Hypothesis

Pathogenesis of peroxisomal deficiency disorders (Zellweger syndrome) may be mediated by misregulation of the GABAergic system *via* the diazepam binding inhibitor

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Abstract

Background: Zellweger syndrome (ZS) is a fatal inherited disease caused by peroxisome biogenesis deficiency. Patients are characterized by multiple disturbances of lipid metabolism, profound hypotonia and neonatal seizures, and distinct craniofacial malformations. Median live expectancy of ZS patients is less than one year. While the molecular basis of peroxisome biogenesis and metabolism is known in considerable detail, it is unclear how peroxisome deficiency leads to the most severe neurological symptoms. Recent analysis of ZS mouse models has all but invalidated previous hypotheses.

Hypothesis: We suggest that a regulatory rather than a metabolic defect is responsible for the drastic impairment of brain function in ZS patients.

Testing the hypothesis: Using microarray analysis we identify diazepam binding inhibitor/acyl-CoA binding protein (DBI) as a candidate protein that might be involved in the pathogenic mechanism of ZS. DBI has a dual role as a neuropeptide antagonist of GABA(A) receptor signaling in the brain and as a regulator of lipid metabolism. Repression of DBI in ZS patients could result in an overactivation of GABAergic signaling, thus eventually leading to the characteristic hypotonia and seizures. The most important argument for a misregulation of GABA(A) in ZS is, however, provided by the striking similarity between ZS and "benzodiazepine embryofetopathy", a malformation syndrome observed after the abuse of GABA(A) agonists during pregnancy.

Implications of the hypothesis: We present a tentative mechanistic model of the effect of DBI misregulation on neuronal function that could explain some of the aspects of the pathology of Zellweger syndrome.

Background

Clinical background of Zellweger syndrome

Zellweger syndrome (ZS) is a class of inherited metabolic disorders caused by a deficiency in peroxisomal biogenesis (reviewed in [1,2]). The incidence of ZS is about 1/25,000 – 1/50,000 births. ZS patients are characterized by profound hypotonia and neonatal seizures, which – accompanied by feeding difficulties and psychomotor retardation – lead to an early death, usually in the first year of infancy. Another characteristic symptom is a distinct craniofacial malformation complex, consisting of high forehead, low/broad nasal bridge, epicanthus, high

arched palate, and micrognathia. In addition, the severe disturbance of many peroxisomal metabolic pathways in ZS leads to a variety of defects in almost every organ, most prominently the liver (hepatomegaly in 78% of patients, fibrosis in 76%), the kidney (renal cysts in 98%), and the brain. In the latter, white matter abnormalities and a unique neuronal migration defect in the cerebral hemispheres, the cerebellum, and the inferior olivary complex are the most striking symptoms.

Biochemically, ZS patients are characterized by the absence of functional peroxisomes and an almost complete disruption of peroxisomal beta-oxidation, leading to the accumulation of branched and very long-chain fatty acids, abnormal bile acids, and leukotrienes. In addition, the biosynthesis of docosahexaenoic acid (DHA) and plasmalogens is impaired, resulting in a drastic decrease of these important lipid components of the central nervous system. The metabolism of pipecolic acid and the peroxisomal steps of isoprenoid biosynthesis are also impaired to some extent.

Pathogenetic mechanism of Zellweger syndrome

ZS is caused by mutations in any one of about a dozen components of the peroxisome assembly machinery [3]. The cell biology of the disease has been elucidated in considerable detail during the last decade. However, it is still completely unknown, how the deficiency in peroxisomal metabolism leads to the severe phenotype and extremely poor prognosis of ZS patients.

Several mechanisms have been suggested:

➢ Accumulation of branched and very long-chain fatty acids could lead to a structural disruption of cell membranes, particularly in the central nervous system (reviewed in [4]).

➢ The disturbance of lipid metabolism could also lead to a more subtle disturbance of membrane micro-domains (rafts, caveolae) involved in intercellular signaling.

➢ Deficiency of major myelin constituents, such as plasmalogens, DHA, and cholesterol, might lead to impaired neuronal development (see for example [5] for a review of the beneficial effects of DHA supplementation in ZS patients).

➢ Increased levels of leukotrienes due to beta-oxidation deficiency could precipitate an inflammatory process that might be involved in the pathogenesis of the brain defects [6,7].

➢ Studies in a mouse model of ZS have shown that reduced levels of the ether phospholipid platelet activating factor (PAF) and consequent impairment of glutamatergic signaling might cause the neuronal migration defects by an unknown mechanism [8].

