Alleviation of seipinopathy-related ER stress by triglyceride storage

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Mutations affecting the N-glycosylation site in Berardinelli–Seip lipodystrophy (BSCL)-associated gene BSCL2/seipin lead to a dominantly inherited spastic paraplegia termed seipinopathy. While the loss of function of seipin leads to severe congenital lipodystrophy, the effects of seipin N-glycosylation mutations on lipid balance in the nervous system are unknown. In this study, we show that expression of seipin N-glycosylation mutant N88S led to decreased triglyceride (TG) content in astrocytoma and motor neuron cell lines. This was corrected by supplementation with exogenous oleic acid. Upon oleic acid loading, seipin N88S protein was relocated from the endoplasmic reticulum (ER) to the surface of lipid droplets and this was paralleled by alleviation of ER stress induced by the mutant protein. This effect was not limited to seipin N88S, as oleic acid loading also reduced tunicamycin-induced ER stress in motor neuron cells. Furthermore, both seipin N88S and tunicamycin-induced ER stress were decreased by inhibiting lipolysis, suggesting that lipid droplets protected neuronal cells from ER stress. In developing zebrafish larvae, seipin N88S expression led to TG imbalance and reduced spontaneous free swimming. Importantly, supplementation with exogenous oleic acid reduced ER stress in the zebrafish head and increased fish motility. We propose that the decreased TG content contributes to the pathology induced by seipin N88S, and that rescuing TG levels may provide a novel therapeutic strategy in seipinopathy.

INTRODUCTION

Seipin is an endoplasmic reticulum (ER)-resident protein whose precise function is unknown. Its loss of function results in a severe form of congenital generalized lipodystrophy, Berardinelli–Seip syndrome (BSCL) (1). In addition, mutations affecting its N-glycosylation lead to a dominantly inherited motor neuron disease SPG17 or Silver syndrome, also termed as ‘seipinopathies’. In yeast, seipin has been shown to regulate lipid droplet morphology and inheritance (2–4) and in mammals, it is essential for the induction of adipogenesis (5,6). Thus, its function appears to be linked to neutral lipid deposition in cells and tissues. However, it is not known if seipin function in lipid droplet regulation is linked to the molecular mechanisms of seipinopathy.

Seipin is an integral membrane protein with a proposed hairpin structure (7,8). The most conserved part of seipin is the central region consisting of two transmembrane domains and an ER luminal loop (1). In humans, there are two isoforms that differ in the length of the N-terminus; the numbering of the glycosylation site refers to the short variant. In the ER membrane, seipin forms oligomers (9,10). Mutations in the N-glycosylation site lead to aggregation of the protein with concomitant induction of ER stress in both cell and mouse models (9,11). ER stress has been suggested to underlie the disease pathogenesis in seipinopathy: when seipin N-glycosylation mutant N88S was expressed under the Thy-1 promoter in a mouse model, the mice developed symptoms of a motor neuron disease. In the mice, ER stress markers were upregulated, although significant neuronal loss was not detected (11).
Seipin has a high level of expression in the brain, but its function in the central nervous system (CNS) has not been characterized. Lipodystrophy due to seipin deficiency is associated with mild mental retardation, suggesting that seipin has a role in neuronal functions (1). The functional significance of lipid droplets in the CNS has been largely neglected, probably because neutral lipids (i.e. triglycerides (TG) and cholesteryl esters) are low at the tissue level. Recent studies have, however, demonstrated that several lipid droplet-associated proteins and enzymes that participate in glycerolipid synthesis are abundantly expressed in the brain (12,13). In general, both yeast and mammalian cells respond to various stress stimuli by inducing the formation of lipid droplets (14). The physiological significance of this phenomenon is poorly understood.

In this study, we set out to investigate a possible involvement of lipid imbalance in seipinopathy. We observed that in addition to ER stress, seipin N88S expression leads to a reduced TG content in cells of CNS origin as well as in the developing zebrafish brain. We show that restoration of TG storage resolves the ER stress, possibly by allowing misfolded seipin protein to be sequestered from the ER to the surface of lipid droplets. We discuss these findings in relation to the pathogenesis of seipinopathy and their potential implications for other neurodegenerative conditions.

