changes

The biochemical hallmark of GA-1 is the accumulation of GA, 3-OH-GA, and glutaconic acid in blood. In the urine of GA-1 patients, these metabolites may be severely elevated (aciduria) to slightly elevated or even normal, which make the diagnosis of GA-1 difficult. High levels of glutaric acid may be found in

skeletal and heart muscle and aqueous humor of the eye [15]. Cultured skin fibroblasts showed lack of GCDH activity [7].

Kolker and coworkers [16] determined in a single postmortem investigation of a GA-1 patient that concentrations of 3-OH-GA and GA in urine were elevated (156-224 mmol/mol and 1998-3608 mmol/mol creatine respectively). These levels were also elevated in blood plasma and cerebrospinal fluid (CSF) (plasma, GA = 16.9μmol/L, 3-OH-GA = 30.6μmol/L; CSF, GA = 39.7μmol/L, 3-OH-GA = 4.5μmol/L). In a unique experiment the levels of these and other metabolites were measured in various brain regions. The levels of 3-OH-GA and GA in the putamen were 62 and ~50nmol/g protein respectively. Assuming ~13% protein content [17], this is equivalent to 0.48μmol/g wet brain weight for 3-OH-GA. In another case, Goodman [2] reported a frontal cortex concentration of 1.04 μmol/g for GA.

Research focusing on the concentrations of various organic acids and metabolites in blood and CSF has yielded mixed and often conflicting results. The concentrations of organic acids in the CSF are directly related to their rate of production in the brain. The concentrations of GA in the urine, plasma, and CSF were determined in 5 cases [18]. In all cases there were inconsistencies found when comparing the concentrations of GA from the three sample sources. While urine GA concentration may be decreased through diet, CSF and plasma concentrations remain unchanged. The exact relationship between sample concentrations is not known, because of this, a diagnosis of GA-1 cannot be made solely on the basis of urine GA concentration.

Diagnosis of GA-1 can be made after repeated blood and urine analysis by high performance liquid chromatography, gas chromatography (GC), mass spectrometry (MS) , and enzymologically [19]. Recently, PCR has been used to identify a specific GCDH mutation that occurs in this unique population [11]. This method has proven extremely effective in identifying carriers of GA-1 and affected infants at birth. It is a definite improvement when compared to previous methods of mass spectrometry (MS) and gas chromatography (GC) [6].

1.4 Imaging Studies

Imaging techniques such as ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI) have the ability to identify common features seen in the brains of GA-1 patients. Stereotypical neuroimaging findings for cases of GA-1 include enlarged ventricles, frontotemporal atrophy, widening of the Sylvian fissure, delayed myelination or white matter injury, basal nuclei atrophy and calcification, chronic subdural effusions and hematomas [13, 20-25]. CT and MRI studies have also identified basal nuclei or periventricular hypodensities [22]. In one study [20] a complete lack of "operculation" of the temporal lobes was observed. Since this is an event that normally takes place during the last trimester of gestation, this data that the toxic effects of GA-1 may start in utero. The only findings that can be identified by imaging prior to onset of clinical signs is macroencephaly (macrocephaly by overt observation) and widening of the Sylvian fissures [19]. In many possible and confirmed cases the deterioration of the

caudate and putamen may not be prominent enough to make a diagnosis of GA-1 [26].

1.5 Pathological studies

1.5.1 Striatal anatomy and biochemistry

In humans, the caudate and putamen are subcortical forebrain gray matter structures situated near the frontal horns of the lateral ventricles. Collectively they form the striatum. The caudate head bulges into the lateral ventricle frontal horn and the long, narrow body and tail curve along the ventricle ending along the temporal horn opposite the hippocampus. The putamen is an irregular disc shape, lying more lateral and separated from the head of the caudate nucleus by the fiber bundles of the internal capsule. Due to the presence of "striatal bridges" this separation is incomplete. This is most evident at rostral levels where the head of the caudate joins the putamen by means of these gray matter bridges [27]. Ventrally the two structures merge to form the nucleus accumbens. In rodents, the caudate and putamen are not separate structures.

Input into the striatum is from three main sources: glutamatergic input from the thalamus and cerebral cortex and dopaminergic from the substantia nigra. Excitatory amino acid receptors are mainly localized on

the medium spiny neurons, which are the main efferent cells of the striatum. They project to the globus pallidus and substantia nigra and use GABA as well as various peptide neurotransmitters. These projections act as a main signaling pathways for both sensory and motor function [27]. Large cholinergic neurons also form a significant minor population of cells that project locally within the striatal region [27].

Research into the neurodevelopment of the rodent striatum is valuable because it can give insight into possible human neurodevelopment and may explain susceptibility of the striatum in GA-1. In situ studies have focused on messenger ribonucleic acids (mRNAs) encoding for various subunits of the NMDA receptor in the developing rat CNS. One such study demonstrated that the four NR2 transcripts (NR2A, B, C, and D) displayed distinctive expression patterns while the NR1 transcripts were expressed in virtually all neurons. The different pattern of gene expression which occur in rats until P20, indicate a temporal role in the development of the CNS and that the functions properties of the NR2 subunits provide a basis for the NMDA channel diversity in the brain [28].

1.5.2 Histological Studies

The neuropathology of 10 cases affected with GA-1 described in the literature [2, 7, 15, 16, 21, 24, 29, 30]. A summary of the gross pathology and histological findings are summarized in Table 1. In general, the

pathological findings confirm the imaging data. The striatum of GA-1 cases demonstrate enlarged ventricles, a severe loss of neurons, gliosis, and calcification in the basal nuclei, frontotemporal atrophy, widening of the Sylvian fissure, and white matter spongiosis. . Mild spongiform changes of in the cerebral cortex and white matter and migration or maturation abnormalities are also seen [24].

Table 1. Gross pathology and histological finding in 10 cases of GA-1

Author	Age	Gross Pathology				Histological findings	
		Brain Weight (g)	Open Sylvian Fissure	Enlarged lateral ventricles	Striatal Atrophy	Neuron loss & gliosis, striatum	White matter spongiosis
Goodman et al. 1977	10y	1620g (↑)	-	-	putamen	-	-
Leibel et al. 1980	3.5y	1475g (↑)	-	-	caudate, putamen	-	-
Bennett et al. 1986	10.5m	NA	-	-	-	"fatty change", caudate	-
Chow et al. 1988	2.5y	1025g (N)	-	+	caudate, putamen	+	+
Chow et al. 1988	7y	NA	-	+	caudate, putamen	+	+
Chow et al. 1988	9y	1345g (↑)	-	+	caudate, putamen	+	+
Bergman et al. 1989	13m	NA	-	+	?caudate, putamen	putamen only	+
Soffer et al. 1992	6.5y	1450g (↑)	+	+	caudate, putamen	+	+
Kimura et al. 1994	10m	800g (N)	+	+	caudate, putamen	+	+
Kolker et al. 2003	14y	1532g (↑)	+	+	caudate, putamen	+	+

Note: Table adapted from Soffer et al. 1992.

NA = not available; ↑ = heavy for age; N = normal for age; + = present; - = absent

1.6 Human diseases with similar pathology

In GA-1, damage to medium spiny neurons in the striatum leads to the movement disorders. There are many other familial diseases of the basal nuclei that are similar in onset and pathology to GA-1.

Infantile bilateral striatal necrosis (IBSN) is a sporadic and familial disease of bilateral caudate and putamen necrosis affecting children under the age of three [31]. It too usually develops in conjunction with a febrile illness and causing necrosis of the entire neuronal population of the striatum.

Huntington's disease is an autosomal dominant disorder usually affecting people in their fourth decade of life and is very rare in children. Neuropathologic changes include chronic neuron loss and astrocytosis of the caudate, putamen, globus pallidus, and other regions of the brain [32].

Wilson's disease is an autosomal recessive disorder in which excessive amounts of copper accumulate in the body. Neuron loss occurs in the putamen, globus pallidus, subthalamic nucleus, substantia nigra, and dentate nucleus. These diseases along with others [33] resemble GA-1 but differ slightly in anatomical structures and cell populations affected. In addition, the pathogenesis of all of these diseases is poorly understood and available treatments for some disorders are inadequate [34-36].

Hypoxic-ischemic encephalopathy (HIE) in neonate is a disorder of excessive neuronal excitation. HIE is triggered by a disruption in the re-uptake of glutamate from the synapse and excitation of post-synaptic glutamate receptors.

Together hypoxia and ischemia lead to a cascade of events that result in cell death by necrosis and/or apoptosis [37].

In addition, MRI and neuropathology studies have demonstrated selective patterns of injury to sub-regions of the basal nuclei in children. Some examples are kernicterus and certain mitochondrial encephalopathies which cause damage to the putamen and globus pallidus, and near-total perinatal asphyxia which causes damage to the putamen and the thalamus. Locations within the neurotransmitter-specific circuitry of the basal nuclei motor loop may be an explanation of the differential vulnerability of nuclei that are millimeters from each other. In severe hypoxic-ischemic encephalopathy, excitatory glutamatergic pathways into the putamen and thalamus are overactive but the globus pallidus may be protected by its inhibitory neuronal activity. In contrast, high resting neuronal activity in the globus pallidus may make it more vulnerable to less intense, sub-acute oxidative stresses from mitochondrial toxins such as bilirubin or from genetic mitochondrial disorders [38].

The basal nuclei, including the caudate, putamen, and globus pallidus is an area that continues to be a focus for many investigators, likely due to the plethora of movement disorders that involve the striatum.

1.7 Mechanism of damage

The leading biochemical hypothesis behind the pathogenesis of GA-1 is that GA, 3-OH-GA, and possibly quinolinate act through NMDA receptors to

produce a neurotoxic effect [39]. This neurotoxicity leads to the destruction of medium-sized neurons and gliosis of the caudate and putamen. Quinolinic acid has also be hypothesized to play a significant role in this excitoxicity [19]. The neurotoxicity of these metabolites has been demonstrated in a variety of in vitro models. In addition to the neuroexcitotoxicity of GA and 3-OH-GA, Kolker et al. [40] indicate that these metabolites indirectly regulate glutamatergic and GABAergic (γ-amino butyric acid) neurotransmission. This results in an imbalance of inhibitory and excitatory neurotransmitters. A second theory is that secondary amplification loops such as nitric oxide production, a decrease in energy metabolism, and reduction of cellular creatine phosphate levels potentiate the neurotoxic properties. In addition, maturation dependent changes in the expression of neuronal glutamate receptors may enhance the toxicity of GA and 3-OH-GA.

The majority of research concentrating on GA-1 focuses on the loss of neurons within the striatum via necrosis, however, in the review of drug induced apoptosis, Olney et al. [41] demonstrated the vulnerability of the developing brain to various agents that affect both the NMDA and the $GABA_A$ receptor. NMDA blockers and $GABA_A$ promoters injected during the time of peak neurogenesis in the developing rodent brain cause widespread apoptotic neurodegeneration. Ethanol has been shown to be an NMDA antagonist as well as to mimic the effects of GABA and cause a strong pattern of apoptosis. This may be a leading factor in understanding developmental disorders such as fetal alcohol syndrome (FAS). Abuse of other drugs such as barbiturates, benzodiazepines,

anticonvulsants, and anesthetics may cause similar developmental disorders due to their effects of NMDA and GABA receptors. Although apoptosis may play a role in neuron loss, the ability to delineate the cause of death of a particular neuron is not possible if it no longer exists. Since human research especially with regards to GA-1 is based on autopsy studies the ability to differentiate between apoptosis and necrosis is impossible.

1.8 Current methods of treatment of GA-1

More than 90% of affected children will go on to develop severe neurological disabilities [10]. Several treatments are used but none is fully protective. Carnitine supplementation and low lysine and tryptophan diets [42] along with riboflavin supplements, GABA analogues (baclofen and vigabatrin), and vitamin E [34] are the most effective treatment at the moment. There is significant evidence that vigorous treatment and supplementation of carnitine improves outlook for patients. However there is still questions about the effectiveness of the low lysine and tryptophan diet. Generally, most people are in agreement that for a treatment to be affective it must be implemented diagnosis prior to the onset of symptoms [13]. In Manitoba, perinatal deoxyribonucleic acid (DNA) testing has been used to screen for infants that carry the specific allele (either heterozygous or homozygous) for GA-1 [11,34]. Although, this new technique allows us to identify children that may become affected with GA-1, an early intervention with

current methods of pretreatment is not be enough to prevent the effects of this disorder in these children.

A case report of an infant diagnosed at 25 months of age with GA-1 showed a typical pattern of clinical manifestation, onset, and treatment [19]. Treatment with riboflavin, L-carnitine, vigabatrin and baclofen produced some symptomatic relief. This action was implemented after prior treatment and was more beneficial than the use of other drugs such as nitrazepam, sodium valproate for seizure control and low protein diet.

A GA-1 female patient was diagnosed at 6.5 months of age. Despite a good biochemical response to a reduction of dietary lysine and tryptophan there was no response to riboflavin therapy [15]. Her neurological condition deteriorated until her death at 10.5 months. High levels of glutaric acid were found in skeletal and heart muscle and aqueous humor of the eye [15].

Kolker et al. [43] described a case report of a GA-1 patient who had an acute encephalopathic crisis despite early intervention. L-carnitine supplementation and a lysine and tryptophan reduced diet was begun at 1 month of age (prior to crisis). At age six months the patient was hospitalized after a respiratory tract infection. Despite efforts the encephalopathic crisis worsened and led to bilateral striatal destruction (diagnosed by MRI). This report indicates that current therapeutic interventions, whether they are pharmacological or dietary, are insufficient for high-risk patients. This case report further stresses that need for new therapeutic approaches to GA-1 [43]. This variety in treatment effectiveness is the prime reason that an animal model must be produced in order to test possible treatments.