The importance of lipid metabolism for the pathogenesis of ZS is underlined by the phenotype of peroxisomal single enzyme-deficient patients. Disruption of peroxisomal beta-oxidation at the level of multifunctional protein type 2 leads to the same pathological syndrome as ZS, although the patients have a slightly better prognosis [9]. Patients with acyl-CoA:dihydroxyacetone phosphate acyltransferase deficiency have rhizomelic chondrodysplasia punctata, which shares many of the key symptoms with ZS, including hypotonia, feeding problems, growth retardation, and early neonatal death [9]. On the other hand, defects of many other peroxisomal enzymes (catalase, glutaryl-CoA oxidase, phytanoyl-CoA hydroxylase, alanine:glyoxylate aminotransferase, 2-methylacyl-CoA racemase, pipecolic acid oxidase, and mevalonate kinase) as well as deficiencies in non-peroxisomal metabolism do not lead to ZS.

The most important recent data on the pathogenesis of ZS come from the analysis of three mouse models, that are deficient in different components of the peroxisomal biogenesis machinery (Pex2, Pex5, and Pex11beta, respectively [10-12]). All three mice show an almost exact phenocopy of the human ZS, including severe hypotonia at birth, impaired neuronal migration in the same brain areas, and early neonatal death. However, some important pathologies such as hepatomegaly, renal cysts, and bone abnormalities are not recapitulated. Mice disrupted in Pex11alpha are externally indistinguishable from wild-type animals [13]. Detailed analysis of these ZS models has demonstrated that DHA and isoprenoid/cholesterol biosynthesis are unlikely to be central to the pathogenic mechanism [14-16]. A more detailed study of Pex5 knockout mice further established that changes in very long chain fatty acid, docosahexaenoic acid, or plasmalogen levels in brain are unlikely to be responsible for the neuronal migration defects [17]. Most strikingly, however, the Pex11beta knockout mouse (which has intact peroxisomes) dies of ZS, but shows almost normal peroxisomal metabolism, including normal levels of very long-chain fatty acids and plasmalogens, thus confuting the major pathogenic hypotheses listed above [12]. This conclusively establishes that the obvious biochemical disturbances are concomitant but not causal to the most serious symptoms of ZS patients.

In addition, analysis of a mouse model of peroxisomal beta-oxidation deficiency indicates that the most severe ZS-like symptoms are independent of the characteristic
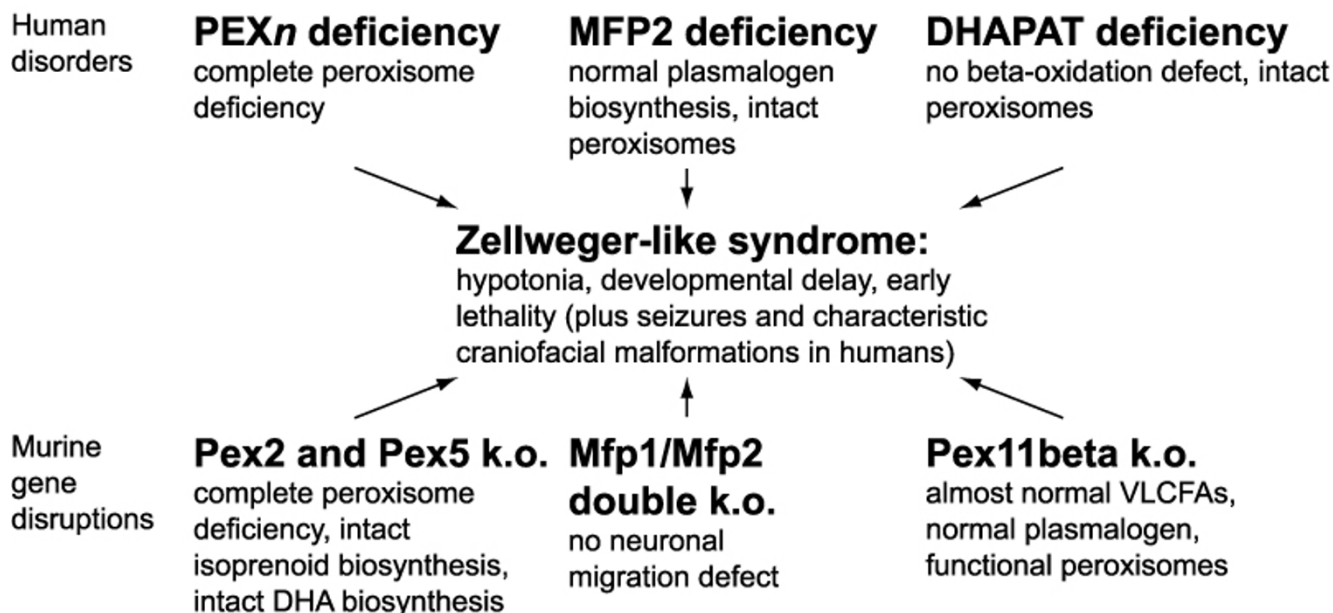
Figure 1
Molecular conditions leading to Zellweger-like syndromes in human and mouse. Biochemical and pathological consequences that are not common to all disorders are indicated. PEX*n* = any of the peroxisome biogenesis proteins that can be mutated in classical Zellweger syndrome. MFP1/2 = multifunctional proteins of peroxisomal beta-oxidation. DHAPAT = dihydroxyacetone phosphate acyl transferase. VLCFAs = very long-chain fatty acids. DHA = docosahexaenoic acid.

