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An autoantibody inhibitory to glutamic acid decarboxylase in the neurodegenerative disorder Batten disease

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Mutations in the *CLN3* gene are responsible for the neurodegenerative disorder Batten disease; however, the molecular basis of this disease remains unknown. In studying a mouse model for Batten disease, we report the presence of an autoantibody to glutamic acid decarboxylase (GAD65) in *cln3*-knockout mice serum that associates with brain tissue but is not present in sera or brain of normal mice. The autoantibody to GAD65 has the ability to inhibit the activity of glutamic acid decarboxylase. Furthermore, brains from *cln3*-knockout mice have decreased activity of glutamic acid decarboxylase as a result of the inhibition of this enzyme by the autoantibody, resulting in brain samples from *cln3*-knockout mice having elevated levels of glutamate as compared with normal. This elevated glutamate in the brain of *cln3*-knockout mice co-localizes with presynaptic markers. The decreased activity of GAD65 and increased levels of glutamate may have a causative role in astrocytic hypertrophy evident in *cln3*-knockout mice, and in altered expression of genes involved in the synthesis and utilization of glutamate that underlie a shift from synthesis to utilization of glutamate. An autoantibody to GAD65 is also present in sera of 20 out of 20 individuals tested who have Batten disease. Postmortem tissue shows decreased reactivity to an anti-GAD65 antibody that may be due to loss of GAD65-positive neurons or due to the reactive epitope being blocked by the presence of the autoantibody. We propose that an autoimmune response to GAD65 may contribute to a preferential loss of GABAergic neurons associated with Batten disease.

INTRODUCTION

Neuronal ceroid lipofuscinoses (NCLs) are the most common group of progressive neurodegenerative diseases in children, with an incidence as high as one in 12 500 live births, and with about 440 000 carriers in the USA (1,2). These disorders are autosomal recessive and have similar early symptoms and disease progression. Initial diagnosis is often based on visual problems, behavioral changes and seizures. Progression is characterized by a decline in mental abilities, increased severity of seizures, blindness, loss of motor skills and premature death. The NCLs are characterized pathologically by the accumulation of autofluorescent hydrophobic material in the lysosomes of neurons and other cell types; however, the mechanism driving these cellular alterations and the manner in which they relate to the neurodegeneration in NCLs are unknown. The NCLs have traditionally been divided into subtypes based on the age of onset and pathology, with the following CLN genes being responsible for each disease: infantile NCL (Haltia–Santavuori disease), CLN1; late infantile NCL (Jansky–Bielschowsky disease), CLN2; juvenile NCL (Batten disease), CLN3; adult NCL (Kufs disease), CLN4; and two variant late infantile forms, CLN5 and CLN6. The gene products of CLN1 and CLN2 have been identified as a lysosomal protein thiolesterase and a lysosomal pepstatin-insensitive protease, respectively (3,4). Recently, the gene products of CLN5 and CLN8, the latter of which is associated with epilepsy with progressive mental retardation (EPMR), were identified as novel proteins of unknown function (5,6). Although the CLN3 gene responsible for Batten disease was positionally cloned in 1995 (7), with

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most affected individuals harboring a major deletion of the gene, the function of this protein remains elusive. Recent studies have demonstrated that CLN3 co-localizes with synaptic vesicle proteins in neuronal cells, suggesting a potential role in neurotransmission (8,9).

We have detected in the serum of cln3-knockout mice and individuals with Batten disease the presence of an autoantibody to glutamic acid decarboxylase (GAD65) that is inhibitory to this enzyme's ability to convert glutamic acid to γ-aminobutyric acid (GABA). Furthermore, we report elevated levels of glutamic acid in the brains of a cln3-knockout mice resulting from decreased activity in GAD65, presumably due to the inhibitory action of the GAD autoantibody that is associated with brain tissue. Mouse models for Batten disease, namely the cln3-knockout mouse and the mnd mouse, show a preferential loss of GABAergic neurons (10,11). We propose that the autoantibody to GAD65, which leads to high levels of glutamate and may contribute to a preferential loss of GABAergic neurons, may be significant in the mechanism of neurodegeneration in Batten disease.

RESULTS

An autoantibody to GAD65 that inhibits the activity of glutamic acid decarboxylase in cln3-knockout mice

We used serum drawn from normal and homozygous cln3-knockout mice as a source of primary antibody and probed whole-brain and cerebellar extracts from mouse. Figure 1A demonstrates that serum at a 1 : 1000 dilution from the cln3-knockout mouse reacts with one predominant band and weakly with several other protein bands, confirming the likely presence of autoantibodies to proteins present in both whole-brain and cerebellar extracts; in contrast, serum at a 1 : 1000 dilution from normal mice does not react with any proteins. The predominant band with which cln3-knockout serum reacts is approximately 65 kD in size and corresponds to the predicted molecular weight of an isoform of glutamic acid decarboxylase, GAD65. We performed western analysis on recombinant GAD65 with serum from normal and cln3-knockout mice. Figure 1B demonstrates that serum from the cln3-knockout reacts with

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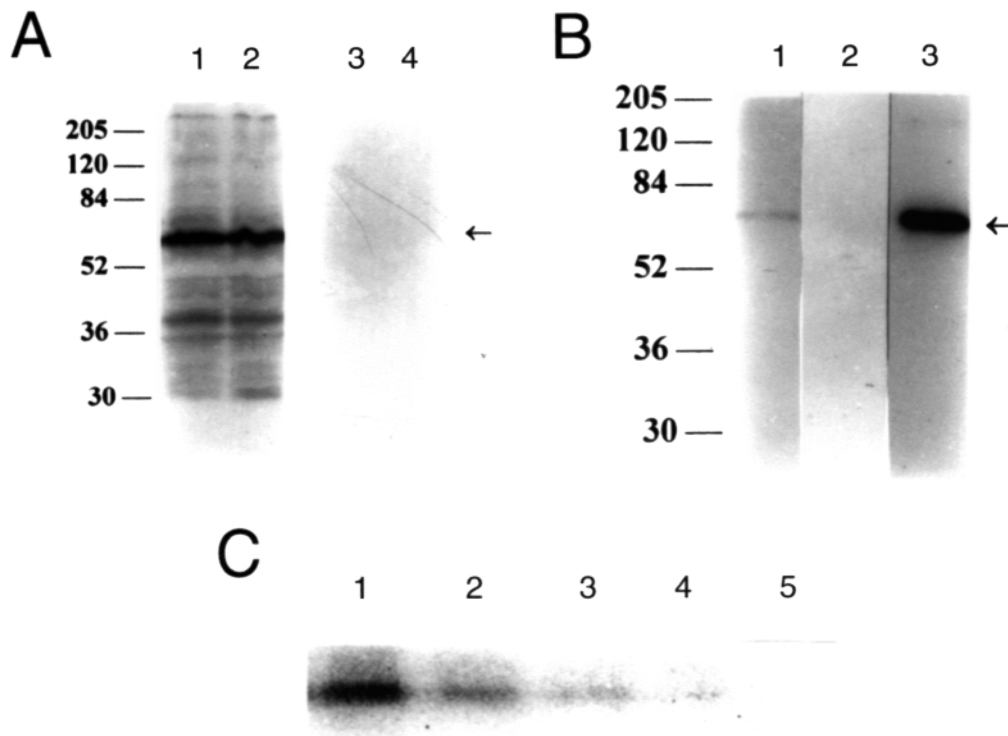