RESULTS

Effect of seipin N88S expression on cellular neutral lipid storage

To analyze the effect of seipin N88S mutation on cellular lipid balance, we transiently expressed the short variant of GFP-tagged wild type (wt) or N88S seipin in U251MG astrocytoma and NSC-34 motor neuron cells. We observed that both forms of the protein form large oligomers that are visible as high-molecular weight bands in immunoblots (Fig. 1A), in accordance with previous findings (9,10). When analyzing the cellular neutral lipid content under standard growth conditions, we observed that seipin N88S but not wt seipin overexpression in astrocytoma cells led to a significant reduction in the TG levels, when compared with control (Fig. 1B). A similar reduction in TG was achieved when seipin was depleted by small-interfering RNAs (siRNA) (Supplementary Material, Fig. S1A and B). Thus, seipin N88S expression induced a metabolic phenotype that resembled that of seipin loss-of-function. We observed a reduced TG storage also in motor neuron cells expressing seipin N88S, despite their intrinsically low TG content (Fig. 1C). Supplementation of the culture medium with exogenous oleic acid strongly increased the TG content in both astrocytoma and motor neuron cells, and corrected the storage defect observed in cells expressing seipin N88S (Supplementary Material, Fig. S1C and D). Accordingly, formation of lipid droplets upon oleic acid loading in control and seipin N88S expressing astrocytoma cells was similar, as judged by the quantification of the fraction of cell area occupied by lipid droplets using microscopy (12.0 ± 1.0% in seipin N88S expressing and 12.4 ± 1.3% in non-expressing cells, number of cells 10 to 11) (Supplementary Material, Fig. S1E).

Seipin N88S is recruited to lipid droplets upon oleic acid loading

We next analyzed the subcellular localization of wt and N88S seipin in U251MG astrocytoma cells. We found that under standard culture conditions, wt seipin showed mainly a reticular ER pattern as judged by extensive co-localization with the ER-resident protein calreticulin (Fig. 2). In the case of seipin N88S, in addition to the ER localization, also cytoplasmic punctae were observed (Fig. 2). This is in agreement with previous findings (8,15,16). Interestingly, we found that overnight incubation with oleic acid resulted in even more pronounced segregation of seipin N88S from the ER, leading to decreased colocalization with calreticulin (Fig. 2). Concomitantly, the fraction of cells exhibiting a punctuate seipin N88S staining pattern increased from 32% to 46% (visual inspection of 169–175 cells from 10 separate fields). This effect was not observed with wt seipin which remained localized to the ER even in the presence of oleic acid loading (Fig. 2).

We found that the punctuate structures harboring seipin N88S in both astrocytoma and NSC-34 motor neuron cells were lipid droplets, as visualized by oil red O staining (Fig. 3A). Furthermore, sucrose density gradient centrifugation of astrocytoma cells confirmed that in oleic acid loaded cells, a fraction of seipin N88S was recovered in the light-density top fraction containing lipid droplets (6.44 ± 0.46% of seipin in top fraction, compared with 0.41 ± 0.41% in the absence of oleic acid loading, variation between duplicate gradients) (Fig. 3B). Within a cell, not all the lipid droplets acquired seipin N88S. We noted that the lipid droplets containing seipin N88S were somewhat larger than those without the protein (by microscopy analysis, the average area of seipin N88S-positive versus negative lipid droplets was 0.78 ± 0.02 versus 0.63 ± 0.02 µm², number of lipid droplets 124–142 from nine cells).

Induction of lipid droplet formation or inhibition of lipolysis alleviates seipin N88S or tunicamycin-induced ER stress in motor neuron cells

Previous studies have shown that seipin N88S expression induces ER stress in cell and mouse models (9,11). This was also readily evident in NSC-34 motor neuron cells, where a robust increase in the expression of CCAAT/enhancer binding protein (CHOP) upon seipin N88S expression was observed when compared with wt seipin or control plasmid expression (Fig. 3C). As oleic acid loading led to the relocation of seipin N88S away from the ER, we considered whether it might have some effect on ER stress induced by the protein. We found that upon oleic acid loading, CHOP levels in seipin N88S expressing cells were significantly reduced (Fig. 3C), suggesting that induction of neutral lipid storage alleviated ER stress. In order to more directly test whether this effect was due to the presence of TG containing lipid droplets, we analyzed CHOP levels in cells where TG hydrolysis was inhibited by silencing the expression of adipose TG lipase (ATGL) with siRNAs (Supplementary Material, Fig. S2A). In motor neuron cells, lipid droplets were practically undetectable under normal growth conditions (Fig. 4A). In contrast, upon ATGL silencing, a bright lipid droplet staining
emerged in >50% of the cells (Fig. 4A), in agreement with observations in other cell types (17).