1.9 In vitro experiments

In vitro studies have focused on the neurotoxicity of GA and 3-OH-GA which are thought to act through NMDA mediated receptors. There are various other acids that cause abnormalities of the basal nuclei, cortical atrophy and white matter impairments. One of these is methylmalonic acid, which like GA and 3-OH-GA is thought to act through the NMDA receptor [44].

Corticostriatal and hippocampal rat brain slice cultures exhibited massive cell death when treated with glutamate, kainate, and NMDA (glutamate receptor agonists). These effects could be avoided with the preincubation of receptor antagonists (MK 801 and CNQX). Short-term incubation with GA, 3-HO-GA, or glutaconic acid did not lead to significant cell death in either culture. In addition, long-term incubation with GA or glutaconic acid did not result in significant tissue loss. In contrast, the long-term incubation of the culture in 3-OH-GA resulted in a dose-dependent neurotoxic effect in both corticostriatal and hippocampal cultures similar to the effects seen with the glutamate receptor agonists. This effect could also be avoided with the pre or coincubation with MK 801 or CNQX. An interesting note is that pathophysiological concentrations taken from human cases were used in a variety of these experiments and demonstrated no cell swelling or cell death. It was not until concentrations of greater than two to five times higher were used did the cultures exhibit extensive cell death [45].

Incubation of primary neuronal cultures from 7-day-old chick embryo telencephalons with GA and 3-OH-GA decreased cell viability. The effectiveness of this was dependent on metabolite concentration and length of incubation. Maximum decrease in cell viability was seen at extremely high concentrations (5mmol/L) for both metabolites. Data showed that GA was more effective at decreasing cell viability at lower concentrations while 3-OH-GA had a greater maximum decrease at higher concentrations when compared to GA. The destruction caused by both metabolites was potentiated by MK 801 and ifenprodil and NR2B antibodies. A variety of other antagonists proved to be ineffective [39, 46].

Using these same neuron cultures, it was demonstrated that GA and 3-OH-GA also increased toxicity in parallel to the increasing expression of the NR2B subunit on the NMDA receptor. It was concluded from these studies that GA and 3-OH-GA act as false neurotransmitters and activate the NR2B subunits in particular [47]. One interesting study demonstrated that short-term incubation of chick embryo telencephalons cell cultures in GA caused partial tolerance to 3-OH-GA and NMDA mediated cell damage. The NR2B subunit is of particular importance in this GA induced effect, resulting in reduced generation of reactive oxygen species (ROS) and reduced calcium ion (Ca^{2+})increase [48].

Chemiluminescence of glutaric acid, which is a measure of free radial production, has also been shown to be significantly increased brain homogenate culture. GA demonstrated a reduction of total radical-antioxidant potential (TRAP), which represents the antioxidant capacity of the tissue. Taken together these data demonstrate that GA produces significant oxidative stress in the in vitro

rat brain [49]. This experiment was replicated in vivo using 3-OH-GA and it was observed that 3-OHGA significantly increased chemiluminescence and nitric oxide metabolites. This was in contrast to TRAP, which was decreased. The data indicate a stimulation of lipid peroxidation and free radical production, and a reduction of the tissue antioxidant defenses is caused by3-OH-GA. If these findings also occur in the human condition, it may be presumed that oxidative stress is involved in the brain damage observed in GA-1 [50].

When the cellular concentration of high-energy phosphate compounds (creatinephosphate, CP; adenosine triphosphate, ATP; adenosine diphosphate, ADP; adenosine monophosphate, AMP) were measured after a mixed cortical cell culture (cells taken from neonatal rates P1) were exposure to 3-OH-GA they showed a significant reduction in CP. This reduction occurred in a dose dependent manner, while the concentrations of ATP, ADP, and AMP remained unchanged. Such reduction could be prevented with the pre incubation with MK 801, a non-competitive NMDA receptor antagonist or the co-incubation with a high concentration of creatine. The reduction of CP levels may result in a lack of energy within the cell and cause the opening of NMDA receptor associated ion channels. The opening of these ion channels could lead to membrane depolarization and impairment of cell functions [51].

Since the acute encephalopathic crisis seen in GA-1 is typically precipitated by a febrile illness, it may be useful to look at the role of inflammatory cytokines in this disease. One study investigated the effects of interleukin-1β and interferon-γ on 3-OH-GA toxicity in rat cortical and

hippocampal astrocyte cultures. Cytokine pretreatment potentiated the neurotoxic effects of 3-OH-GA. Co-treatment of cytokines also induced the expression of astrocytic inducible nitric oxide synthase (iNOS). NOS inhibition prevented iNOS-mediated potentiation of 3-OH-GA toxicity, however, it failed to protect the culture from direct neurotoxicity of 3-OH-GA. This effect could be blocked by MK 801, again supporting a role for NMDA receptor stimulation [52].

Studies focusing on reactive oxygen species (ROS) demonstrated slightly elevated levels after exposure to 3-OH-GA. ROS production and neuronal damage caused by the incubation of 3-OH-GA with neuronal cultures from chick embryo telencephalons was reduced with a pretreatment of creatine. However, creatine was only effective if given more than six hours prior to exposure to 3-OH-GA. This is still a significant finding keeping in mind that this disorder can be identified at birth and early intervention is possible [53].

Previous studies has shown that in vitro, GA can inhibit the uptake of [3H]glutamate leading to elevated concentrations of the excitatory neurotransmitter in the synapse. This has the potential of having excitotoxic effects on neural cells [54].

In vitro administration of GA has been shown to significantly reduced brain carbon dioxide (CO_2) production and adenosine triphosphate (ATP) as well as multiple oxireductase complexes. These results indicate that GA impairs brain energy production and if these results occur in vivo in the human, it may contribute to GA-1 neuropathology [55]. Inhibition of brain glutamate decarboxylase by glutarate, glutaconate and beta-hyrdroxyglutarate may be a

possible explanation for the destruction seen in GA-1 patients. Glutamate causes the opening of the NMDA receptor and the subsequent excitation of the cell. If glutaric acid impairs the function of the glutamate decarboxylase enzyme this would lead to the accumulation of glutamate in the synapse. This excess of glutamate would be able to continually stimulate the NMDA receptor without getting broken down. This would result in over-excitation of the NMDA receptors, an influx of Ca^{2+} and lead to excitotoxic cell death [56].

Research using cortical tissue slices of rat brain show that 3-OH-GA is the pertinent neurotoxin in GA-1 when compared to the effects of GA and glutaconic acid. 3-OH-GA was shown to produce an indirect activation of NMDA receptors and reduced cellular ATP levels. As other studies have suggested [28, 39] the activation of the NMDA receptor may be due to the selective expression of the NR2B subunit [57].

Directly opposed to the excitatory actions of glutamate is γ-amino butyric acid (GABA), which is formed from the decarboxylation of glutamate by a family of cytosolic and membrane-bound glutamate decarboxylase (GAD) enzymes and is the main inhibitory neurotransmitter in the CNS. In the adult, blockade of GAD-derived GABA rapidly leads to convulsions and death. Activation of GABA during development directs the migration of post-mitotic neuroblasts and stimulates their motility via Ca^{2+} signaling mechanisms. These findings suggest that GABA can affect embryogenesis of the CNS through effects on cell migration and proliferations [58].

Heyes [59] hypothesizes a role of quinolinic acid in the neuropathology of GA-1. The GCDH enzyme normally converts tryptophan into quinolinic acid and according to the law of mass action the production of quinolinic acid should be increased in GA-1. Quinolinic acid is a potent neurotoxin causing convulsions when injected into the CNS of experimental animals and could play a part in the neuropathology of GA-1.

1.10 In vivo experiments

At present there is currently no satisfactory in vivo model of GA-1. There have been in vivo and in vitro as well as genetic knockout experiments preformed but none have provided the accurate selective neuronal loss required for a model of GA-1. Although the in vitro experiments have shown the excitoxicity of the majority of the metabolites that build up in GA-1, when transferred over to the in vivo models they fail to provide an adequate model. An excellent review of the relevant models of acute striatal necrosis can be found [60].

In vivo experiments are beginning to be used to test the effects of the various metabolites that accumulate in GA-1. The most common is the use of a stereotaxic intrastriatal injection technique to investigate the behavioral and neurotoxic effects of these metabolites. One group performed this type of experiment using 3-OH-GA in rats. Cannulaes were implanted and the animals were given 3 days to recover prior to 3-OH-GA exposure. They demonstrated that 3-OH-GA induced an increase in convulsion frequency and duration. They

showed on Nissl-stained brain sections from treated rats pale lesions in the striatum. These effects were prevented by pretreatment with MK-801, an NMDA receptor antagonist and stimulation of $GABA_A$ receptors by muscimol. They concluded that 3-OH-GA induces convulsions and striatal damage via an imbalance in the excitatory glutamatergic and the inhibitory GABAergic neurotransmission. This results in over excitation of neurons and appears to support the NMDA receptor-mediated hypothesis [61]. These same effects were seen when testing the neurotoxic effects of methylmalonic acid [62]. Though these studies demonstrated that metabolites can cause increases in convulsions they failed to clearly describe the type and/or population of cell loss. The destruction is not limited to specific neuronal loss and is therefore not an accurate model of GA-1.

In vivo work has also been done to study the effects of GA [63]. Adult rats received unilateral intrastriatal injections of GA via a prior implanted cannula. GA induced rotational behavior and clonic convulsions in a dose-dependent manner. Rotational behavior was prevented by intrastriatal pretreatment with DNQX (AMPA receptor blocker) and muscimol ($GABA_A$ receptor agonist), but not by MK-801 (NMDA receptor antagonist). Convulsions were prevented by intrastriatal pre-injection of muscimol. This study provides evidence for a participation of glutamatergic and GABAergic mechanisms in the GA induced behavioral alterations. However, there was no investigation of the pattern of cellular loss, which is essential for a working animal model.

Since GA-1 is a genetic disease in human, the logical modeling approach was to knockout the GCDH gene in mice. Koeller et al. [64] described the genomic mapping and cloning of the mouse GCDH (Gcdh) gene and cDNA along with its chromosomal localization. The Gcdh cDNA is 1.75 kb long and contains 438 amino acids. The amino acid sequences of mouse, human, and pig GCDH are highly conserved. The mouse gene was mapped by backcross analysis to chromosome 8 within a region that is homologous to a region of human chromosome 19, where the human gene was previously mapped[64].

The targeted deletion of the Gcdh gene in embryonic stem cells produced a mouse that had no functional GCDH. These Gcdh-/- mice have biochemical phenotypes similar to human GA-1 patients, including elevated levels of GA and 3-OH-GA. However, the mice have only mild motor impairment and they do not develop the progressive dystonia seen in human patients. Pathologically, Gcdh-/- mice do not exhibit the neuron loss or gliosis in the striatum but they do demonstrate diffuse spongiform myelinopathy similar to that seen GA-1. Exposing these mice to metabolic stress (injections of poly-inosine/cytosine or a-interferon) to mimic the cytokine response to a viral infection, which often precipitates the encephalopathic crisis seen in humans failed to produce any neurologic effect. The conclusion of this knockout study was that the lack of similarity in neurologic phenotype and striatal pathology of GA-1 is due to intrinsic differences between the striata of mice and humans [65]. In addition, analysis of the expression of the Gcdh in the knockout model demonstrated that

there is no link between the amount of expression and the specific neuropathology seen in GA-1 [66].

1.11 Summary

GA-1 is an inheritable defect in activity of GCDH that leads to accumulation of GA and 3-OH-GA and causes catastrophic damage to the striatal neurons leading to movement abnormalities, seizures and early death. The histopathological changes have been described in 10 human cases but the pathogenesis of the brain damage is not understood.

The effects of GA, 3-OH-GA and their metabolites on neurons are reasonably well understood in vitro. There are still questions that need to be answered with regard to the effects of these metabolites in the developing human brain. This is difficult when the knockout mouse does not have the same deficits seen in the human condition. And so far their in vivo effects have not led to an animal model that clearly mimics the human disorder.

Since the disorder can be identified at birth in this population [34] we hope to develop an intervention that could reduce brain damage incurred by this disorder. Current therapeutic strategies are insufficient and cannot reliably and effectively reduce, delay, or prevent onset of this disorder. There are many gaps in the present understanding of neuropathogenesis of striatal injury in this disorder.

2.0 Hypotheses and goals

Hypothesis 1: Neuropathological changes in the Manitoba/Ontario aboriginal cohort of GA1 are similar to those reported in other ethnic backgrounds. Goal 1: To characterize the neuropathology (i.e. neuronal loss, atrophy, and immuno reactivity) of the human disorder GA-1. Herein we describe the detailed neuropathological findings in the brains of 5 children and 1 adult. This cohort is of particular interest because all patients share the same genetic background with a range of survival times.

Hypothesis 2: GA or 3-OH-GA will cause selective medium neuron loss when injected into the striatum of rat brain. There will be an age-dependent difference in sensitivity that will help explain why humans are affected in infancy. An intraperitoneal injection of lipopolysaccharide (LPS) will cause additional damage and lead to a more representative model. Goal 2: To create an animal model that mimics the histopathology of human GA-1. This is necessary to develop therapeutic interventions that might be used to decrease the brain damage in GA1.