neuronal migration defects [18,19]. Figure 1 summarizes the molecular conditions leading to Zellweger-like syndromes in human and mouse.

Identification of candidate genes by microarray analysis
We suggest that a regulatory defect is central to the severe disease phenotype of ZS patients. Even a slight disturbance of specific pathway intermediates, such as seen in the Pex11beta knockout mouse, could trigger a catastrophic regulatory response that escalates by inappropriate activation of feedback loops. Such a mechanism has recently been identified in peroxisome-deficient yeast, where a slight increase in cytosolic aminoadipate levels is suggested to lead to a full-blown activation of the lysine biosynthetic pathway *via* a positive feedback mechanism [20].

We decided to use microarray data to identify possible players involved in such a misregulation mechanism in human ZS. Microarrays provide an unbiased survey of gene expression levels in human tissues and cells under various experimental conditions. Many hundred data sets, examining most of the predicted genes of the human genome, are now publicly available for exploratory analysis. Based on the known clinical data of ZS patients, we defined three criteria for candidate genes:

1. A candidate should be regulated positively or negatively in concert with lipid metabolism genes, because changes in acyl-CoA derivatives are the only common factor in ZS and the ZS-like single enzyme deficiencies.

2. To be active in a regulatory pathway, a candidate should be involved in a signaling pathway or in transcriptional regulation, so that feedback regulation is possible.

3. Because the most serious symptoms of ZS affect the brain, an ideal candidate would be involved in brain or neuron function.

Using these criteria we identified only a single candidate gene, Diazepam Binding Inhibitor, DBI, in all data sets available in the Stanford Microarray Database http://genome-www5.stanford.edu/MicroArray/SMD/. Co-regulation of DBI with lipid metabolism is found consistently in experiments representing the two current microarray methodologies, Affymetrix oligonucleotide arrays [21] and spotted two-color cDNA arrays [22] (Table 1). DBI is also among the most highly upregulated genes in lovastatin-treated squamous cell carcinomas [23].

Table 1: Ten genes that have expression profiles most similar to diazepam binding inibitor, DBI, in two large-scale microarray expression profiling studies, as quantified by Pearson correlation coefficient *r* (the top 10 neighbors of DBI are shown for each sample set, corresponding to *r* > 0.73 for the fibroblast samples). Genes that are mentioned twice refer to two independent clones on the array.

50 human fibroblast samples [22]		174 human carcinoma samples [21]	
FDPS	farnesyl diphosphate synthase	DKFZP564B167	unknown function
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase	**IDI1**	isopentenyl diphosphate isomerase
SC4MOL	sterol-C4-methyl oxidase	**INSIG1**	insulin induced gene 1
INSIG1	insulin induced gene 1	UAP1	UDP-N-acetylglucosamine pyrophosphorylase
H2AV	histone H2A variant	**FASN**	fatty acid synthase
FADS1	fatty acid desaturase	**CYP51**	lanosterol 14-alpha-demethylase
EBP	sterol-delta8,delta7-isomerase	C6orf34	unknown function
SQLE	squalene epoxidase	**SC4MOL**	sterol-C4-methyl oxidase
INSIG1	insulin induced gene 1	SORD1	sorbitol dehydrogenase
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase	SYBL1	synaptobrevin-like 1

Bold genes are involved in lipid metabolism. The expression profiles can be explored and compared at http://genome-www.stanford.edu/fibroblast/figures.shtml (fibroblast samples) and http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch (carcinoma samples).