Figure 1. (A) Western analysis shows that identical concentrations of serum from a cln3-knockout but not normal mice react with proteins contained in whole-brain and cerebellar extracts of mice. Whole-brain and cerebellar protein extracts were separated on a 10% SDS–PAGE gel, transferred to nitrocellulose. Lanes 1 and 3: Whole brain extracts. Lanes 2 and 4: Cerebellar extracts. Molecular weight markers are indicated on the left of the figure. Lanes 1 and 2 were probed with a 1 : 1000 dilution of serum drawn from a cln3-knockout mouse, and react with several proteins – most notably, there is a strong reaction with a protein of approximately 65 kD in size (as indicated by the arrow). Lanes 3 and 4 were probed with a 1 : 1000 dilution of serum drawn from a normal mouse. (B) Western analysis indicates that cln3-knockout serum but not normal serum reacts to recombinant GAD. Recombinant GAD protein was separated on a 10% SDS–PAGE gel, transferred to nitrocellulose and probed with a 1 : 1000 dilution of serum drawn from a cln3-knockout mouse (lane 1), a 1 : 1000 dilution of serum drawn from a normal mouse (lane 2) and a 1 : 1000 dilution of a monoclonal antibody to GAD (lane 3). Molecular weight markers are indicated on the left of the figure. (C) Pre-adsorption of the serum with GAD65 blocks reactivity to GAD65. Recombinant GAD protein was separated on a 10% SDS–PAGE gel, transferred to nitrocellulose and probed with a 1 : 1000 dilution of serum drawn from a cln3-knockout mouse (lane 1) and a 1 : 1000 dilution of serum drawn from a cln3-knockout mouse that was pre-incubated or pre-adsorbed with 1, 2, 5 and 10 ng recombinant GAD65 before probing of the sample (lanes 2–5). Similar blocking of reactivity was obtained with a control monoclonal mouse anti-GAD (StressGen, Canada).

recombinant GAD65, whereas serum from a normal mouse does not. The control indicates that a monoclonal antibody to GAD65 reacts with the same band. These results have been reproduced on 12 independently derived cln3-knockout mice. Both normal littermates and heterozygous cln3/normal littermates do not appear to have an autoantibody to GAD65.

To identify the epitope of GAD65 that is recognized by the autoantibody, we performed competition studies whereby sera are pre-absorbed with a synthetic peptide to the epitope that is recognized by the autoantibody. These studies have shown that we can block the reactivity of the autoantibody to GAD65 with GAD65 protein (Fig. 1C). This has independently confirmed the specificity of the autoantibody to GAD65, and demonstrates that GAD65 is in fact the autoantigen.

The cln3-knockout mouse was constructed by the Mitchison group (10) on a 129S6/SvEv genetic background. In addition, we have obtained serum from an alternative cln3-knockout mouse, constructed by the Katz group (12) on a C57BL6 genetic background, which also contains an autoantibody to GAD65 (data not shown). This suggests that the autoimmune response that we report is specific for deletion of a functional CLN3 gene product. Western analysis against either brain extracts or recombinant GAD65 using sera from cln3-knockout mice at 1 week of age to 12 months of age all give the same qualitative results that we present in Figure 1 (data not shown). Clearly, cln3-knockout mice have an early autoimmune response to GAD65. Furthermore, mouse models for variant late infantile NCL (nclf mouse) and Niemann–Pick type C disease (NPCl) do not have autoantibodies to GAD65 in serum (data not shown), suggesting that the phenomenon is specific for Batten disease mouse models.

Autoantibodies to GAD have been detected in individuals with late-onset insulin-dependent diabetes mellitus (IDDM), which does not show a neurodegenerative course; however, these autoantibodies do not apparently inhibit GAD activity (13). In contrast, the autoantibodies to GAD detected in individuals with stiff person syndrome, which does result in neurodegeneration, have the ability to inhibit GAD activity (13). We compared the ability of serum from normal and cln3-knockout mice to inhibit GAD activity in mouse brain extracts (Table 1). The serum from cln3-knockout, but not normal, mice inhibited GAD activity by up to 45%. The data suggest that the GAD-inhibitory element of cln3-knockout serum is the autoantibody to GAD65. It is pertinent to add that there was no increase in GAD activity in serum from a cln3-knockout mouse as compared with normal, and that cln3-knockout serum did not inhibit activity of a selection of other enzymes, such as creatine kinase.

Table 2. GAD activity and total free glutamic acid detected in a cerebellar extract from normal and cln3-knockout mice

	Wild type (cln3 $^{+/+}$)	cln3-knockout (cln3 $^{-/-}$)
GAD activity (c.p.m./μg cerebellum protein/h)	812 ± 101	560 ± 48
Glutamic acid level (pmol/mg cerebellum)	1598 ± 411	2779 ± 296

Decreased GAD-activity, increased levels of presynaptic glutamate and the presence of IgGs in the cerebellum of cln3-knockout mice

The fact that cln3-knockout mice have an autoantibody to GAD65 that inhibits GAD activity appears to be significant. GAD activity in total cerebellar extracts from cln3-knockout mice is decreased by about 33%; in the same extracts, levels of free glutamic acid, the substrate for this enzyme, are increased by about 70% when compared with normal mice (Table 2). It should be noted that levels of GAD65 as determined by western analysis of extracts from whole brain and cerebellum of normal and homozygous cln3-knockout mice show no significant difference (data not shown). An important question regarding the accumulation of glutamate in the brain would be where exactly is the accumulation occurring at the cellular level. In Figure 2, we show by immunohistochemistry that elevated glutamate in cln3-knockout cerebellum, as compared with normal, co-localizes with the presynaptic proteins synaptophysin and amphiphysin (Fig. 2A, B), but not post-synaptic PSD-95 (Fig. 2C).

To link the inhibition of GAD activity, elevated glutamate and the presence of an autoantibody to GAD65, we have probed extracts of both whole brain and cerebellum by western analysis with an antibody that specifically recognizes IgGs. Figure 3A shows an elevation above that of endogenous blood-borne IgGs to be present in cln3-knockout mice, as compared with normal. We postulate that a likely component of the brain-bound IgG is the autoantibody to GAD65. In addition, we consistently see reactivity to recombinant GAD with cerebrospinal fluid (CSF) from cln3-knockout, but not normal mice (data not shown), but we cannot be sure that our

Table 1. GAD activity in cerebellar extracts is inhibited by serum from cln3-knockout mice

	Volume of serum added				
	None	2 μl		4 μl	
		Wild-type	cln3-knockout	Wild-type	cln3-knockout
GAD activity[a] (c.p.m./μg cerebellum protein/h)	812 ± 101	843 ± 99	633 ± 55	851 ± 111	467 ± 37
Percentage inhibition of GAD activity by cln3-knockout serum	–		25%		45%

[a]GAD activity incubated in the presence of serum from either wild-type or cln3-knockout mice. Six independent incubations for each data point.