3.0 Experiments

3.1 Human GA-1 study

Neuropathological and molecular findings in a glutaric acidemia type 1 cohort

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Abstract

Glutaric acidemia type 1 (GA-1) is an autosomal recessive disorder with deficiency of glutaryl-CoA dehydrogenase (GCDH). Glutaric acid (GA) and 3-hydroxyglutaric acid (3-OH-GA) accumulate in blood, urine, and cerebrospinal fluid. GA-1 is characterized by macrocephaly and acute encephalopathic crisis occurring between 6 and 18 months of age. This neurological event is associated with acute striatal damage resulting in severe dystonic movement disorders, psychomotor retardation, seizures, and early death. Ten autopsy cases have been previously described. We present the neuropathological features of five children and one adult (8 months – 40 years), all North American aboriginals with the identical homozygous mutation. Four had macroencephaly. All had mildly enlarged ventricles and striatal atrophy. Severe loss of medium-sized neurons and mild loss of large neurons in the caudate and putamen occurs within a few months of the first encephalopathic crisis and does not appear to be progressive. Overall, this cohort displays similar pathologic characteristics to those previously described with one exception: no spongiform changes were observed in the white matter. Reverse transcriptase-PCR to detect the GCDH mRNA in frozen brain samples revealed normal and truncated transcripts similar to those seen in fibroblasts. As well, all brain regions demonstrated markedly elevated concentrations of GA (377-2120 nmol/g protein) and 3-OH-GA (28-74 nmol/g protein) with no evidence of striatal specificity or age dependency. The pathogenesis of selective neuronal loss

cannot be explained on the basis of regional genetic and/or metabolic differences. Alternate hypotheses must be developed in order address the issue of selective striatal vulnerability and neuron loss.

Introduction

Glutaric acidemia type 1 (GA-1) is an autosomal recessive disorder of amino acid metabolism caused by the deficiency of functional glutaryl-CoA dehydrogenase (GCDH) activity [1], an essential enzyme in the catabolic pathways of L-tryptophan, L-lysine, and L-hydroxylysine [2-4]. Lack of functional GCDH activity leads to the accumulation of glutaric acid (GA) and 3-hydroxyglutaric acid (3-OH-GA) and glutaconic acid in the blood, urine, and cerebrospinal fluid (CSF) [2, 3, 5]. Previous research has suggested that GA and 3-OH-GA may act through excitotoxic NMDA receptors to produce a neurotoxic effect, although current work suggests that 3-OH-GA is most likely the offending agent. In one autopsy case 3-OH-GA was relatively more abundant in the striatum [16, 40]. This neurotoxicity leads to the selective loss of medium-sized neurons in the caudate and putamen.

GA-1 affected children are clinically characterized by macrocephaly, progressive dystonia and choreoathetosis, seizures, gait disturbances, developmental delay, and mental retardation [6, 7]. Neurological abnormalities usually appear abruptly between 6 and 18 months of age, often in conjunction with a febrile illness. Once striatal injury occurs, brain damage is irreversible. In most circumstances, the damage leads to profound neurologic sequelae and in many instances to death in early childhood. However, some individuals survive for years despite severe neurologic impairment. In addition, a minority of affected

individuals may remain asymptomatic or with minimal neurologic findings. The brains of children affected with GA-1 exhibit enlarged ventricles, mild atrophy of frontal and temporal cortex, and widening of the Sylvian fissure. There are only ten published autopsy reports of GA-1 [2, 7, 15, 16, 21, 24, 29, 30]. Atrophy and severe neuronal loss affecting the caudate and putamen are always present. Spongiform change in the white matter has been frequently described.

This disease is overrepresented among North American aboriginals (Ojibway-Cree) in a genetic isolate with a relatively severe phenotype in northeastern Manitoba and related communities in northwestern Ontario in central Canada [11]. Although the phenotype is severe, the relative amount of GA and 3-OH-GA in blood and urine tends to be quite minimal [6]. More than 25 affected children, many the products of consanguineous unions, have been identified since 1970. In this population the GA-1, carrier frequency is approximately 1 in 10. All affected individuals in this population are homozygous for a splicing mutation, a G to T transversion at the +5 position of intron 1 in the gene encoding GCDH (IVS-1$^{+5\ g>t}$) [9, 12]. This splicing mutation may allow for normal splicing with a resultant transcript of the expected size as well as a truncated transcript resulting from activation of a cryptic splice site 26 base pairs upstream in exon 1. This cryptic splicing leads to a frame shift and premature termination of the transcription 9 amino acids into exon 2. Because the disorder can be identified at birth [34], our goal is to develop an intervention strategy that

could prevent the episode causing acute striatal injury and thus minimize brain damage in affected children. There are many gaps in the present understanding of neuropathogenesis of striatal injury in this disorder. Herein we describe the detailed neuropathological findings in the brains of 5 children and 1 adult with GA-1, all with the same mutation, presumably inherited from a single founder. This is of particular interest because the range of survival times and the availability of frozen tissue for genetic analysis might offer additional insight into pathogenesis of the disorder.

Materials and Methods

This is a retrospective neuropathological study of patients diagnosed with GA-1. All patients in this study were examined and diagnosed by clinical geneticists at the Children's Hospital / Health Sciences Centre in Winnipeg, Canada. All were of North American aboriginal background and all had phenotypes consistent with GA-1. There have been 13 known deaths in this cohort between 1978 and 2002. The age of death ranged from 8 months to 40 years. Complete autopsies were performed on six cases. In the majority, the cause of death was related to an intercurrent respiratory infection (e.g. bronchopneumonia). For each case, one or two anonymized control cases with no neurological disease matched for age and gender were obtained from the autopsy archives. This study was conducted with approval of the local Health Research Ethics Committee.

Archived paraffin blocks, glass slides, and hospital records were retrieved for all cases. The brains had been reasonably well sampled with 8-20 tissue blocks per brain available for microscopic examination. All slides were examined by one neuropathologist (MRD). Age and sex matched controls were identified and similar levels of the striatum were taken for each case. Sections from striatal blocks were stained with hematoxylin and eosin. Immunohistochemical staining was done to detect glial fibrillary acidic protein (polyclonal anti-GFAP) (1/1200 dilution; Dako), activated microglia (anti-HLA-DR) (1/250 dilution; Dako), lymphocytes (anti-CD3) (1/100 dilution; Dako), and synaptic vesicle protein synaptophysin (1/25 dilution; Dako). Neurons were identified with the use of anti-NeuN (1/800; Chemicon). To identify inhibitory interneurons, antibodies to calbindin (1/100 dilution; Chemicon) and □-aminobutyrate (anti-GABA) (1/125; Chemicon) were used. To identify noradrenergic axons, anti-tyrosine hydroxylase (dilution 1/75; Chemicon) was used. Choline acetyltransferase (ChAT) (1/250 dilution; Chemicon) was used to identify large cholinergic neurons. A fluorescent secondary antibody (Cy-3) was used with the calbindin primary antibody. GFAP, HLA-DR, CD3, and synaptophysin antibodies were detected using the Envision detection system. GABA, ChAT, NeuN, and TH antibodies were detected with biotinylated secondary antibodies, streptavidin horseradish peroxidase and 3, 3'- diaminobenzidine (DAB). Appropriate negative controls were used in all cases.

Neuron counts were performed on hematoxylin and eosin stained sections. This was chosen because the neurons have a fairly characteristic morphology and because we found the immunostaining to be inconsistent. Counts were made in the dorsal and ventral regions of both the caudate and putamen at an ocular magnification of 400x under normal light microscopy. The size of the ocular reticule counting square was 250μm x 250μm. The counts consisted of neurons contained within six adjacent focal areas in each of the four regions stated above. Only neurons that could be unambiguously identified based on cytologic details were counted. NIH image analysis software was used to quantify density of DAB precipitation, indicating magnitude of immunoreactivity with antibodies against GFAP, HLA-DR, and synaptophysin. Images used for NIH analysis were taken in the dorsal and ventral regions of the caudate and putamen at 10x objective magnification. Two images were obtained from each region; their densities were then averaged to give a better representation of immunoreactivity in each area.

Neuron counts and immunohistochemical labeling data were tested for normal distribution. Paired T-tests were then used to compare differences between cases and age-matched controls using StatView 5 Software (SAS; Cary, NC). Regression analyses were used to test for age-dependent effects.

Reverse transcription (RT)- PCR was performed on total RNA isolated from frozen brain tissue stored at –70°C following autopsy of four

cases (Patients # 1,3,4,5). The method has been previously described for analysis of other cells from this cohort [12]. Briefly, following reverse transcription of total RNA from homogenized frozen tissue, two overlapping fragments of the complete GCDH cDNA were generated by separate standard PCR reactions. PCR conditions were 2□l cDNA in 50□l of 50 pmol of each primer, 200□M of each dNTP, 5□l of 10XPCR reaction buffer (Perkin Elmer-Cetus) and 1 □l Amplitaq (8 units) (Perkin Elmer-Cetus) for 35 cycles at 95°C/3 minutes; 95°C/1 min; 58°C/1 min; 72°C/1 min with a final 10 min cycle at 72°C. Products were analyzed on 6% acrylamide minigels with ethidium bromide (10mg/ml) staining. RT-PCR for GAP-DH was used as a positive confirmation of mRNA integrity and isolation. Similar analyses were performed on normal tissues obtained from non-age matched patients with similar post-mortem delay, because of our supply of material is limited. All analyses were done blinded.

Analysis of organic acid (GA and 3-OH-GA) concentrations was performed on frozen brain tissue from four GA-1 and three control cases. Samples were shipped by curier on dry ice to Germany. The analyses were performed in a blinded manner. The methods for this analysis is described in detail in previous studies [16, 67]. Again, due to limited supply, the controls were not matched.

Results

The ages of the patients with GA-1 ranged from 8 months to 40 years. In 4/6 cases the brains were much larger than expected for age while the body weights and heights were well below average (see details in Table 1). A brief description of clinical and neuropathologic findings follows in order of ascending age:

Case 1. Male with 6 out of 7 older siblings also affected by GA-1. Developmental delay and seizures were noted at 6 months (in 1994). At 8 months he presented with vomiting, diarrhea, fever, and severe dehydration. He became rapidly comatose and CT scan showed hemorrhage in the right temporoparietal region. He died 3 days later. Autopsy revealed widespread ischemic damage in the cerebrum and cerebellum as well as hemorrhagic infarction in the right parietal and temporal cerebrum due to venous sinus thrombosis, a complication of sepsis and dehydration. In addition, there was mild symmetrical dilation of the lateral ventricles and atrophy of the striatum. There was severe loss of medium-sized neurons in the caudate and putamen, with dorsal areas more severely affected than ventral. Immunohistochemical stains demonstrated astrocyte hypertrophy and microglial activation.

Case 2. This male presented at 10 months age (in 1978) with acute bacterial pneumonia accompanied by fever and lethargy. Cerebrospinal protein was elevated. Despite antibiotic treatment he continued to be febrile and three weeks later he developed episodes of opisthotonus, limb stiffening, and lethargy. Metabolic disorders and diffuse hypoxic cortical

injury were considered. CT scan showed enlarged lateral ventricles and Sylvian fissures. On phenobarbital there was slight improvement of his neurologic status but he suffered respiratory arrest due to aspiration of vomit approximately 6 weeks after presentation. Autopsy revealed pneumonia. The external surface of the brain appeared normal but the ventricles were enlarged. There was widespread loss of medium-size neurons with marked astrocytic proliferation, microglial activation, and focal dystrophic calcification in the striatum. Mild lymphocyte infiltration was present focally in the periventricular white matter.

Case 3. This male whose parents were known heterozygotes for the GA-1 mutation presented at 7 months of age (in 1990) with developmental delay and seizures. He became severely impaired, was treated with phenobarbital, required placement of a feeding tube, and was placed in nursing care. At 16 months he developed fever and died suddenly. Autopsy showed acute glottitis and pneumonitis. The external appearance of the brain was unremarkable. The caudate and putamen were decreased in size and the ventricles were mildly enlarged. Microscopically, the striatum exhibited loss of medium-size neurons and gliosis along with scattered calcospherites and moderate glial swelling. In addition, there were scattered pyknotic neurons in the cerebral cortex.

Case 4. This male was found on newborn genetic screening to have GA-1 (in 1999). Beginning at around 6 months of age he developed dystonic and athetoid limb movements and was noted to be delayed in

development. He generally failed to thrive and had multiple respiratory infections. At 15 months he could not sit but had some head control and visual interaction. At 18 months during a febrile illness he stopped breathing. Autopsy revealed laryngitis and dehydration. The brain exhibited mild temporal and frontal atrophy and pronounced widening of the Sylvian fissures. The caudate nuclei were small, yellowish, and firm. There was symmetric lateral ventricle enlargement (Figure 1). The dorsal striatum exhibited severe neuronal loss with pronounced reactive astrocytes. Sections from the anterior frontal cortex exhibited scattered lymphocytic cuffs around venules.

Case 5. This female with developmental delay and hypotonia in infancy. At 6 months age (in 1983) she developed seizures and choreoathetoid movements. CT scan showed short temporal lobes. Thereafter she had severe mental retardation and spastic quadriplegia. Her brother, 3 years younger, was similarly affected, as was a paternal uncle. Unable to walk, she developed severe scoliosis. She died of acute pneumonia at age 7 years. Her brain exhibited mild gyral flattening, slightly enlarged ventricles, and severe striatal atrophy. The caudate and putamen showed a near total loss of medium size neurons (Figure 2) and chronic reactive astrogliosis. There were small foci of spongiform change in the frontal cortex.

Case 6. This male, born in 1953, had a poorly documented early history. It is likely that he became symptomatic in the first year of life. He

resided in a nursing care facility and had spastic quadriplegia with athetosis. His cognitive function was not well documented. Seizures were not reported. He developed aspiration pneumonia and treatment was complicated by pseudomembranous colitis from which he died at age 40 years. His brain was large. The lateral ventricles were moderately enlarged and there was marked atrophy and neuronal loss involving the caudate and putamen. In addition, subtle chronic astrogliosis, demonstrable with modified phosphotungstic acid hematoxylin (PTAH) stain, was evident in the inferior olivary nuclei but there was no obvious neuron loss.