Properties of Diazepam Binding Inhibitor

DBI is a particularly promising candidate for the regulatory pathway responsible for the brain and morphological defects associated with ZS. It has a dual function as a secreted GABA(A) receptor neuroregulatory peptide and as an acyl-CoA binding protein involved in lipid metabolism (reviewed in [24,25]). DBI is expressed almost ubiquitously, including in a variety of glial and neuronal cell types in many brain regions [26-32]. In the brain DBI is processed to various neuropeptides (triakontatetraneuropeptide TTN = DBI17-50, octadecaneuropeptide ODN = DBI33-50, eikosaneuropeptide ENP = DBI51-70, and others [33]) with slightly varying activity. DBI was first identified by its ability to displace the GABA(A) agonist diazepam and hence is able to antagonistically modulate the GABA(A) receptor in the brain. GABA is the most important inhibitory neurotransmitter in the central nervous system, and the GABA(A) receptor is the main target of benzodiazepine sedatives, such as diazepam. Increases in DBI serum levels were found in epileptic patients [34]. In contrast, DBI was found to be the only identifiable protein downregulated in both schizophrenic and Alzheimer's disease patients [35].

In addition to GABA(A) receptor, DBI and its processing products bind to the ubiquitous mitochondrial benzodiazepine receptor pBR and play a crucial role in the acute stimulation of steroidogenesis [36-38]. A third type of DBI receptor mediates rapid release of intracellular calcium, but remains to be characterized in detail [39,40].

In its role as an acyl-CoA binding protein, DBI specifically binds to medium and long-chain acyl-CoA esters (but not to fatty acids, acyl carnitines, cholesterol and a number of nucleotides; [41]) and is involved in fatty acid-mediated

regulation of gene expression and the formation of intracellular acyl-CoA pools (reviewed in [42]).

In vitro studies have identified functional sterol response elements (SREBP binding sites) and peroxisome proliferator gamma response elements (PPARgamma binding sites) in the DBI gene promoter [43,44].

Finally, DBI has a stimulating effect on the pancreatic cholecystokinin and insulin system [45,46], that might be related to the pancreatic islet hyperplasia seen in many ZS patients.

Benzodiazepines, GABAergic signaling, and Zellweger syndrome

Neonatal epileptic seizures and floppy infant syndrome are two important symptoms that point to an involvement of defective GABAergic signaling in ZS pathogenesis. However, the most striking connection between Zellweger syndrome and DBI-mediated misregulation of GABAergic signaling is provided by the report of severe developmental malformations in babies born to mothers that had used benzodiazepines such as diazepam during pregnancy. Laegreid et al. [47-49] have described a "benzodiazepine embryofetopathy" that involves extreme hypotonia, craniofacial abnormalities (epicanthus, low nasal bridge, abnormal ears, high arched palate), feeding difficulties, and delayed neuromotor development (table 2). The similarity to ZS was so striking that one of the first patients described by Laegreid et al. [47] was later recognized to be a genuine ZS case [50,51]. However, two others of their patients were tested biochemically and did not show accumulation of very long and branched-chain fatty acids, a biochemical hallmark of ZS. One of these two died at 11 weeks of age, and upon autopsy was found

Table 2: Comparison of selected symptoms in "fetal benzodiazepine syndrome", Zellweger syndrome and peroxisomal beta-oxidation (multifunctional protein-2) deficiency.

	"benzodiazepine embryofetopathy" [48]	Zellweger syndrome [2]	MFP2 deficiency [9]
hypotonia/floppy-baby syndrome	100% (8/8)	99% (94/95)	98% (41/42)
epicanthic folds	100% (8/8)	92% (33/36)	present[1]
feeding difficulties	88% (7/8)	96% (74/77)	91% (10/11)
mental retardation	86% (6/7)	100% (45/45)	yes
short upturned nose with low nasal bridge	75% (6/8)	100% (23/23)	present
micrognathia	63% (5/8)	100% (18/18)	present
Highly arched palate	50% (4/8)	95% (35/37)	present
abnormal ears	50% (4/8)	98% (39/40)	present
high forehead	present	97% (58/60)	present
neonatal seizures	no	92% (56/61)	95% (36/38)
neuronal migration defects	yes, in single case examined	yes, characteristic	88% (15/17)

[1] 79% (30/38) MFP2-deficient patients have a combination of dysmorphic features [9]. Numbers of affected and examined cases are indicated in parentheses.

to have slight cortical dysplasia and single-cell neuronal heterotopias in white matter [48].