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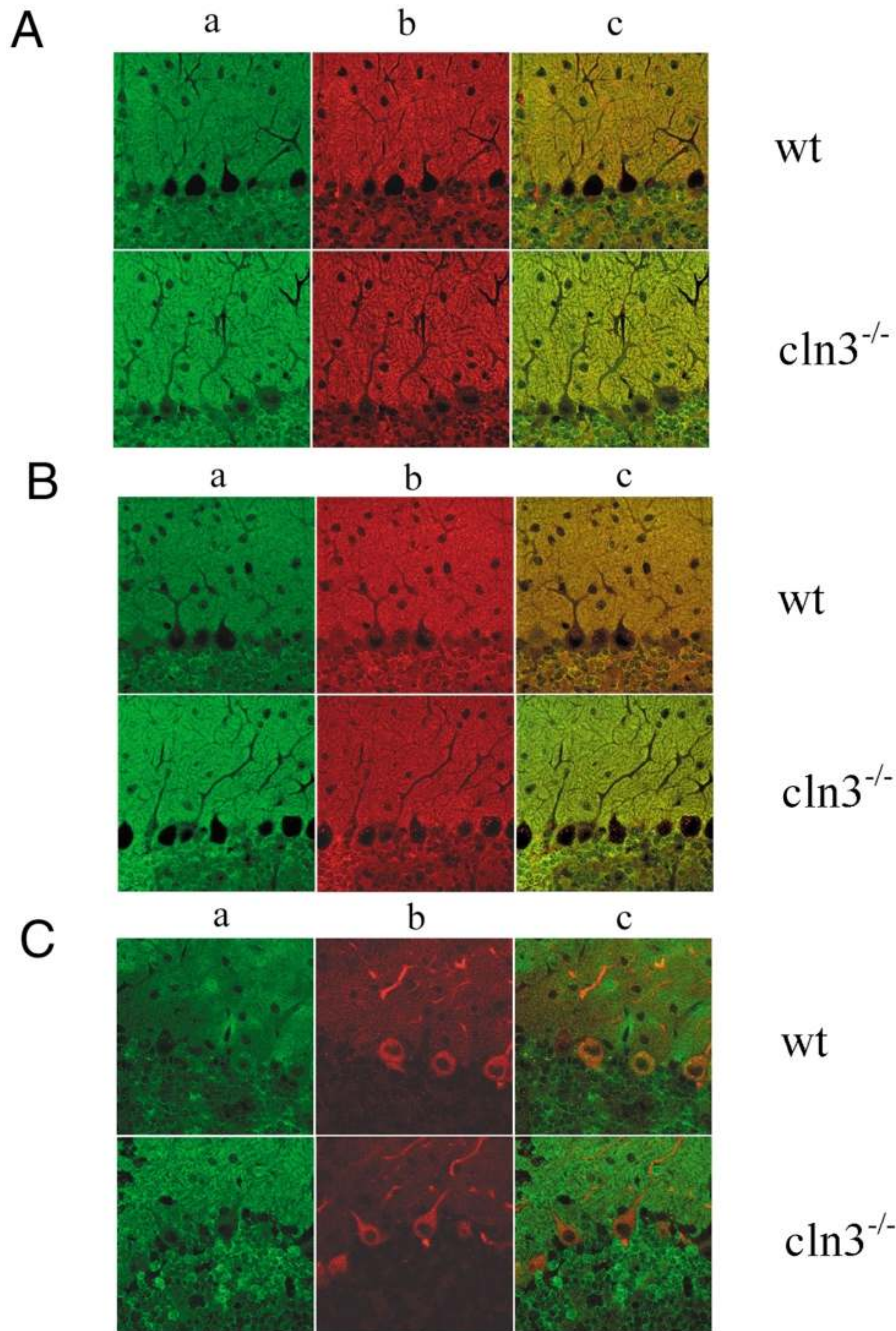

Figure 2. Elevated glutamate in cln3-knockout brain co-localizes with presynaptic markers. Representative dual-channel confocal micrographs reveal co-localization of glutamate with (A) presynaptic synaptophysin and (B) presynaptic amphiphysin, but not (C) postsynaptic PSD-95. (a) (green channel) glutamate; (b) (red channel) synaptic marker; (c) a merged glutamate and synaptic marker images. Comparable results were obtained when switching the fluorochrome used to label each secondary antiserum (data not shown).

CSF samples are serum-free. Collectively, these results indicate that autoantibodies to GAD65 are the most likely cause of decreased GAD activity and increased glutamic acid levels in the whole brain and cerebellum of a cln3-knockout mouse.

Immunohistochemistry (IHC) for IgG did not show immunoreactivity for IgG, except for staining confined to the lumina of blood vessels in any experimental mice brain tissues; and suggests that the IgGs present by western analysis (Fig. 3A) are below the level of detection by this method or that their immunoreactivity is blocked. However, IHC for GFAP showed reactive astrocytosis in thalamic nuclei, hippocampal formation, globus pallidus, cerebral white matter and neocortex

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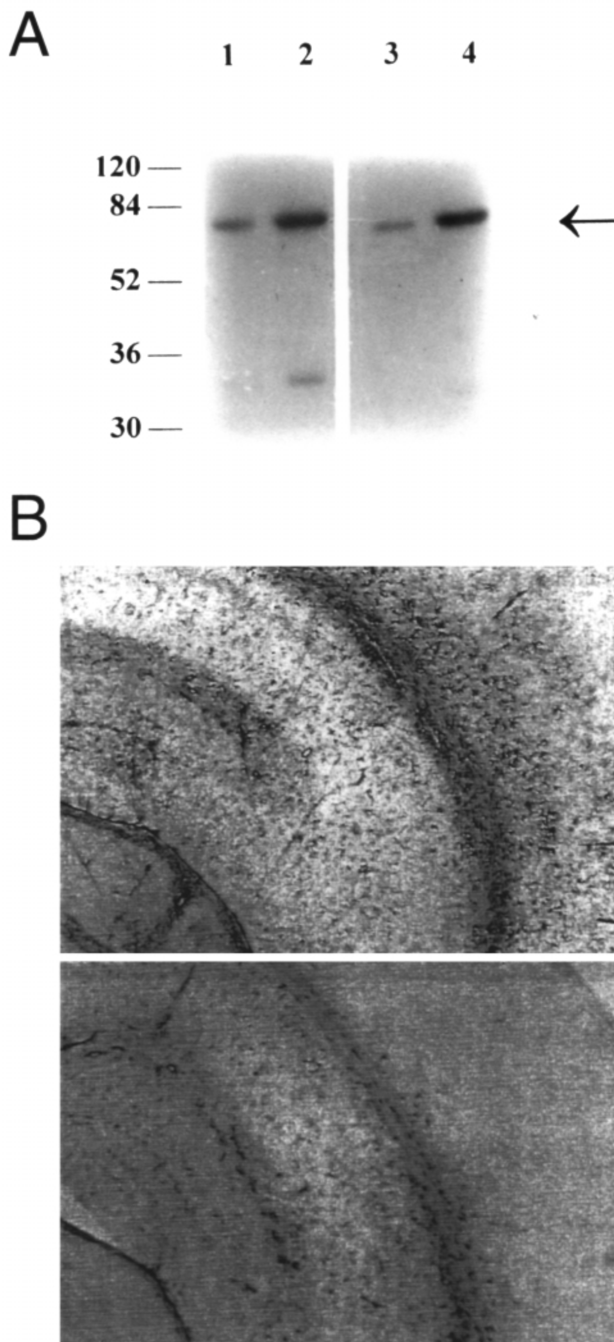