It is important to note that there was no spongiform change in the white matter in any case, in contrast to previous reports [29]

Quantitative comparison of neurons between control and GA 1 cases showed statistically significant ($p< 0.05$) loss of medium sized neurons in the dorsal caudate, ventral caudate, dorsal putamen, and ventral putamen (Figure 3). The dorsal regions of both the caudate and putamen more severely affected, although this was not statistically significant. When it could be identified, the nucleus accumbens appeared to be less severely affected. Where the tail of the caudate could be identified near the hippocampus, neurons were spared. There was no age-dependent trend (Figure 4) in the quantity of neurons in the GA-1 cases suggesting that maximal neuron loss had occurred within 2 months of onset of symptoms, which was the time of death after neurologic crisis in Case 1. Large neurons, which are normally much less abundant than the medium-sized

neurons, were significantly fewer in the ventral putamen with similar trends in all areas of the striatum (Figure 5).

Immunostaining for GFAP (Figure 6 and 7) demonstrated the presence of reactive astrocytes in all areas of the striatum. A dorsal/ventral gradient was also seen (Figure 6). Analysis of reactive microglial activation (Figure 8 and 9) demonstrated HLA-DR immunoreactivity in only the two youngest cases, suggesting that it only persists a few months after the acute episode.

The loss of GABA and calbindin immunoreactivity confirmed that the population of medium sized neurons is decreased in GA-1 (not shown). The relative absence of NeuN labeling confirmed that neurons were lost and not simply atrophic. Qualitative inspection of ChAT and TH immunoreactivity in large cholinergic and dopaminergic neurons indicated little if any difference between GA-1 cases and controls (not shown). Synaptophysin immunoreactivity in striatum was not significantly different between cases and controls (not shown).

Presence of GAP-DH amplification product in both GA-1 cases and controls indicated the presence of undegraded mRNA despite long postmortem delays to autopsy and the lengthy interval between autopsies and molecular study. However there was limited frozen tissue stored. In particular, striatal tissue was only available from patients 3 and 5 and thus proper quantitation of the relative proportion of normal and mutant GCDH transcripts was not possible. Nonetheless, we observed abundant mutant

and normal sized GCDH transcripts in the frontal cortex, white matter, and cerebellum, the most abundant frozen tissues available, and these were not obviously different from striatum (data not shown).

Organic acid analysis demonstrated markedly elevated of concentrations of GA and 3-OH-GA with no evidence of striatal specificity or age dependency when compared to controls. There was only a slight elevation of GA in one control brain which likely can be considered as a nonspecific change due to delayed freezing, re-thawing etc. (Table 2).

Table 1. Clinical and autopsy data concerning GA-1 patients

Case	Sex	Age at time of neurologic crisis	Age at Death	Body Weight/ Height (a)	Brain Weight (g) (expected Mean ±2SD) (b)	Delay in Autopsy hours (c)	Frozen Tissue Sample Areas
1	M	6 months	8 m	9.4 kg, 75th; 73 cm, 50th	1320 (770±60)	20	Cerebellum
2*	M	10.5 months	12m	9.1 kg, 15th; 75 cm, 25th	980 (850±110)	11	None
3	M	7 months	16 m	7.7 kg $<5^{th}$; 79 cm, 25th	1176 (950±110)	48	Caudate, Cerebellum, Frontal Cerebrum
4	M	5.5 months	18.5 m	8.1 kg, $<5^{th}$; 74 cm, $<5^{th}$	1104 (1000±115)	18	Frontal Cerebrum
5	F	6 months	7 y 7 m	21 kg, 25th; 114 cm, 5th	1300 (1250±90)	40	Striatum, Frontal Cerebrum
6	M	<1 year	40 y	43 kg, $<5^{th}$; height not recorded	1635 (1520±250)	5.5	None

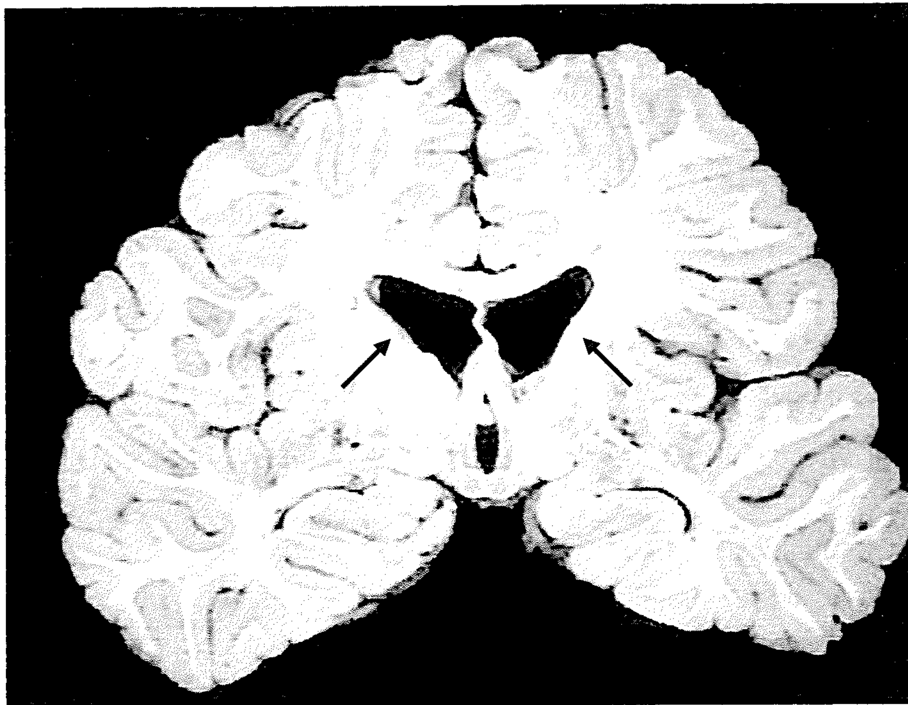
(a) Body weights and heights are shown along with percentile rank. (b) Brain weights and average brain weight with confidence intervals are shown. (c) Time delay to autopsy is shown to aid interpretation of RT-PCR and biochemical results. *Not confirmed by DNA analysis but clinical course typical and strong family history of GA-1

Table 2. Analysis of organic acids in brain tissue.

Brain and Sample Areas	**GA** *nmol/g*	**3-OH-GA** *nmol/g*
Patient 1 - cerebellum	2124	74
Patient 3 - cerebellum	802	55
Patient 3 - thalamus	1096	36
Patient 3 - caudate	596	36
Patient 3 - frontal	776	28
Patient 4 - frontal	377	36
Patient 5 - frontal	599	29
Patient 5 - caudate/ internal capsule	831	42
Control 3wk - frontal	n.d.	n.d.
Control 6m - frontal	n.d.	n.d.
Control 5m - frontal	17	n.d.

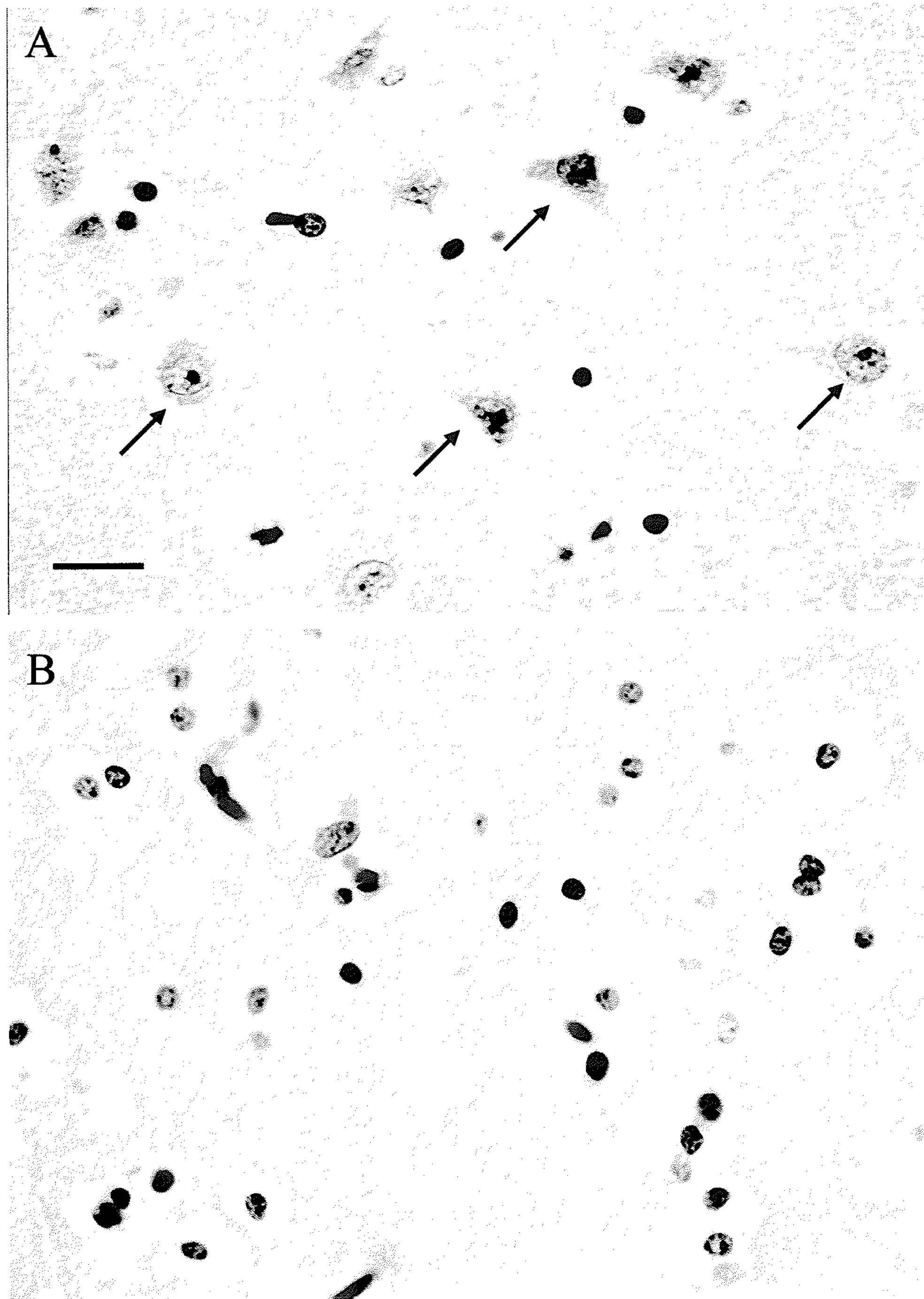
Analysis of organic acids in brain tissue samples from four GA-1 and three control cases (nmol/g protein). n.d. = not detectable.

Figure 1



Photograph showing coronal slice of the brain of case 4. There is flattening of the caudate "bulge" into the ventricle (arrows) and mild enlargement of the lateral ventricles.

Figure 2



Photomicrographs of the striatum showing (A) normal neuron density (neurons indicated by arrows) in age matched control and (B) severe loss of neurons in the same area of GA-1 case 5. (hematoxylin and eosin stained sections, 40x objective magnification, Bar = 20μm).

Figure 3

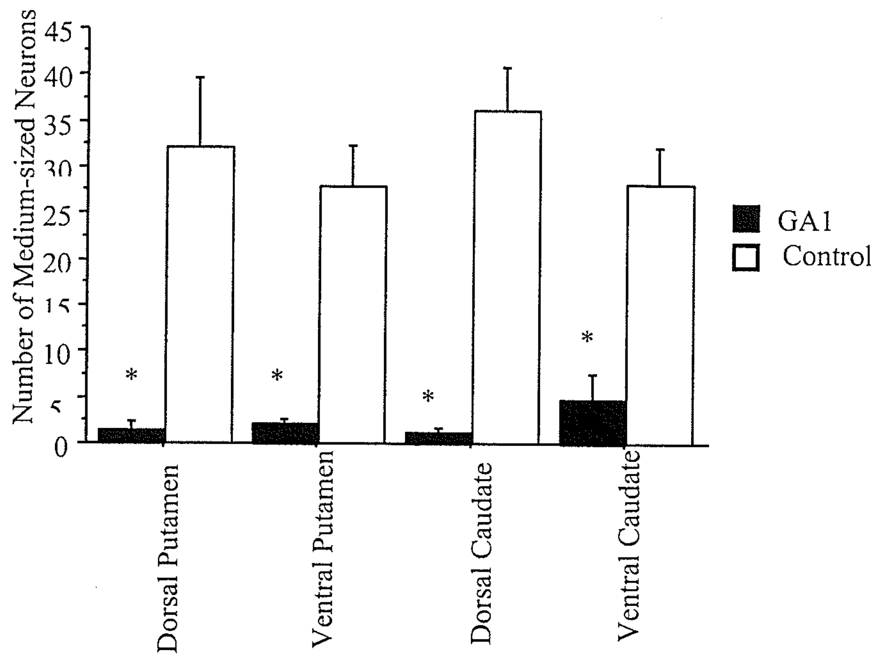
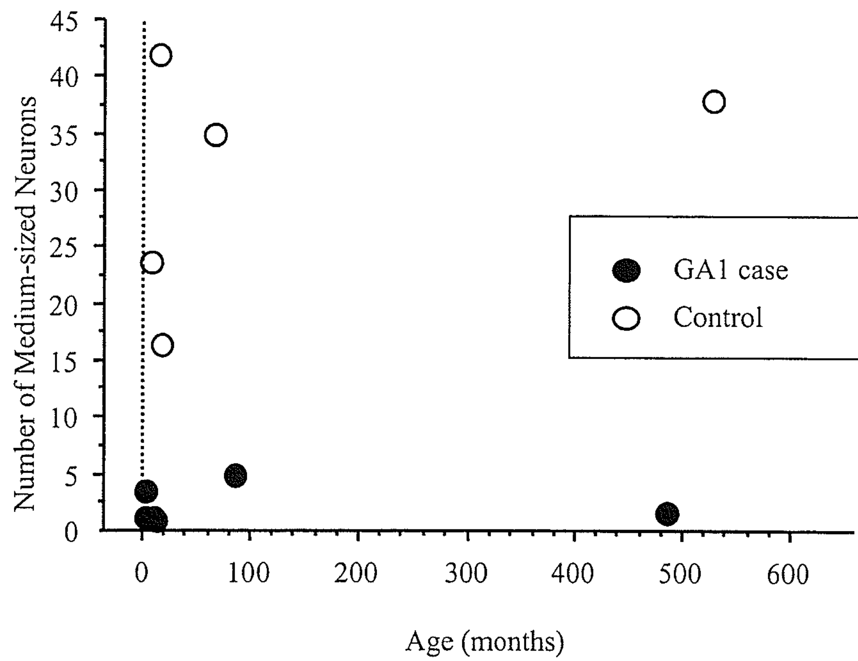