The work by Laegreid et al. was heavily criticized for its epidemiological validity (e.g. [52]) and later studies did not find a significant correlation between benzodiazepine use during pregnancy and birth defects (reviewed in [53]). However, some reports have linked diazepam to oral clefts, distal limb defects, microcephaly, and cardiovascular anomalies (reviewed in [53]). In addition, it is well established that high doses of diazepam lead to craniofacial malformations in rodents [54-57] and mice carrying targeted disruptions of the GABAergic pathway consistently develop a cleft palate, a rather common feature of Zellweger patients [58,59].

We are not aware of any other drug-induced teratogenic syndrome that mimics ZS. It is quite unlikely that all of Laegreid's patients just had a mild form of ZS, as they did neither show the characteristic biochemical abnormalities nor the disease progression with age. Also, consistent with the fact that benzodiazepine exposure is largely terminated at birth, they did not show the neonatal seizures that affect ZS patients.

Presentation of the hypothesis

We suggest the following tentative mechanistic model for the pathogenesis of DBI misregulation in ZS (figure 2):

Peroxisome deficiency leads to an accumulation of peroxisomal metabolic intermediates (metabolite X), most likely acyl-CoA derivatives that are also disturbed by the single enzyme defects that cause ZS-like syndromes. Directly or indirectly, metabolite X causes an inactivation and down-regulation of DBI and a decrease in DBI release,
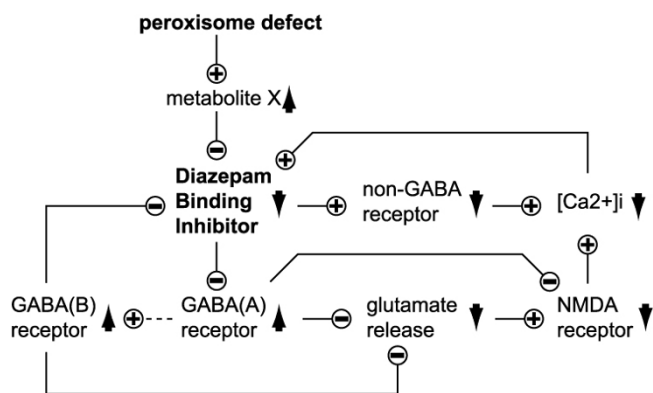

Figure 2
Possible regulatory network leading to disturbed GABAergic signaling in peroxisome-deficient patients. Arrows indicate the predicted direction of change in the patients. See text for details.

possibly by replacing its physiological acyl-CoA ligands. Decreased activity of DBI affects two intertwined feedback loops. In the first loop, decrease of DBI releases the inhibition of GABA(A) receptor [60]. Overactivation of GABA(A) receptor inhibits glutamate release [61] and consequently causes a decrease in NMDA receptor-mediated glutamatergic signaling [62]. This in turn causes a lack of calcium release in the target cells and a deficiency of DBI secretion and expression [63]. The second loop starts by a decrease in DBI action on its non-GABA(A) receptor and a subsequent lack of DBI-stimulated calcium release [39,40,64]. Both pathways converge on the decrease in intracellular calcium, that results in an

inhibition of DBI release [65] and closes the loop. A hypothetical ancillary loop could involve positive crosstalk between the over-activated GABA(A) receptor and its counterpart GABA(B), which is known to inhibit DBI release [66]. The two loops are not necessarily present in the same cells, and each of them may involve several adjacent cells. Also, different processing products of DBI act on intracellular calcium using different pathways [67], and may lead to additional differentiation of the DBI misregulation effect. The main feature is that both loops tend to enhance the down-regulation of DBI, thus amplifying and maintaining a small initial disturbance caused by the peroxisomal defect. Both loops involve a deficiency of NMDA receptor-mediated calcium release, in agreement with observations in ZS mouse models [8]. Gressens et al. (2000) found that administration of GABA at a concentration of 0.25 mg/kg twice a day had no effect on neuronal migration in peroxisome-deficient and control mice. This finding might indicate that the neuronal migration defect is mediated by the direct effect of DBI-derived peptides on intracellular calcium through its non-GABA(A) receptors.