Figure 3. cln3-knockout mice have an elevated level of IgG in the brain as compared with normal mice. (A) Western analysis with an antibody highly specific to IgG shows that whole-brain and cerebellar extracts from cln3-knockout mice have an elevated level of IgGs as compared with normal mice. Whole-brain and cerebellar protein extracts were separated on a 10% SDS–PAGE gel, transferred to nitrocellulose. Lanes 1 and 3: Whole brain extracts. Lanes 2 and 4: Cerebellar extracts. Each lane was probed with 1 : 3500 anti-mouse IgG conjugated to HRP. The arrow indicates the presence of IgG. Molecular weight markers are indicated on the left of the figure. (B) Reactive astrocytosis in cln3-knockout but not normal mouse brain. Top: cerebrum, cln3-knockout mouse. Bottom: cerebrum, normal control. GFAP immunostaining demonstrates early hypertrophy of fibrous astrocytes in the deep cerebral cortex, white matter and alveus (right), fibrae external hippocampi and gyrus dentatus (middle left), and subpial region of the diencephalon (bottom left) of cln3-knockout mouse. The two samples were taken from 14-month old mice and processed identically in every step. Original magnification $\times 40$.

of cln3-knockout mice (Fig. 3B). These astrocytes were not immunoreactive to CD44 and vimentin, and the astrocytic hypertrophy could not be appreciated with hematoxylin–eosin (HE).

Autoantibodies to GAD contribute to Batten disease pathology

Demonstrating that autoantibodies to GAD65 are present in individuals with Batten disease would provide an important correlation with the data derived from the mouse models. To date, we have tested sera from 20 unrelated individuals with Batten disease, all of which have demonstrated autoantibodies to GAD65. Figure 4A shows eight examples of individuals with Batten disease bearing an autoantibody to GAD65, in contrast to controls. Furthermore, studies on other neurodegenerative diseases have shown that numerous control individuals do not have autoantibodies that are reactive to GAD (13,16,17). Interestingly, the reactivity to other brain proteins is minimal, although an apparent increase in this very weak reactivity is seen with the age of the individual and the severity of the disease (data not shown). However, a greater number of individuals need to be examined to substantiate this observation with regard to reactivity of sera to other brain proteins.

Morphologic examination of a juvenile NCL (Batten disease) case reveals scattered neurons, particularly in the deep cortex (parietal), in which there is diffuse perikaryal and apical dendritic staining with an antibody to IgG (data not shown). An antibody to GFAP reveals astrocytic gliosis, with astrocytic processes particularly prominent in the deep cortex in association with neuronal loss. In contrast, the Batten disease cortex stained with an antibody to GAD consistently reveals decreased staining compared with 'normal' controls (Fig. 4B). We interpret this decreased immunoreactivity to GAD in the Batten disease case as a result of decreased antigenic availability of the endogenous GAD, either due to the presence of the autoantibody to GAD blocking potential antigenic sites or due to loss of GAD neurons and their arborizations. Based on the early selective loss of GABAergic neurons reported in Batten disease mouse models, and apparently in Batten disease individuals (reviewed in 14) and the fact that a high percentage of GABAergic neurons are GAD-positive (15), we favor the latter interpretation – that there is a loss of GAD neurons and their arborizations.

Altered gene expression and protein levels of genes and proteins associated with the regulation of glutamate and its utilization

Using Affymetrix Mu19A, B and C high-density oligonucleotide arrays (see Materials and Methods), we have compared the expression of 19 000 genes in the cerebellum of normal and cln3-knockout 10-week-old male mice. For the purpose of this study, we have summarized the expression levels of those genes involved in glutamate metabolism, namely mGluR1, GluRε3, glutamine synthetase, glutaminase, glutamate dehydrogenase, alanine aminotransferase and glutamine aminotransferase (Table 3). Microarray analysis demonstrates that expression of the metabotropic glutamate receptor (mGluRl) and the