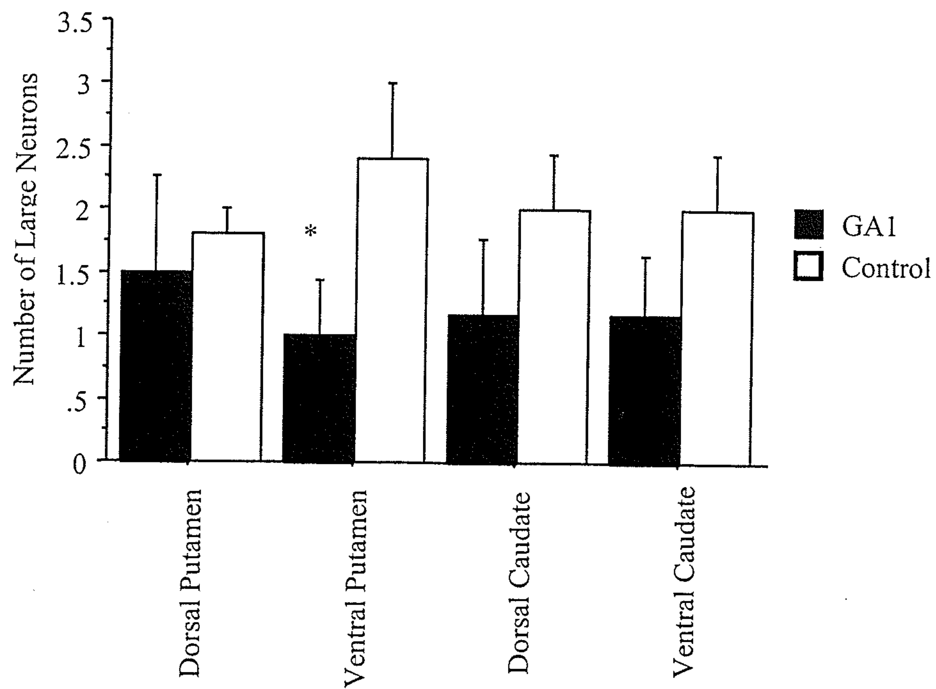
Figure 3: Bar graph showing counts of medium sized neurons (mean ± SE) in all areas of the striatum. A significant loss of medium sized neurons was seen in dorsal and ventral areas of the caudate and putamen; paired t-test vs. age matched controls, * = $p < 0.008$.

Figure 4



Scatter plot showing neuron density as a function of age. Note that the magnitude of neuron loss is similar regardless of the age of the GA-1 patient.

Figure 5



Bar graph showing counts of large neurons (mean ± SE) in the striatum. There was a marginally significant loss in the ventral caudate; paired t-test vs. age matched controls, $p = 0.0422$.

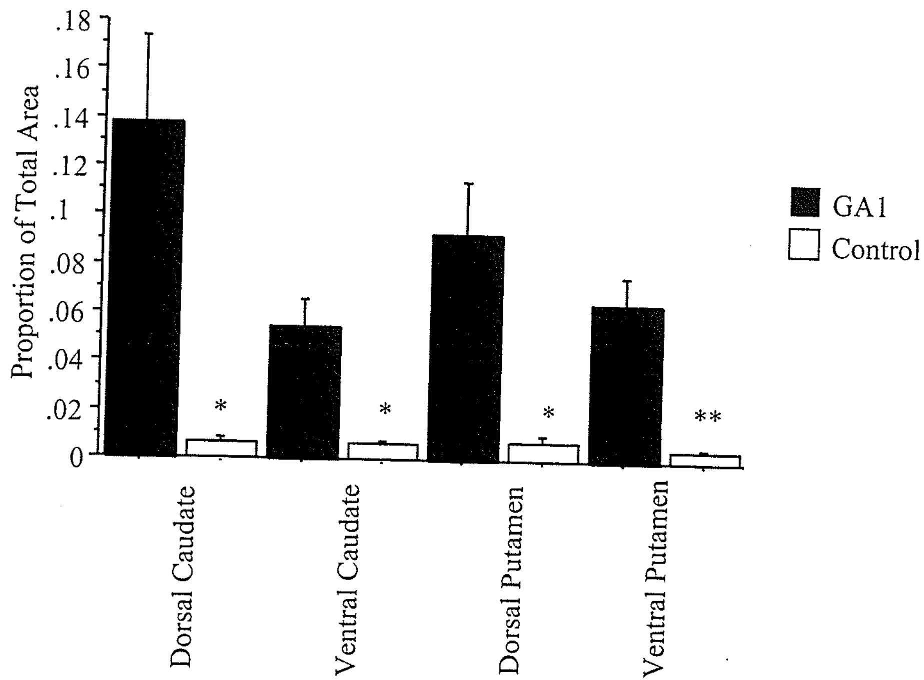
Figure 6

A

B

Photomicrographs showing scant immunoreactivity for GFAP in dorsal caudate of a control case (A) and abundant GFAP positive reactive astrocytes in GA-1 case 1 (B). (DAB detection of anti-GFAP with hematoxylin counterstain, Bar = 50 μm).

Figure 7



Bar graph showing the proportionate area with DAB precipitate (measured by NIH image analysis; mean ± SE) when labeled with primary antibody against GFAP; paired t-test vs. age matched controls * = $p < 0.016$, ** = $p < .005$.

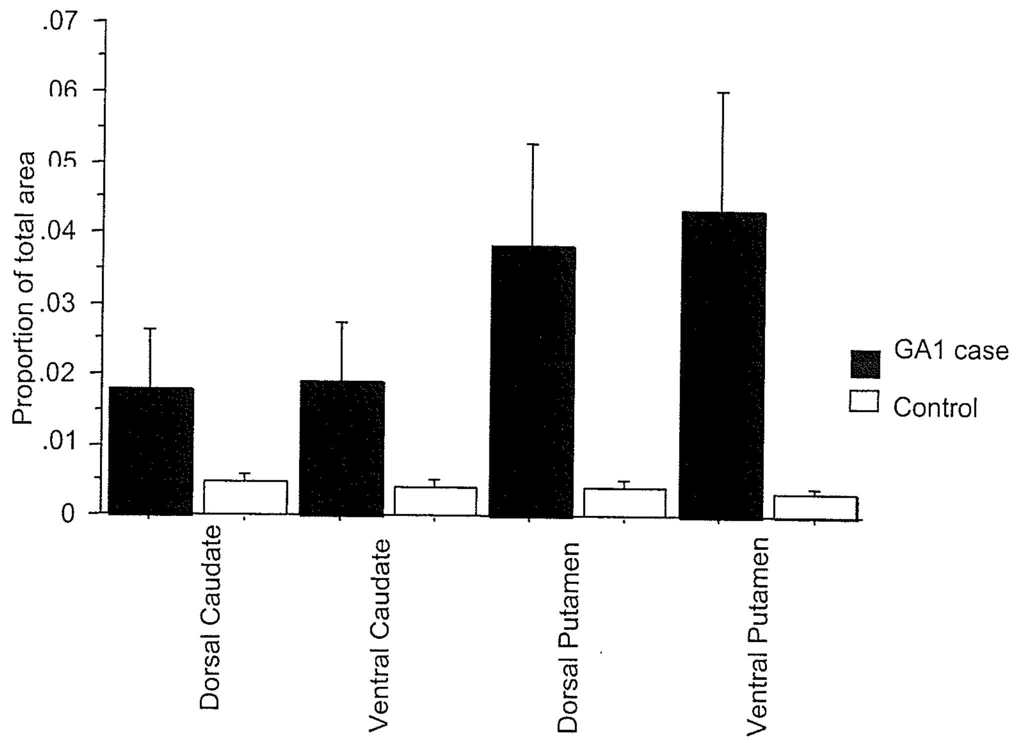
Figure 8

A

B

Photomicrographs showing HLA-DR immunolabeling of reactive microglia in the putamen. The age matched control case (A) exhibits no cells while the GA-1 sample (case 1, B) exhibits abundant activated cells. (DAB detection of anti-HLA-DR with hematoxylin counterstain, Bar = 50 μm).

Figure 9



Bar graph showing the proportionate area with DAB precipitate (measured by NIH image analysis; mean ± SE) when labeled with primary antibody against HLA-DR. Differences in the dorsal and ventral areas of the putamen only approached statistical significance ($p = 0.0725$ and 0.0699 respectively; paired t-test vs. age matched controls) because immunoreactivity was only observed in the two youngest cases.

Discussion

The neuropathological changes associated with GA-1 described herein are essentially similar to those previously described in affected children of other ethnic backgrounds [21]. In ten previously described patients ranging in age from 10 months to 15 years [2, 7, 15, 16, 21, 24, 29, 30] atrophy involving the striatum is consistently described, with sparing of the caudate in one case [24]. In one case there was gliosis in the pallidum but no obvious neuron loss [21]. In seven cases, spongiosis of the white matter was observed.

Despite genetic homogeneity and similarity in the extent of neuron loss from the striatum, the population described herein exhibited a wide range in duration of survival. The neuron counts demonstrated marked loss of medium sized neurons in the striatum (with the exception of the nucleus accumbens and tail of the caudate) that likely occurs within, at most, a few months of the first encephalopathic crisis. It is important to emphasize the apparent lack of progression over the lifespan. This observation supports the idea that a single severe insult during infancy creates the bulk of striatal injury. Quantitation of large neurons has never been done in clinical cases of GA-1 and these cells have been presumed to be unaffected. If this population of neurons was unaffected, in atrophied striatum one would actually demonstrate an increased density. Our results indicate that least focally there is a significant loss of large neurons. Some large neurons are densely encrusted with GABAergic and enkephalinergic

terminals [27], which may give some indication of neuronal vulnerability. The subtle dorsoventral gradient of neuron loss seen in the cases might be explained in different ways. First, the neuron characteristics as well as the afferent and efferent connections to the striatum exhibit dorso-ventral and rostro-caudal differences [27]. This could influence the effect of the toxic agent. The persistence of synaptophysin, a pre-synaptic vesicle protein, suggests the preservation of input axons to the striatum. Second, the dependence on GCDH could vary regionally, although our RT-PCR data suggest that there are no major regional differences in GCDH expression with regard to truncated and intact transcripts. Third, blood flow through lenticulostriate arteries could, in the face of the excitotoxic stress/hyperactivity, be diverted away from the dorsal regions leading to a compounding hypoxic/ischemic injury.

Reactive astrocytes persist many years post injury. Reactive microglia did not exhibit lasting activation; they dissipated within 6 months of the encephalopathic crisis. Two of the cases (#2 and #4) had small collections of lymphocytes in the cerebrum. Although not a common finding in incidental childhood death, this abnormality could simply reflect a septic state rather than a specific component of GA-1 brain damage. Unlike many previous reports, there was no spongiform white matter degeneration in these cases. Whether this reflects a cohort difference or simply a timing difference is not clear.

Although the aboriginal patients described here excrete very low concentrations of GA and 3-OH-GA in urine [6], they have very high concentrations of both organic acids in the brain. The GA concentrations are greater than those in the only previously published case but the 3-OH-GA concentrations are similar [16]. Substantial postmortem delays or true phenotypic differences could explain the difference. Based on our data, the hypothesis that the brain is biochemically different, with regard to GCDH, from the rest of the body seems to be supported [40]. However, we could not confirm the prior observation of higher concentrations of GA and 3-OH-GA in the striatum.

There are several other conditions that exhibit overlapping neuropathological features of neuron loss from the striatum. These include neonatal hypoxic ischemic encephalopathy [37], familial infantile striatal necrosis [31], Huntington disease [68], neuroacanthosis, and Wilson disease [33]. The molecular substrate of these diseases and other disorders is quite varied and it is not clear why the striatal neurons are specifically vulnerable.The precise biochemical agent that causes neuron damage is not yet identified but, the leading hypothesis is that GA and its metabolites act via NMDA receptors (as a glutamate analogue) causing neuronal excitotoxicity (reviewed in [40] . However, there is only one study that addresses the issue of prolonged exposure to either of these excitotoxic reagents [48]. Injections of GA or 3-OH-GA into the brain of rats are claimed to cause neuron loss [61-63] but our attempt to replicate those

experiments did not yield changes that mimic the human abnormality [69]. The loss of GABAergic neurons and the inhibitory neurotransmitter could facilitate neuronal excitotoxicity [41, 58]. The nature of selective neuron loss is similarly elusive in Huntington disease, which has been studied much more extensively [35].