Misregulation of DBI and the consequent disinhibition of GABAergic signaling should lead to diverse defects in various brain areas. A region of special interest is the inferior olive, which is malformed or absent in classical ZS. Olivary axon collaterals do not only innervate non-GABAergic neurons in the cerebellar nuclei, but also GABAergic nucleo-olivary cells, thus establishing a direct feedback loop to the inferior olive [68]. Purkinje cells, which show characteristic heterotopias in the cerebellum of ZS patients are also GABAergic [69,70].

Furthermore, it has been demonstrated recently that excessive GABA(A) receptor activation by neurosteroids or benzodiazepines initiates a slow form of neuronal death in cultured hippocampal neurons [71]. This could be the basis of the increased neuronal apoptosis observed in ZS. It should also be noted that the suggested mechanism for ZS brain disturbances bears conceptual similarity to that for fetal alcohol syndrome, where a combination of NMDA blockade and GABA(A) over-activation leads to widespread apoptosis in the developing brain [72].

The observation of cleft palate in GABA signaling-deficient mouse models indicates that disregulation of the GABA(A) pathway might also be responsible for the malformation syndromes observed in ZS patients and in benzodiazepine embryofetopathy [58,59].

DBI is an especially suitable candidate for ZS pathogenesis because it is shown to integrate signals from a variety of lipid-related pathways (fatty acids, sterols, steroids). Its restricted binding properties narrow down the search for a specific metabolite X that is responsible for the initial repression of DBI, and the feedback loops explain how even a slight disturbance that is common to all ZS-like syndromes can tip the balance towards a disinhibition of the GABAergic system, resulting in the severe disorders that are found in all patients.

Testing the hypothesis
The recent availability of several peroxisome-deficient mouse models provides an excellent opportunity to directly test the GABA/DBI model of Zellweger pathogenesis. This would involve a detailed analysis of DBI isoform levels in wild type and mutant brains, as well as a study of GABA agonist and antagonist effects, both *in vivo* and in neuronal explants. Additional *in vitro* studies could be used to determine the molecular details of DBI regulation and processing, to test the existence of the feedback loops postulated by the model. After the general mechanism has been established, identification of "metabolite X" would be crucial and could be achieved, e.g., by examining the effect of fractionated brain extracts on a DBI reporter cell line.

Implications of the hypothesis
Currently, no successful therapy for peroxisome deficient patients is available. Identification of a GABA component to the pathogenesis of Zellweger syndrome has obvious implications for the treatment of affected patients. Straightforward pharmacological intervention with well-known GABA antagonists could be used to counteract the misregulation and to interrupt the pathological feedback loop. In addition, even if the molecular details of the proposed mechanism turn out to be incorrect, consideration of an amplification of minor metabolic changes by positive feedback changes could influence the search for "metabolite X", which by implication of the present model would be a low-concentration intermediate that binds DBI, and hence is probably a Co-enzyme A thioester.

Conclusions
We suggest that peroxisomal deficiency leads to an unidentified disturbance of lipid metabolism that causes misregulation of the diazepam binding inhibitor, DBI. This misregulation affects two independent systems and is amplified by specific feedback loops: The DBI/GABA(A) system and the DBI/calcium system. The independence of the two systems agrees with the observation in peroxisomal beta-oxidation-deficient mice, where hypotonia and early death occur without neuronal migration defects. The activation and subsequent escalation of feedback loops would explain why several defects with a widely varying biochemical background lead to the same complex of symptoms.

Our rather simplistic model necessarily ignores many details that complicate the picture, e.g. we do not consider the regional and developmental heterogeneity in the GABAergic system (e.g. excitatory GABA action in early development and the enormous plasticity of the central nervous system that will result in important compensatory regulation phenomena. Also, relatively little is known about the functional distribution of DBI in the central nervous system and its autocrine vs. paracrine effects.

In fact, the DBI hypothesis is not able to explain *all* observations in peroxisome deficiencies. However, it seems that the DBI hypothesis is able to explain a larger part of the observations than previous ideas, e.g. the Pex11beta k.o. phenotype seems to follow naturally from the suggestion that slight initiating changes in a metabolite might lead to an escalating response via feed-back mechanisms. It is very well possible that the DBI hypothesis is incomplete and/or over-simplified (for example, explaining some differences between Zellweger and Zellweger-like syndromes might require postulating different mechanisms of action for DBI in these conditions) but we hope that even a partially correct hypothesis may be able to point research into a useful direction, e.g. concerning the importance of small perturbations amplified by misactivated feedback mechanisms.

The existence of several mouse models of ZS should allow for rapid testing and refinement of our idea that misregulation of DBI is involved in the pathogenesis of Zellweger syndrome.

Competing interests
None declared.

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