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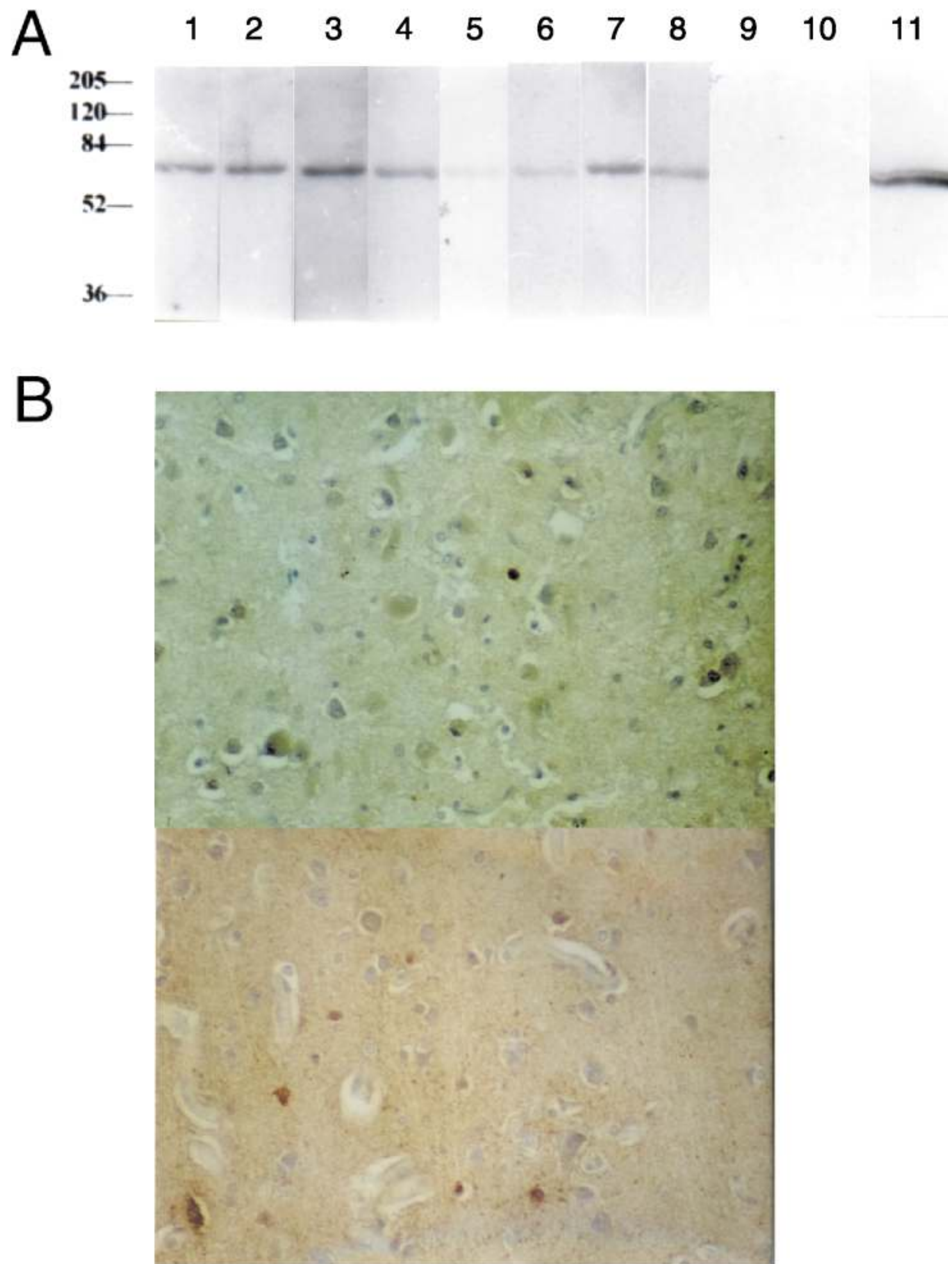

Figure 4. (A) Western analysis shows that sera drawn from individuals with Batten disease have an autoantibody to GAD. Recombinant GAD protein was separated on a 10% SDS–PAGE gel, transferred to nitrocellulose and probed with: a 1 : 1000 dilution of serum drawn from eight individuals confirmed to be homozygous for a 1.02 kb deletion in CLN3 and having Batten disease (lanes 1–8), a 1 : 1000 dilution of serum drawn from two individuals with no known neurologic disorder (lanes 9 and 10) and a control monoclonal antibody to GAD65 (lane 11). Pre-adsorption of sera with GAD65 as performed in Figure 1C resulted in the blocking of reactivity to GAD65 (not shown). Molecular weight markers are indicated on the left of the figure. (B) Decreased immunostaining of GAD in Battern disease brain. Top: frontal cortex, Batten disease patient, 29 years old, 16.6 hours postmortem. Bottom: frontal cortex, congenital heart disease patient, 33 years old, 21 hours postmortem. GAD immunostaining demonstrates diffuse reduction of neuropil reactivity of Batten disease patient, as well as reduced numbers of immunoreactive perikarya. Original magnification $\times 180$.

ionotropic glutamate receptor (GluRε3) are decreased – most likely at the transcriptional level – in cln3-knockout mice as compared with normal mice (Table 3). This is a likely result of the elevated levels of glutamate in the cerebellum. Levels of glutamine synthetase are decreased post-transcriptionally as measured by western analysis (data not shown), with expression of glutaminase and glutamate dehydrogenase decreased, and alanine aminotransferase and glutamine aminotransferase increased. Collectively, these changes indicate a shift at the molecular level in glutamate/glutamine metabolism toward glutamate utilization, rather than glutamate synthesis. This shift is summarized in the model shown in Figure 5. It is

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Table 3. Altered levels of expression of a selection of genes involved in the synthesis and utilization of glutamate, and some other examples that have large alterations but are unrelated to this pathway, as determined by comparative expression in normal and cln3-knockout cerebellum using oligonucleotide arrays

Gene/protein	Mean fold change in expression in cln3-knockout relative to normal mice (average difference)
mGluR1	-13.3 ± 1.3
GluRε3	-38.6 ± 16.2
Glutamine synthetase	No change
Glutaminase	-27.0 ± 0.55
Glutamate dehydrogenase	-13.5 ± 11.2
Alanine aminotransferase	$+25.3 \pm 1.9$
Glutamine aminotransferase	$+26.1 \pm 5.1$
Galectin-3	$+93.2 \pm 8.1$
Galectin-1	$+87.9 \pm 21.0$
CIDE-A	$+161.2 \pm 41.5$
DAP1	$+122.7 \pm 12.9$
G-protein δ3 subunit	-30.4 ± 3.8
Stefin 3	$+109.2 \pm 18.3$

Change in gene expression has subsequently been confirmed by RT–PCR (not shown). The complete dataset from this experiment can be viewed at http://www.urmc.rochester.edu:80/research/microarray/index.html

pertinent to mention that this same study revealed that 163 genes had a greater than 10-fold increase and 57 genes a greater than 10-fold decrease in expression in 10-week-old cerebellum of cln3-knockout mice as compared with wild-type mice, and that these changes should also be considered in the context of the disease. Moreover, many of the alterations in gene expression could easily be linked to the pathology of Batten disease, such as increased expression of genes associated with an immune response or inflammation (galectins 1 and 3), cell-death-associated genes [CIDE-A, (cell death activator) and DAP1 (death-associated protein)], whereas changes in expression of genes involved in signaling (G-protein γ3 subunit) and proteolysis (stefin 3), to name but a few, will need further consideration (Table 3). The complete dataset from this experiment can be viewed at http://www.urmc.rochester.edu/research/FGC/resource.html

DISCUSSION

The fact that cln3-knockout mice bear autoantibodies to GAD65 that associate with brain has profound implications on our understanding of the etiology and progression of Batten disease. We have demonstrated that the GAD65 autoantibody is