With the advent of tandem mass spectrometry or DNA-based testing for newborn screening, GA-1 can now be identified presymptomatically at birth. Current treatments for newborns with GA-1 involve a low-protein or a low lysine /low tryptophan diet with riboflavin, carnitine, and creatine supplements [34, 51]. Our hypothesis of a single insult opens the possibility that pre-symptomatic detection and the use of neuroprotective agents could be tried to limit brain injury. Because there are age-dependent changes in the distribution of NMDA receptors and GABAergic neurons in the rat striatum at ages corresponding to human infancy [28, 70] the cells may be especially vulnerable in infancy. Whether preservation of neurons, for example with a pharmaceutical agent, in infancy could allow them to mature into a less vulnerable phenotype is not known. An animal model of GA-1 that mimics the human neuropathology is required to test possible treatment interventions. However, a useful animal model is not yet available; a GCDH knockout mouse mimicking the genotype exists, but it does not possess the phenotypic characteristics associated with GA-1 [65]. Clearly more work is required to fully understand the pathogenesis of this disorder.

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3.2 Animal GA-1 Study

Attempt to establish a rat model of striatal injury in glutaric acidemia type 1

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Abstract

Glutaric acidemia type 1 (GA-1) is an autosomal recessive disease caused by the deficiency of glutaryl-CoA dehydrogenase enzyme. The metabolic block results in accumulation of glutaric acid (GA) and 3-hydroxyglutaric acid (3-OH-GA) in blood and cerebrospinal fluid (CSF). Neuropathological changes consist of severe neuronal loss in the caudate and putamen. An animal model is necessary to test possible intervention strategies. We attempted to create a rat model of GA-1. We hypothesized that intrastriatal injections of GA or 3-OH-GA could be used to create specific neuron loss. Adult, 3-week, and 2-week-old rats were given "High" and "Low" concentration injections. High concentrations caused dense necrotic lesions in striatum. Low concentration injections showed a dose-dependent effect ranging from negligible damage, to white mater axonal damage, to small areas of neuron loss in the vicinity of the needle entry site. A separate group of animals were given an injection of lipopolysaccharide (LPS) to create an inflammatory response and mimic the stereotypical human onset prior to 3-OH-GA administration. In no case were the injections associated with selective neuronal loss. We conclude that the simple model of a single GA or 3-OH-GA injection into rat brain does not replicate the neuropathological findings in humans. It therefore cannot be depended upon as a model in which to test pharmacologic interventions.

Introduction

Glutaric acidemia type 1 (GA-1) is an autosomal recessive, inborn error caused by the deficiency of glutaryl-CoA dehydrogenase enzyme (GCDH, enzyme commission number, EC 1.3.99.7; OMIM *231670). GCDH is involved in the degradative pathway of the amino acids L-tryptophan, L-lysine and L-hydroxylysine [71]. The metabolic block results in excretion of glutaric acid (GA), 3- hydroxyglutaric acid (3-OH-GA) as well as glutaconic acid in urine, blood [5, 14], and to a lesser extent cerebrospinal fluid (CSF) [18]. Urine organic acid analysis shows excretion of variable amounts of GA and 3- OH-GA. The disorder causes an acute devastating neurologic syndrome in infants that is characterized by sudden onset hypotonia, dystonia, and encephalopathy often in conjunction with a febrile illness. Death in infancy is not uncommon. Survivors often have dystonic movements, seizures, and developmental delay.

Our understanding of the neuropathology of this disorder is limited to few published studies. Autopsy features of children with GA-1 have been described in 10 cases [2, 7, 15, 16, 21, 24, 29, 30] along with 6 additional cases examined by our own group (paper submitted). The striking feature is striatal injury characterized by the degeneration of medium-sized GABAergic neurons in the caudate nucleus and putamen. White matter vacuolization has been described in a few cases.

There is in vitro evidence that GA and 3-OH-GA might act as endogenous neurotoxins by over stimulation of NMDA receptors,

interference with mitochondrial energy metabolism, and accumulation of reactive oxygen species [39, 45-47, 52, 53]. In the Manitoba variant of GA-1, presymptomatic diagnosis is possible through DNA based newborn screening [12, 34]. In theory, a pharmacologic intervention might prevent the encephalopathic crisis and striatal damage. An animal model is necessary to test possible interventions. Injection of GA into the striatum of adult rat brains has been shown to cause rotational behavior and seizures [63]. In similar studies using 3-OH-GA injections, convulsions and striatal lesions were reported but poorly characterized [61]. A genetic knock out mouse model has been created, that mimics the biochemical phenotype by showing excess accumulation of GA and 3-OH-GA in urine and brain parenchyma. The brain however exhibits no striatal changes and only minor spongiform white matter changes (increasing with age), and generally lacks the characteristic damage described in humans [65].

We attempted to create a technically simple rat model of GA-1 that could be used to investigate possible pretreatments for the disorder. We hypothesized that intrastriatal injections of GA and 3-OH-GA could be used to create specific neuron loss, mimicking the findings in humans. In addition, we pretreated a group of animals with lipopolysaccharide (LPS) to generate an inflammatory stress response [72] in an attempt to recreate the typical course of events seen in the majority of human cases.

Methods

Adult, 3 week, and 2 week old male Sprague-Dawley (N=112) rats were maintained on a 12:12h light/dark cycle with ad lib access to tap water and standard lab chow. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care. The animal care protocol was approved by the University of Manitoba. GA and 3-OH-GA were obtained from Sigma (St Louis, MO, USA) and Dr. H. J. ten Brink (VU University Medical Center, Amsterdam) respectively. They were dissolved in sterile double distilled water (DDH_2O). The pH was 2.1 and 2.3 respectively at high dose concentration. LPS was provided by Dr. D. Nance (University of Manitoba, Canada) [72].

Animals were anesthetized using a mixture of 2% halothane with oxygen inhaled via a nose cone and then placed in a stereotactic frame. "High concentration" intrastriatal injections (i.c.) of GA and 3-OH-GA (1 and 4 μmol dissolved in 2μl DDH_2O or .5 and 2 M/L respectively) were administered intrastriatally to adult (n=8, stereotactic coordinates, A/P=0mm, M/L=4mm, V=5mm from the dura), 3 week (n=10, A/P=0mm, M/L=3.5mm, V=4.5mm), and 2 week old rats (n=15, A/P=0mm, M/L=3mm, V=4mm) using a 10μL Hamilton syringe and a 30 gauge needle. Doses were infused over a period of 2 minutes.

"Low concentration" intrastriatal injections of GA and 3-OH-GA (3, 1.5, 1, 0.75, 0.5, 0.1, 0.05μmol dissolved in 10μL DDH_2O or 0.3, 0.15, 0.1, 0.075, 0.05, 0.01, and 0.005 M/L respectively) were administered over 10 minutes to 3 week old (n=55) and 2 week old (n=8) rats in a

similar location and by similar means. Because the 3-week-old rats have been weaned and their brains more closely resemble those of 6-month-old humans (the onset age in many human cases) they were the focus of this study. The high doses used in this experiment were based on previous experiments [61, 63]. The use of 2-week-old rats allowed us to assess an age response to dosage. Appropriate saline-injected controls were used.

Two separate groups of animals (n=5) each received an intraperitoneal (i.p.) injection of lipopolysaccharide (LPS, 1mg/kg) or saline 3-4 hours prior to the intrastriatal injection of 3-OH-GA (1.5μmol/10μl). This dose was chosen because damage would be apparent but not severe, giving opportunity for either an upward or downward modification by LPS. The experimenter was blinded to the composition of the i.p. injection, however, the subsequent physical state of the rats that received the LPS injections exposed its identity.

Damage to the striatum injection was graded into four categories. Grade "0" damage represented the needle tract characterized by some blood or hemosiderin containing macrophages (depending on the survival period) and rarefaction not more than 50μm in total width. Grade "1" damage included a slight loss of neurons up to 200μm in total width around the needle tract. Grade "2" damage consisted of a larger area of neuron loss extending up to 500μm in total width. Grade "3" damage included an area of neuron loss, as in grade 2, and the presence of a necrotic area.

After 1 or 7 days the rats were euthanized with an overdose of sodium pentobarbital and their brains were fixed with 10% formaldehyde for 3 days, then paraffin embedded. Serial coronal sections (6μm thick every 200μm) stained with hematoxylin and eosin (H&E) were used to assess the extent of damage to the striatum. The short survival period of 1 day was used to screen for doses that could potentially cause selective neuron loss. Brains from 7-day survivors were also used for immunohistochemical investigations. Staining was done to detect glial fibrillary acidic protein (polyclonal anti-GFAP) (1/1200 dilution; Dako) and activated microglia (Lectin from Bandeiraea Simplicifolia, BS-I) (1/150 dilution; Sigma). Neurons were identified with the use of monoclonal mouse anti-NeuN (1/200; Chemicon). GFAP, Lectin, and NeuN antibodies were detected either with peroxidase secondary antibodies or biotinylated secondary antibodies and streptavidin horseradish peroxidase. In all cases, 3, 3'- diaminobenzidine (DAB) was used to visualize the epitopes. In addition, Fluoro-Jade (0.001%, Histo-chem, Inc., Jefferson, AR) staining was done to identify dying neurons [73, 74]. Appropriate negative controls were used in all cases.

Results

The animals tolerated the injections well. Three animals died due to anesthetic overdose. No seizures were observed after any of the GA or 3-OH-GA injections, however slight hind limb peddling motion was noted

in some animals recovering from anesthesia. The animals injected with LPS demonstrated moderate "shivering" for no longer than 2 minutes during recovery from anesthesia. Animals given a pre-injection of LPS were notably "sick". They huddled in the corner of the cage for 3-4h after injection (at the time of 3-OH-GA administration). This gave overt confirmation that our dose of LPS was producing the desired effect.

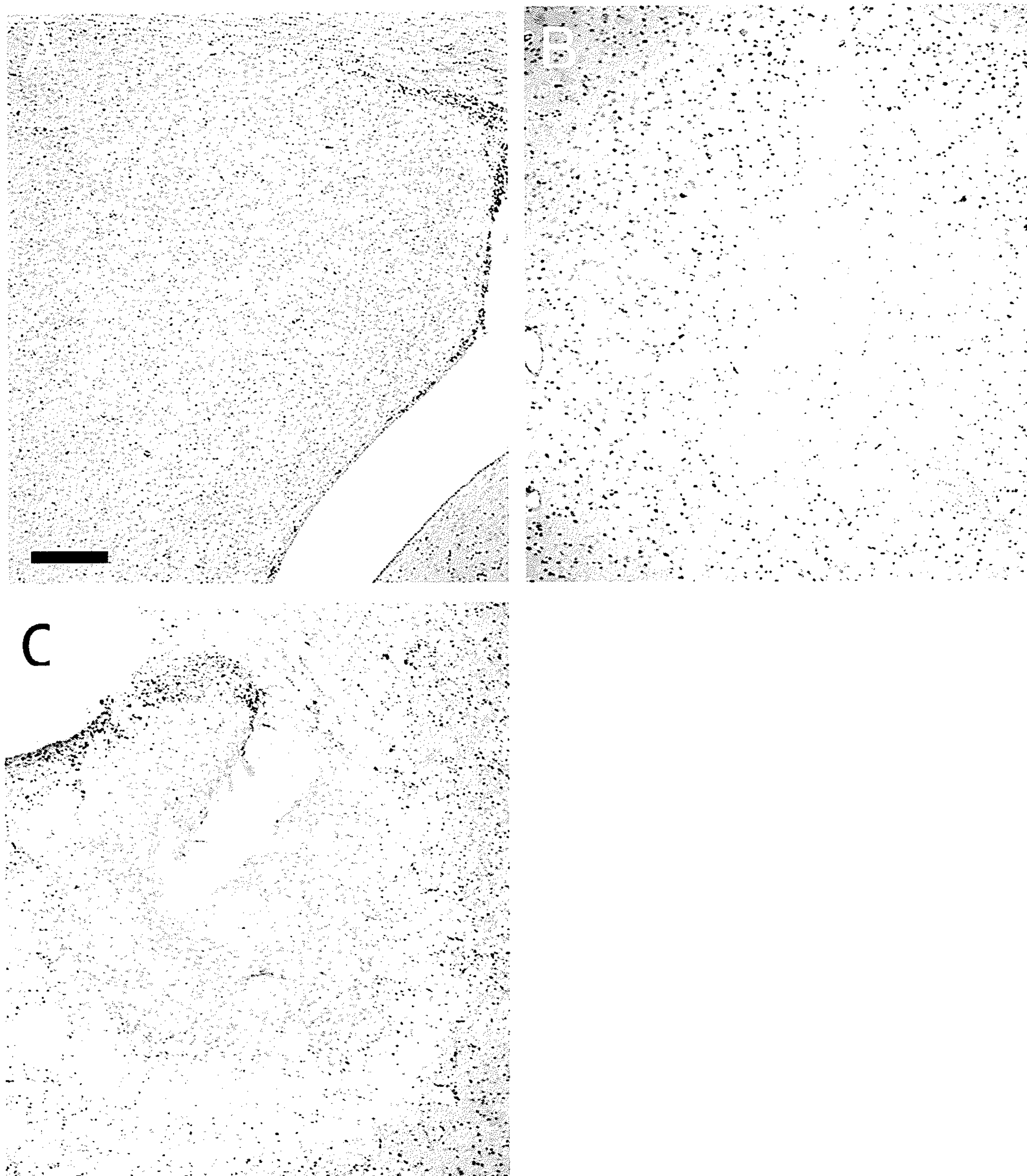
Saline injections in all cases were associated with only needle tract damage. High concentrations of both GA and 3-OH-GA (4 μmol/2μl more so than 1 μmol/2μl) created necrotic lesions in the striatum within 1 day. These often extended laterally through the external capsule to the cerebral cortex. There was no evidence of selective neuronal loss whatsoever (Figure 1), in contrast to previous reports [61]. We concluded that GA and 3-OH-GA were too concentrated and therefore increased the volume of the injection to increase the spread of the chemical.

Low concentration doses (3, 1.5, 1, 0.75, 0.5, 0.1, 0.05μmol/10μl) were used to assess a dose response effect (Figures 2 and 3). The lowest doses created negligible damage with mild edema and white matter axonal injury, high doses created a larger area of damage (up to 750μm diameter), with slight neuron loss and usually with minimal necrosis (Figure 1). Immunostaining with anti-NeuN to demonstrate neurons confirmed that there was no selective pattern of neuron loss in the vicinity of the injections (Figure 4). When the injection was accidentally placed in the lateral striatum, the damage often affected the external capsule much more

than the striatum itself. Fluoro-Jade staining demonstrated injured neurons only in the immediate vicinity of the injection site. In addition, the low doses of both GA and 3-OH-GA caused axonal injury (seen as Fluoro-Jade positive expansions) in the white matter bundles of the striatum (Figure 5). This damage was seen at distances further away from the needle tract than the Fluoro-Jade positive neurons (Figure 5). In some rats, the striatum near the injection site had an increased number of small nuclei. This was due to accumulation of activated microglia, which bound the lectin (Figure 6), and reactive astrocytes, which were GFAP immunoreactive (not shown).

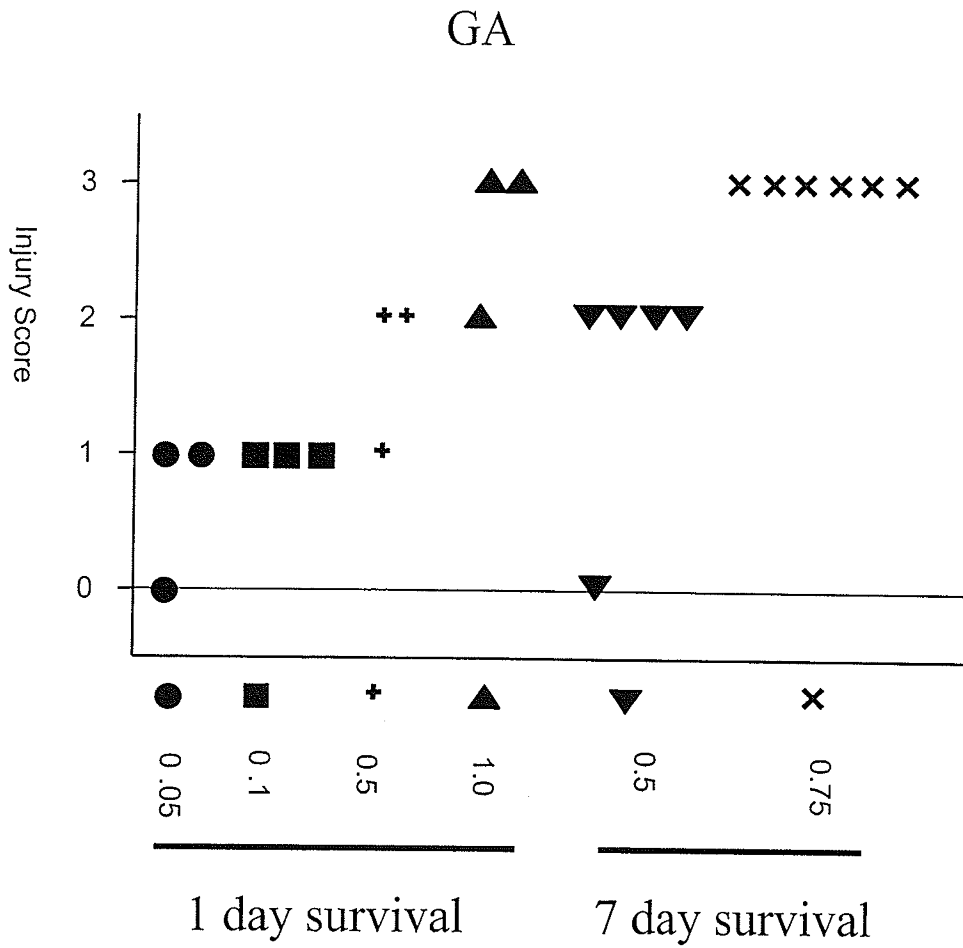
The slides were graded by two separate observers blinded to the nature of the group. There was no significant change in the pattern of damage seen when compared to the rats that received i.p. injections of saline (p=.356, Mann-Whitney U test, Figure 7). The most prevalent finding was a large infiltration of inflammatory cells that were Lectin positive (i.e. microglia and macrophages).

Figure 1



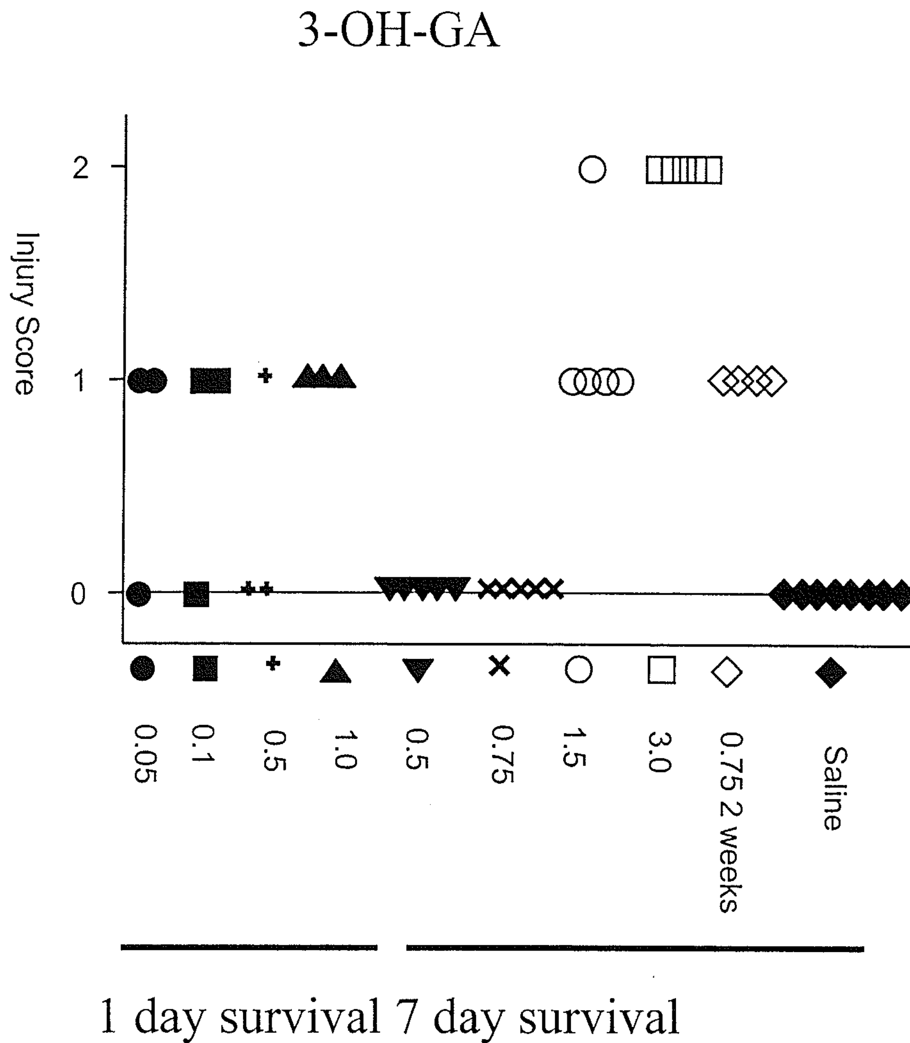
Low magnification photomicrographs showing striatum in 3 week old rat brains. The normal striatum adjacent to the lateral ventricle exhibits homogeneous cellularity (A). Low dose injection of GA (1μmol/10μl) followed by 1 day survival is characterized by pallor in the striatum, which is due to neuron loss and edema (B). High dose of injection of GA (4μmol/2μl) followed by 1 day survival causes a large area of necrosis (C). Hematoxylin and eosin stain, Bar = 250μM.

Figure 2



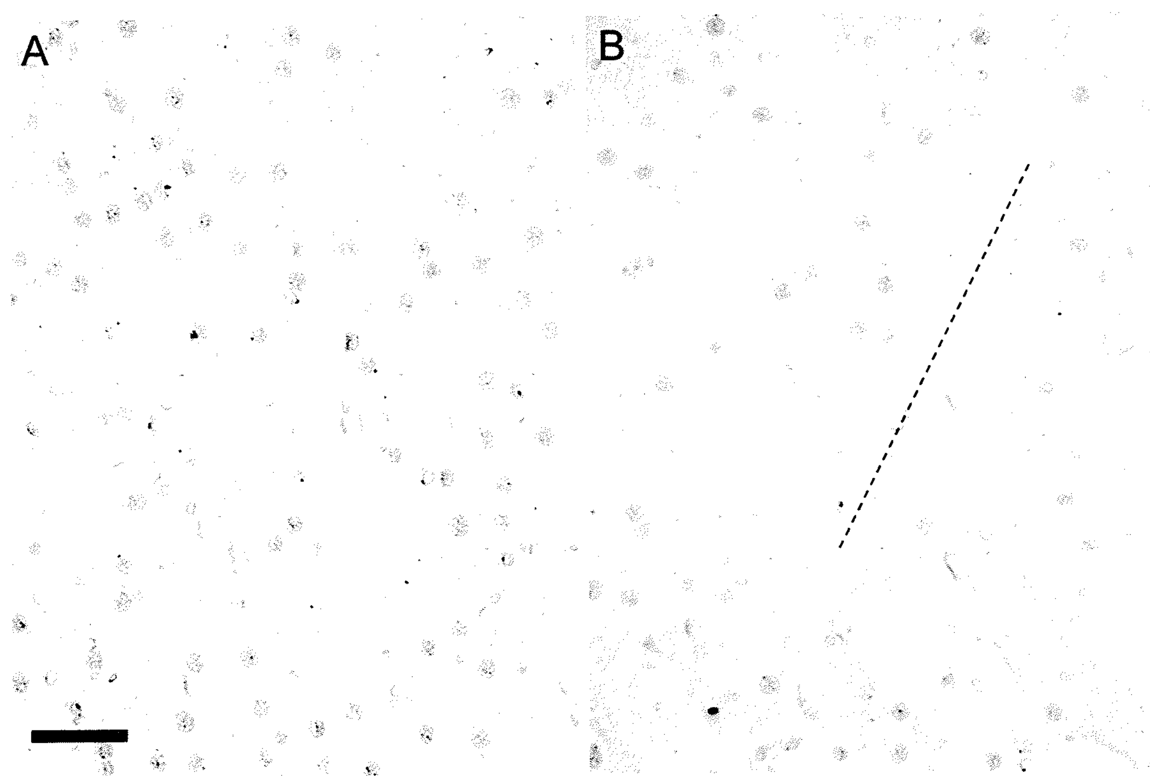
Scatterplot showing injury score for different GA doses (in μmol/10μl) and survival times. All rats were 3 weeks of age. There is a dose response effect in both the 1 and 7-day survival periods (p= 0.0028, Kruskal-Wallis test).

Figure 3



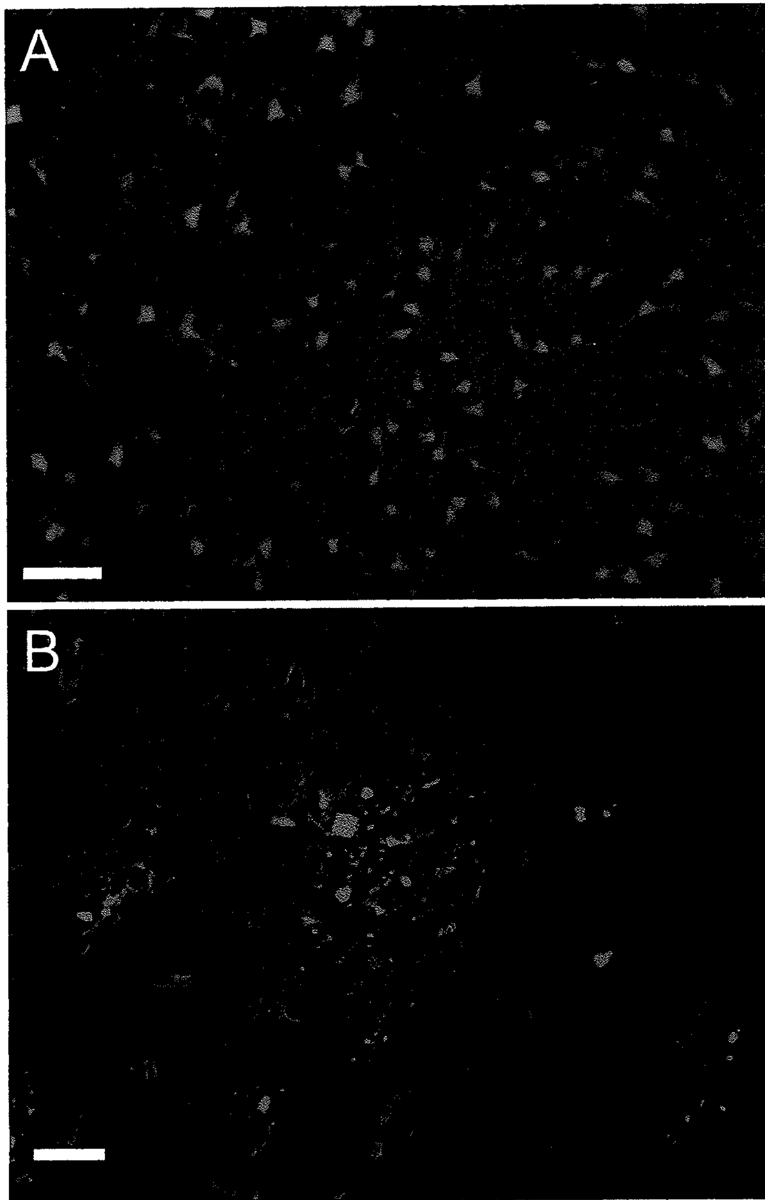
Scatterplot showing injury score for different 3-OH-GA doses (in μmol/10μl) and survival times. All rats were 3 weeks of age at the time of injection with one exception,(labeled as 2 weeks). There is a dose dependent effect in both the 1 and 7-day survival periods ($p < .0001$, Kruskal-Wallis test).