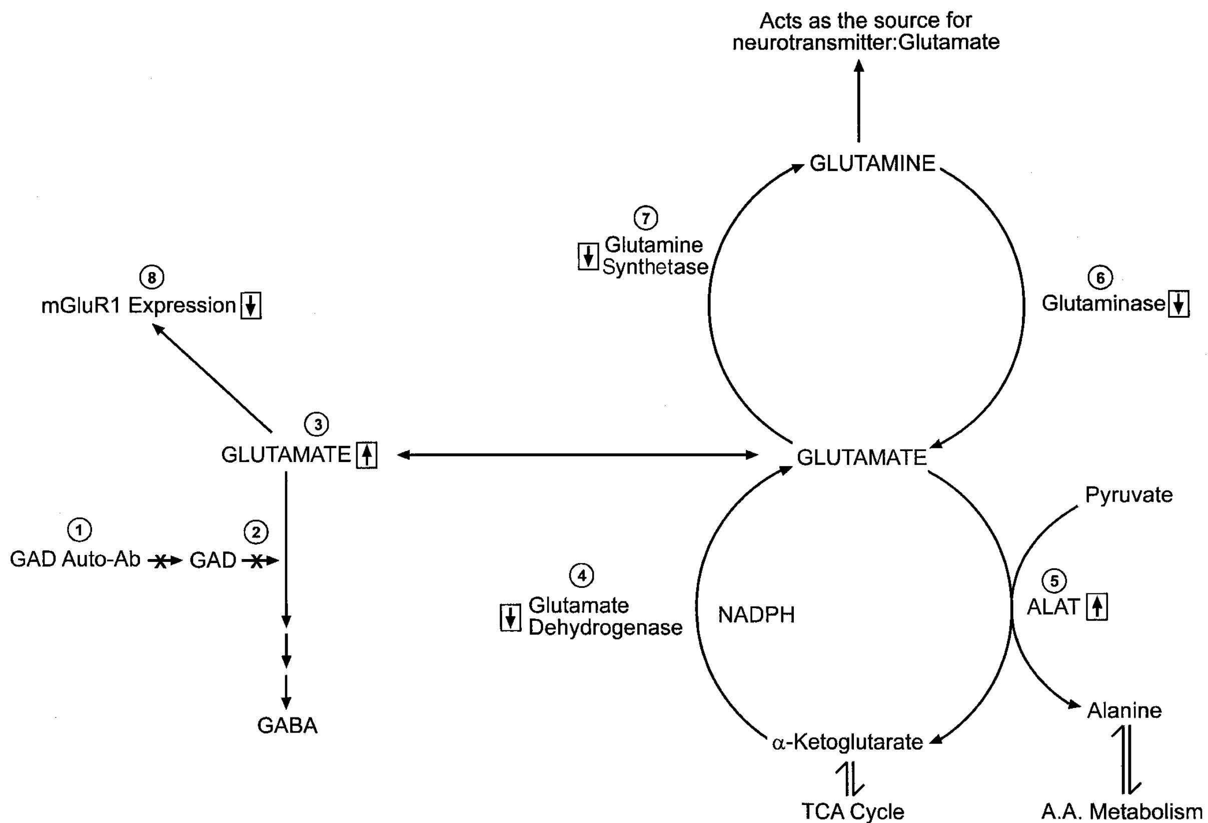

Figure 5. Biochemical model of how cln3-knockout cells attempt to balance aberrant glutamate metabolism. (1) Autoantibody to GAD65 decreases activity of GAD65 (Fig. 1/Table 2). (2) Decreased activity results in decreased levels of GABA and excess glutamate (Fig. 2/Table 2). (3) Increased levels of glutamate lead to the following observations. (4) Downregulation of glutamate dehydrogenase (Table 3). (5) Upregulation of alanine aminotransferase (Table 3). (6) Downregulation of glutaminase (Table 3). (7) Post-transcriptional downregulation of glutamine synthetase (not shown). (8) Downregulation of mGluR1 (Table 3). Collectively, the altered enzyme activities described in steps 4–7 represent a shift from glutamate synthesis to glutamate utilization resulting from the autoantibody to GAD65-mediated inhibition of GAD65 that results in elevated levels of glutamate.

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associated with reactive astrocytosis, and a metabolic shift within the brain for the neurotransmitter glutamate, although evidence of an inflammatory response is apparently absent or below the level of detection. GAD autoantibodies have been shown to be present in individuals with IDDM, stiff person syndrome and cerebellar ataxia, but are not apparent in a large number of other neurological disorders or in numerous control individuals (13,16,17). Autoantibodies to GAD in stiff person syndrome and cerebellar ataxia inhibit the activity of GAD, whereas autoantibodies in IDDM do not (13,16,17).

There is no precedent for understanding what the presence of these autoantibodies to GAD65, and the resultant changes in glutamate metabolism, would have on a child's developing nervous system. Interestingly, stiff person syndrome and cerebellar ataxia, diseases of the mature central nervous system (CNS), result in progressive muscle rigidity and spasms (16–18), both of which occur in the advanced stages of Batten disease. However, it is important to recognize that in stiff person syndrome and cerebellar ataxia, and also IDDM, a convincing pathological correlation remains to be established between the autoantibodies and the disease. The cln3-knockout mouse will prove a valuable research tool in understanding why GAD65 is an autoantigen in Batten disease, and could also serve as a tool in deducing whether the GAD65 autoantibody contributes to IDDM, stiff person syndrome and cerebellar ataxia.

CLN3 has been shown to localize to the late endosome/lysosome (8,9) in non-neuronal cells, and a loss of CLN3 function may impact upon antigen presentation or recognition at this organelle. While in this study the autoimmune response appears to be specific to loss of CLN3, ongoing studies are focusing on the construction of immune-deficient cln3-knockout mice to further dissect the role of the autoantibody in Batten disease and whether a lack of CLN3 does result in loss of self recognition of GAD65. The most compelling functional correlate of CLN3 comes from studies on a yeast homolog, Btnlp. Mutation of Btnlp has been implicated in altered vacuolar (lysosomal) pH (19) and metabolite content, and human CLN3 has been shown to complement the function of Btnlp (20). If there were any correlation in function between yeast Btnlp and human CLN3, altered pH and content in the late/endosome may well impact upon antigenic presentation.

CLN3 has also been shown to co-localize with synaptic vesicle proteins (8,9) in neuronal cell types, and it has been suggested that altered lysosomal/late endosomal pH due to an absence of a functional CLN3 results in altered vesicular pH and a disturbance in vesicular trafficking (21). A recent report indicated that GAD could become associated with membranes and with synaptic vesicles through an interaction with HSC70, suggesting a functional link between GABA synthesis and vesicular GABA transport (22). Therefore, if CLN3 is involved in this trafficking, mutant CLN3 would selectively affect GABAergic neurons – a hypothesis already borne out by the selective loss of this type of neuron in the cln3-knockout and mnd mouse models for Batten disease (10,11). Autoantibodies to GAD from a patient with cerebellar ataxia have been shown to act at presynaptic terminals of GABAergic neurons and alter the release of GABA (23). Interestingly, mice that have been engineered to lack GAD65 have been shown to have a compensation of GAD activity, most likely through GAD67, and only show a slight decrease in GABA (24). However, GAD65-knockout mice are susceptible to spontaneous and stress-induced seizures, which also occur in Batten disease (25). Since glutamate also acts as a neurotransmitter, leakage of the accumulating glutamate may result in increased stimulation of specific neurons, which might result in excitotoxicity. The astrocytic hypertrophy in cln3-knockout mice probably represents a pathophysiologic response, possibly related to elevated levels of glutamate (26). A recent report has shown that glutamate clearance, and therefore glutamate concentration and diffusion in the extracellular space, is associated with the degree of astrocytic coverage (27). Therefore, if the reactive astrocytosis that we report is a direct response to the elevated glutamate, this may impact on glutamate-mediated neurotransmission. Expression of genes involved in glutamate metabolism is altered presumably as a response to the elevated levels of glutamate generated by the autoantibody-mediated inhibition of GAD65. Glutamate dehydrogenase and glutaminase are downregulated, and levels of glutamine synthetase decreased, therefore decreasing further production of glutamate. Expression of alanine aminotransferase and glutamine aminotransferase – enzymes that will drive glutamate into other metabolites – is increased. Overall, a shift from glutamate production to glutamate utilization is observed (Fig. 5).

If mutation in CLN3 does directly result in an autoimmune response, much work is needed to define the role of this response with respect to disease progression, as opposed to the simple loss of a functional CLN3. Does altered function of the lysosome somehow impact upon antigen processing, leading to a loss of self-recognition of GAD65? The discovery of autoantibodies in the brains of cln3-knockout mice raises several additional questions: Do these autoantibodies bind specifically to an antigen in brain or are they passively deposited because of some breach in the blood–brain barrier? What is the source of the autoantibodies? The apparent preservation of GAD neurons, the early appearance of the antibody (1 week) and the failure to identify increased GAD in the serum of cln3-knockout mice indicate that the autoantibodies are not a secondary response to the liberation and vascular dissemination of GAD from dead or dying GAD neurons in the CNS. Further study of the observed autoimmune component in Batten disease could lead to the development and application of novel therapeutic strategies, such as immunotherapy and the cln3-knockout mouse may provide a model for the study of this autoimmune response.

MATERIALS AND METHODS

Animals

129S6/SvEv and homozygous cln3-knockout mice on a 129S6/SvEv background (10) were used for this study, and, unless stated otherwise, all tissue/serum used in this study were derived from 3-month-old animals. Serum from a cln3-knockout mouse on a C57BL6 background (12) was kindly provided by Dr Beverly L. Davidson (Department of Internal Medicine, University of Iowa), and the nclf and npc1 mice were obtained from the Jackson Research Laboratories. All procedures were carried out in accord with NIH Guidelines and the

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University of Rochester Animal Care and Use Committee Guidelines. All animals were housed under identical conditions.

Whole-brain and cerebellar extract preparation

Whole-brain or cerebellum extracts were prepared as described (13) using an Ultra-Turex T8 homogenizer (IKA Labortechnik) at 5000 r.p.m./stroke for 3×5 s strokes in ice-cold 0.32 M sucrose, 10 mM KH_2PO_4, 1 mM EDTA, 20 μM leupeptin, 1 μM pepstatin and 0.5 mM phenylmethylsulphonyl fluoride (pH 7.0). Tissue debris was removed by centrifugation at 800g for 5 min at 4°C. Supernatant was then centrifuged at 25 000g for 25 min at 4°C, and the pellet, representing a crude synaptosomal fraction, was resuspended in ice-cold distilled buffer and used for subsequent glutamic acid decarboxylase activity assays. Supernatant from this last centrifugation step was used for assay of free glutamic acid levels. For the detection of IgGs in brain tissue, the method of Goust et al. (28) was utilized. In summary, pelleted material from brains of perfused animals, following the above homogenization and extraction procedure, were resuspended and thoroughly mixed in ice-cold 0.1 M glycine, 0.1 mM phenylmethylsulphonyl fluoride, 10 mM pepstatin and 0.02% NaN_3 (pH 2.5). Acid eluate for testing for the presence of eluted IgGs was generated by removal of cellular material by centrifugation at 10 000g and analyzed by western analysis.

Western analysis

Extracts of either whole brain or cerebellum, or recombinant human GAD (Kronus, USA) were separated by SDS–PAGE on a 10% gel, and transferred by standard techniques. Filters were blocked overnight with 5% non-fat dried milk in 20 mM Tris–HCL, pH 7.5, 136 mM NaCl and 1% Tween-20 (TBST). For brain/cerebellum extracts, 12 μg total protein, or, for recombinant GAD, 600 ng protein was loaded to each gel. Serum collected from either normal or cln3-knockout mice or monoclonal mouse anti-GAD (StressGen, Canada) was diluted to 1 : 1000 in TBST. For pre-adsorption of sera with GAD65, sera was incubated with recombinant GAD65 as indicated overnight at 4°C. Secondary antibodies, either HRP-conjugated anti-mouse or anti-human, depending on the origin of the serum, were used at 1 : 3500. For assessment of the presence of IgGs in brain and cerebellum, secondary antibody only was hybridized to extracts as a mode of detecting IgGs as a whole rather than for one specific IgG. Immunodetection was performed with an ECL-PLUS kit (Amersham-Pharmacia Biotech, USA) as per the vendor's instructions.

Immunocytochemistry

For these studies, 3-, 6- and 14-month-old normal and homozygous cln3-knockout mice were used. Brains were removed after perfusion fixation with 10% buffered formalin or 4% paraformaldehyde (PFA). Samples of cerebrum, cerebellum and brainstem were processed routinely and embedded in paraffin. One-half of the brains were fixed with 4% PFA and frozen in isopentane. Cryostat and paraffin sections were stained with hematoxylin–eosin (HE). Immunohistochemistry (IHC) with the following dilutions and antibodies was performed: for mouse IgG (Serotec, UK) 1 : 2000, glial fibrillary acidic protein (GFAP; Dako, USA) 1 : 10 000, CD68 (macrophage; Dako, USA) 1 : 800, CD45RB (common leukocyte antigen; Dako, USA) 1 : 200, CD45RO (T cell; Dako, USA) 1 : 75, CD3 (T cell; Dako, USA) 1 : 100, Ricinus Communis Agglutinin (RCA; activated microglia, Vector Laboratories, USA) 1 : 15 000, CD44 (Chemicon International, USA) 1 : 2000, vimentin (Dako, USA) 1 : 800, anti-Alzheimer precursor protein A-4 (Boehringer Mannheim) 1 : 100 and glutamic acid decarboxylase (GAD; Stressgen Biotechnologies, Canada) 1 : 500. The IHC was carried out with the streptavidin–biotin method using 3-amino-9-ethylcarbazole (AEC; Sytec Laboratories, USA) as chromogen. All IHC, except for IgG and GAD, were performed on a Dako (USA) autostainer. The in situ cell death detection kit (Roche, USA) of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) was utilized to identify apoptotic cells; sections of 6-month-old mice were treated according to the vendor's instruction.

From archival autopsy tissue samples, brain sections of one Batten disease case, three normal age-matched controls and three neurological controls (GM1 gangliosidosis, Niemann–Pick type C and adrenoleukodystrophy) were analyzed. IHC for human IgG (Serotec, UK) 1 : 2000, GAD, GFAP and synaptophysin (Biogenex, USA) 1 : 100 were carried out as described previously in triplicate.