Figure 4



Photomicrographs show NeuN immunolabeling in the striatum 7-days after injection of 3-OH-GA (3µmol/10µl). (A) Shows the normal concentration of neurons in striatum contralateral to the site of injection. (B) Shows the patchy neuron loss along the needle tract (dashed line). Bar = 50µM.

Figure 5



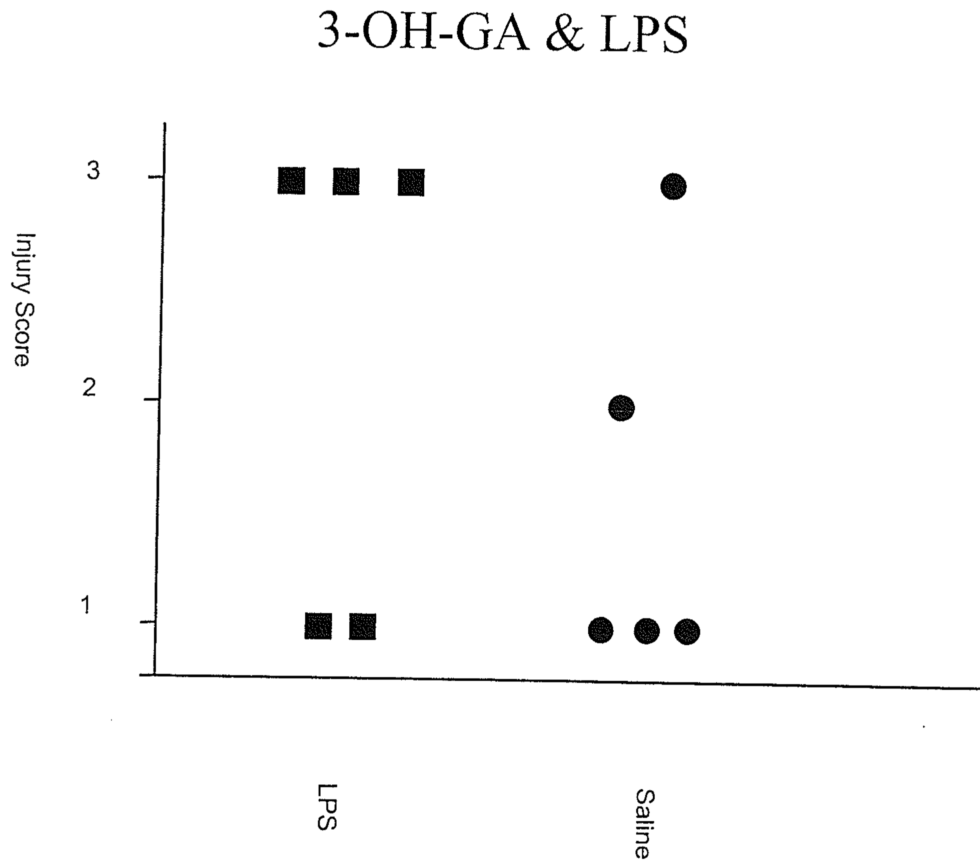
Photomicrographs showing Fluoro-Jade staining of damaged structures in striatum of 3 week old rats 1 day following injection of GA. Following high dose injections (A - 1µmol GA) entire cell bodies exhibit green fluorescence indicative of dying striatal neurons adjacent to the needle tract (Bar = 50µM). Following injections of lower doses (B - 0.75µmol GA) there were only rare dying neurons, however within striatal white matter bundles irregular smaller structures exhibit green fluorescence indicative of swollen, damaged axons (red circle; Bar = 25µM).

Figure 6

A

Low magnification photomicrograph showing lectin labeling (B) of activated microglia at the site of injection and spreading along the white matter tract of the external capsule (7 days post LPS and 3-OH-GA injection). There is no labeling in the contralateral striatum (A). Bar = 250μM.

Figure 7



Scatterplot showing injury score following 3-OH-GA injections (3μmol/10μl) into the striatum of 3 week old rats, with additional intraperitoneal injections of LPS or saline. The survival period was 7 days. There is no significant difference ($p = 0.356$, Mann-Whitney U test).

Discussion

In 1 and 7 day survival experiments we found clear evidence that striatal injury was dose dependent following injections of both GA and 3-OH-GA. However, the lesions were very restricted and there was no indication of widespread selective neuronal injury in the striatum at any dose. With regards to the amount of damage, on an equimolar basis, GA tended to cause more severe injury than 3-OH-GA. Although we increased the volume of the injection to enhance the spread of the chemicals into a greater area of the striatum, it either failed to spread throughout the striatum or did not cause significant damage as it extended into the outlying areas. Sections labeled with Fluoro-Jade, demonstrated significant damage in the white matter bundles within the striatum. Lateral striatal injections also caused substantial damage in the external capsule, more so than the striatum itself. This suggests relatively greater vulnerability of white matter to the chemicals injected. White matter changes were reported in the knockout mouse [65]. Whether this corresponds to white matter spongiosis reported in some human cases of GA-1 [29] is not clear. Expression of NMDA receptors in rat brain changes considerably between 12 days and adulthood [28]. At intermediate doses of 3-OH GA (0.75 μmol), there was no clear indication of an age dependent difference (i.e. 2 week vs. 3 week) although our study was not complete in this regard. Injections of LPS intraperitoneally prior to brain injection of 3-OH-GA also failed to induce the type of damage seen in humans, although

the area of injury tended to be larger. In essence, the lesions do not mimic the changes noted in postmortem studies in humans [29], including our own.

Kolker and coworkers [16] determined in a single postmortem investigation of a GA-1 patient that concentrations of 3-OH-GA and GA in putamen were 62 and ~50nmol/g protein respectively. Assuming ~13% protein content [17], this is equivalent to 0.48μmol/g wet brain weight for 3-OH-GA. In another case, Goodman [2] reported a frontal cortex concentration of 1.04 μmol/g for GA. Assuming 20% extracellular fluid volume in the rat brain, 10μl injections would spread acutely into ~50μl of brain. The largest lesions in our rats were calculated to be ~65μl total volume. In this volume we estimate a concentration of 20μmol/g, a vastly different concentration than determined in the human case. Why the difference? In vivo, these metabolites do not cross the blood brain barrier (BBB) [18]. It is possible that the injections disrupted the blood brain barrier, thereby allowing the chemicals to leave the brain. Considering the anatomical restriction of the lesion, we cannot even exclude the possibility that the lesion is simply related to non-specific pH-related neurotoxicity.

Our single injection model is a modification of that described in two previous reports [61, 63]. In those experiments, the investigators implanted a cannula into the striatum 3 days prior to injection of GA or 3-OH-GA, which were buffered by NaOH to pH 7.4. In the GA experiment, only seizure activity was assessed; it was dose-dependent [63]. In the 3-OH-GA experiment, the size of the striatal lesion after a single 0.4-0.6 μmol

injection was reported as a percentage of the striatal area [61]. The survival was brief (maximum 3 days) and the histologic description rather vague ("pale striatal lesion with cellular loss in the core region"). Based on that report, we estimate the lesions to have had a diameter of ~2mm (several fold larger than we observed). But the lesions decreased in size at 3 days probably "due to a reduction of tissue edema". We are therefore uncertain about the extent of actual neuronal loss in those experiments. However, the damage was reduced by administration of MK-801 and muscimol, consistent with the postulated involvement of NMDA receptors.

We have been unsuccessful in creating a model of selective neuronal damage mimicking the pathological changes described in humans with GA-1. Our results, however, do not exclude the possibility that GA and 3-OH-GA are the main neurotoxins in this disease. Our experiment has several shortcomings, some imposed by the exhaustion of funding. We were unable to explore fully the age-dependent effects of GA and 3-OH-GA. We did not monitor or manipulate temperature to determine whether this variable could be used to better replicate the clinical situation. Furthermore, the inflammatory changes incited by LPS might now mimic completely the clinical situation with regard to cytokines or timing [52]. We did not attempt combination injections of GA and 3-OH-GA. We did not extend survival to the long-term (e.g. 2 months) to determine if there is slow neuron loss, although in the absence of any Fluoro-Jade staining distant from the needle tract this seems

unlikely. We cannot exclude the possibility that relatively greater white matter sensitivity to GA and 3-OH-GA is a rodent specific effect. An extension of this experiment would be to chronically infuse a very low dose of either GA or 3-OH-GA directly into the striatum through an implanted cannula using osmotic mini-pumps. This would allow the BBB to regain its integrity and, possibly, more accurately replicate the human condition of chronic metabolite exposure.

In summary, the simple model of a single GA or 3-OH-GA injection into rat brain does not replicate the neuropathological findings in humans. It therefore cannot be depended upon as a model in which to test pharmacologic interventions. More complex model systems need to be explored for this purpose.

Acknowledgements

We thank Sharon Allen and Susan Janeczko for technical assistance. We thank the Garrod Association of Canada and the Manitoba Medical Service Foundation for grant funding. Dr. Del Bigio holds the Canada Research Chair in Developmental Neuropathology.

4.0 Discussion

GA-1 is a debilitating autosomal recessive disorder caused by the absence of functional GCDH [1]. The build up of GA and 3-OH-GA in the blood, urine, and cerebrospinal fluid (CSF) [2, 3, 5] destroy the striatum and leave children who are fairly normal at birth with progressive dystonia and choreoathetosis, seizures, developmental delay, and mental retardation [6, 7]. Brain damage appears to be localized to the striatum (the caudate nucleus and putamen). In most circumstances the damage leads to death in early childhood but some individuals survive for years despite severe neurologic impairment.

In our human study the hypothesis that the neuropathological changes in the Manitoba/Ontario aboriginal cohort of GA-1 are similar to those reported in other ethnic backgrounds is confirmed. However, this cohort is of particular interest because all patients share the same genetic background with a range of survival times. The neuropathological findings in the brains of 5 children and 1 adult demonstrate marked neuronal loss and gliosis of the striatum. This neuron loss is limited to the medium-sized, GABAergic neurons within the striatum. As well, the severity of neuron loss was the same in patients of all ages, suggesting that the loss does not seem to progress throughout the lifespan.

This supports the hypothesis that there is a single severe insult early in childhood, that, if avoided (by treatment intervention), could potentially protect children from suffering the effects of this disease.

At present there is no satisfactory in vivo model of GA-1. There have been in vivo and in vitro as well as genetic knockout experiments preformed but none have provided the accurate selective neuronal loss required for a model of GA-1 in which treatment interventions can be tested. Although the in vitro experiments have shown the excitoxicity of the major metabolites that build up in GA-1, when transferred over to the in vivo models they fail to provide an adequate model.

The KO mouse model developed produced the genetic genotype of GA-1 but if fails to cause the detrimental effect. Other than the KO mouse model the most common model is the use of a stereotaxic intrastriatal injection technique to investigate the behavioral and neurotoxic effects of these metabolites.

We set out to clearly identify what type of cellular loss occurred when rats were given intracerebral (i.c.) injections of GA and 3-OH-GA and to examine whether these injections led to selective neuron loss. To this extent our second hypothesis 2 is not confirmed. We were unable to establish a dose of GA or 3-OH-GA that caused selective striatal injury. We did however, find that at low doses, both chemicals affected the white matter bundles of the striatum, much more so than the surrounding neurons. In cases where only very minor neuronal loss was seen there were white matter bundles that demonstrated damaged axons at considerable distances from the injection site. Taking this into account we still failed to produce specific neuronal loss and therefore, the simple single injection is not a good model for testing protective strategies.

There are multiple possibilities for these findings. GA and/or 3-OH-GA may not be the toxins causing damage in the human case. This is unlikely

however since multiple studies both human and animal (in vitro and in vivo) demonstrate that both of these chemicals have the potential to cause neurotoxic injury. Another possibility is that this is not the correct type exposure need to evoke selective neuronal loss. This may be augmented by administering a more chronic lower dose in a pro-inflammatory environment such as circulating cytokines. The final possibility is that this may be a case of species difference in which the rat and human brains respond differently to varied amounts of these particular neurotoxins. The lack of a neurological phenotype in the KO mouse may support this idea. A question also arises regarding the lack of age-dependent differences we saw in our experiments. This indicates that the neurons of adult rats are just as vulnerable to these toxins as neurons of young rats. If this is analogous to the human situation treatment to prevent damage would be necessary throughout life because eventually the toxicity will occur and patients would become affected. Taking all information into account, a more complex model system needs to be explored for the creation of an accurate model of GA-1 to test possible pre-treatment strategies.

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