To permit direct comparisons of the relative level of glutamate immunoreactivity, pairs of 40 μm saggital sections, from cln3 null mutant and control mice were incubated overnight together in the same solution of primary antiserum (rabbit anti-glutamate, Sigma, St, Louis, MO at a dilution of 1 : 2000) alone, or in combination with mouse monoclonal antisera against selected pre-synaptic (synaptophysin, 1 : 250; amphiphysin, 1 : 250) or post-synaptic (PSD95, 1 : 100) markers (StressGen Biotechnologies Corp, Victoria, BC, Canada). Subsequently sections were rinsed and incubated with secondary antisera labeled with either Alexa 488 (1 : 500) or Alexa Fluor 568 (1 : 1000) (Molecular Probes, Eugene, OR). Immunofluorescence was initially observed by conventional epifluorescence microscopy (Zeiss Axioskop 2 MOT, Carl Zeiss Ltd, UK) and recorded digitally using a fixed exposure time across all sections (Zeiss Axiocam, Axiovision 3.0, Carl Zeiss Ltd, UK). Subsequently, the extent of colocalization of glutamate immunoreactivity with other antigens was revealed by dual channel confocal microscopy (Zeiss LSM 5 Pascal, Carl Zeiss Ltd, UK) employing sequential acquisition of each channel and maintaining a consistent relative relationship between detector gain and amplitude offset between samples.

From archival autopsy tissue samples, brain sections of one Batten disease case, three normal age-matched controls and three neurological controls (GM1 gangliosidosis, Niemann-Pick-Type C and Adreno-leukodystrophy) were analyzed. IHC for human IgG (Serotec, UK) 1 : 2000, GAD, GFAP and Synaptophysin (Biogenex, USA) 1 : 100 were carried out as described previously in triplicate.

Measurement of GAD activity

Assay of GAD activity was performed on either whole-brain or cerebellar extracts from normal and cln3-knockout mice where

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indicated based on the method reported by Dinkel et al. (13). Essentially, 5 µg total protein was diluted into 20 µl 50 mM KH_2PO_4, 1 mM aminoethylisothiouronium hydrobromide and 0.2 mM pyridoxal phosphate (pH 6.8). To this, 180 µl of this same GAD assay buffer containing 10 µl L-[1-^{14}C]glutamic acid of specific activity 56 mCi/mmol (Amersham Pharmacia, USA) was added, and incubated for 3 h at 37°C. For inhibition studies, 1–8 µl of either normal or cln3-knockout serum was pre-incubated for 90 min before addition of assay mix containing the radiolabeled glutamic acid substrate. During the incubation, $^{14}CO_2$ release from the L-[1-^{14}C]glutamic acid was trapped on a small piece of glass fiber soaked in hyamine hydroxide. Following the incubation, radioactivity associated with the trapped $^{14}CO_2$, expressed as counts per minute (c.p.m.), was measured in a Beckman LS 6000SC liquid scintillation counter.

Amino acid analysis

Whole brain and cerebellum were homogenized as described above, and supernatants were analyzed by the Hewlett-Packard Aminoquant system. Amino acids were derivatized as per the vendor's instructions and separated on column HP 5 µM × 200 × 2.1 mm.

Gene expression studies

For comparative gene expression studies, cerebellum from three 10-week-old normal and cln3-knockout mice were pooled, respectively, and homogenized by standard procedures in Trizol (GibcoBRL) for mRNA extraction. From each sample, 10 µg of total RNA was used to generate a high-fidelity cDNA, which was modified at the 3′ end to contain an initiation site for T7 RNA polymerase as per the manufacturer protocol (SuperChoice, Gibco BRL). Upon completion of cDNA synthesis, 1 µg of product was used in an in vitro transcription (IVT) reaction that contained biotinylated UTP and CTP, which were utilized for detection following hybridization to the microarray as per the manufacturers protocol (ENZO). Subsequently, 20 µg of full-length IVT product was fragmented in 200 mM Tris–acetate (pH 8.1), 500 mM KOAc and 150 mM MgOAc at 94°C for 35 min. Following fragmentation, all components generated throughout the processing procedure (cDNA, full-length cRNA and fragmented cRNA) were analyzed by gel electrophoresis to assess the appropriate size distribution prior to microrarray analysis. Comparative gene expression was performed on Mu19 Affymetrix gene chips as described by the manufacturer at the University of Rochester Microarray Core. Hybridization, staining and washing of all arrays were performed in the Affymetrix fluidics module as per the manufacture's protocol. Streptavidin phycoerythrin stain (SAPE, Molecular Probes) was the florescent conjugate used to detect hybridized target sequences. The detection and quantitation of target hybridization was performed with a GeneArray Scanner (Hewlett Packard/Affymetrix), set to scan each array twice at a factory-set PMT level and resolution. In addition, all arrays were scanned pre and post antibody amplification to address potential issues with respect to the dynamic range of the scanner. All arrays referred to in this study were assessed for 'array performance' prior to data analysis. This process involves the statistical analysis of control transcripts that are spiked into the hybridization cocktail to assess array performance. In addition, several genes have been identified on each array to help assess the overall quality of signal intensity from all arrays. The results of this analysis have demonstrated that all the arrays are within a 0.2-fold difference of each other at baseline. This analysis affords the necessary confidence needed to apply a global scaling approach to data normalization in the subsequent analyses. The Microarray Analysis Suite (Affymetrix) was employed to generate the comparative analysis presented in this study. Distinct algorithms were used to determine the absolute call that distinguishes the presence or absence of a transcript, the differential change in gene expression [increase (I), decrease (d), marginal increase (MI), marginal decrease (MD), and no change (NC)], and the magnitude of change, which is represented as fold change. The mathematical definitions for each of these algorithms can be found in the Microarray Suite Analysis manual in the algorithm tutorial. In brief, the algorithm that defines the presence or absence of a gene takes into consideration the following qualitative and quantitative metrics from the raw data set: the positive/negative ratio, the positive fraction and the log average ratio. The algorithm that defines the differential change in gene expression takes into consideration the following metrics from the raw data set: Max(increase/total, decrease/total), the increase/decrease ratio, the log average ratio change and the Dpos–Dneg ratio. The threshold setting for this decision matrix was set at default levels. Finally, the fold-change calculation is based on the average difference of each probe set owing to the fact that this output is directly related to its expression level. The fold change of any transcript between baseline and experimental value is calculated following global scaling. Lastly, all array within this data set were normalized via global scaling (target intensity 2500), and Super Scoring (SDT = 3) was applied to all probe sets of eight probe pairs or more, which means that any probe pair's average difference that exceeded 3 standard deviations of the mean of all probe sets was not calculated in the average difference metric. All data represented are from the average of all pairwise comparisons (of which there were 16) in the same direction.

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