We found that ATGL silencing led to the recruitment of seipin N88S to the surface of lipid droplets similarly as was observed upon oleic acid loading (Fig. 4B, compare with Fig. 3A). Moreover, ATGL silencing prior to ER stress induction by seipin N88S reduced CHOP levels in cells, in line with the effect of oleic acid loading (Fig. 4C). The effects of increased lipid storage were not restricted to the ER stress induced by seipin N88S expression: when cells were incubated with oleic acid prior to treatment with tunicamycin, a general inhibitor of N-glycosylation, CHOP levels were reduced by at least 50% when compared with cells treated with tunicamycin only (Fig. 4D). Furthermore, ATGL silencing also reduced CHOP levels in tunicamycin-treated cells (Fig. 4C; and Supplementary Material, Fig. S2B). These results suggest that TG storage as lipid droplets may protect neuronal cells from ER stress induced by protein misfolding.

Zebrafish model of seipinopathy

To investigate the in vivo relevance of our findings, we introduced messenger RNAs (mRNAs) encoding fusions of human seipin N88S with GFP or red fluorescent protein (RFP) into zebrafish eggs by mRNA microinjection at a two-cell stage. Using this method, a robust and ubiquitous transient expression of the transgene is achieved (Fig. 5A). During early development, yolk-derived lipids can easily access the brain, as the blood–brain barrier only starts to function at 3 days postfertilization (dpf) (18). This probably contributes to the relatively high TG content in the heads of 2–4 dpf embryos and a sharp decline thereafter (Fig. 5B). Analysis of the TG content of 2–6 dpf fish showed that when compared with GFP expressing zebrafish larvae, TG levels were significantly reduced in the heads of seipin N88S but not wt seipin expressing fish (Fig. 5C). This reduction was paralleled by a marked increase in the TG levels in the trunks of the larvae, suggesting a defect in the mobilization of yolk lipids to the developing brain (Supplementary Material, Fig. S3A and B).

We next studied the effect of oleic acid loading on ER stress in the developing zebrafish. Analysis of protein extracts from three dpf fish heads revealed that the basal level of CHOP protein was already relatively high in control fish (Fig. 5D). This is not surprising, considering that during embryonic brain development, several ER stress and proapoptotic pathways are induced (19). Even tunicamycin treatment failed to significantly increase the basal CHOP levels (data not shown). Similarly, seipin N88S expression did not substantially elevate CHOP levels in three dpf fish heads (Fig. 5D). However, a reduction in ER stress was readily evident by CHOP immunoblotting when the embryos were supplemented with exogenous oleic acid in the swimming water. This indicates that oleic acid administration also reduced cell stress in vivo.

Interestingly, we observed a significant decrease in the spontaneous free swimming of seipin N88S expressing zebrafish larvae when compared with control fish expressing GFP alone (Fig. 5E). This phenotype was not accompanied by morphological abnormalities or loss of the motor neurons (Supplementary Material, Fig. S3C). Also touch-evoked escape and swimming behavior of both wt seipin and seipin N88S expressing larvae appeared normal (Supplementary Material, Videos S1 and S2), suggesting that their expression did not result in gross developmental defects. Addition of exogenous oleic acid increased voluntary free swimming of both control and seipin N88S expressing fish (Fig. 5E). Importantly, in seipin N88S fish, oleic acid treatment compensated for the original motility defect, as indicated by comparing their swimming with that of non-treated control fish. Of note, also wt seipin expression led to reduced swimming of the fish. This defect was, however, not rescued by oleic acid loading (Fig. 5E).
DISCUSSION

Seipin has been implicated in the regulation of lipid droplet morphology and adipogenesis (1). In seipinopathy, the disease pathology involves accumulation of the misfolded seipin protein in the ER, leading to cell stress. Up to date, there have been no studies linking seipin function in neutral lipid storage to the pathology of seipinopathy. We show that under standard growth conditions, the expression of seipin N88S that underlies spastic paraplegia leads to decreased TG levels in motor neuronal and astrocytoma cells as well as in the developing zebrafish brain, mimicking the effect of seipin depletion. While seipinopathy has not been linked to impaired development of the adipose tissue, our results raise a possibility that also the N88S mutation leads to some degree of loss of seipin function. This may be related to the fact that N88S mutation in seipin alters its subcellular distribution, leading to partial localization in cytoplasmic inclusions.

Seipinopathy is a disease affecting motor functions, and the essential pathological features are recapitulated in the mouse model expressing seipin N88S in the CNS (11). The disease phenotype in mice was not associated with neuronal loss or neurodegeneration, but was instead suggested to be due to cell stress affecting axonal transport. In the present work, we generated the first zebrafish model for seipinopathy and found that in the fish, expression of seipin N88S led to a motility defect. Also in this case, there was no associated motor neuron loss. With seipin N88S, the reduction in swimming was paralleled by a decreased TG content in the developing head, possibly due to disturbances in the mobilization of yolk lipids. Importantly, oleic acid supplementation restored the motility defect of seipin N88S expressing fish, suggesting that the impairment of lipid metabolism is a contributing factor in the pathology associated with seipin N88S mutation. It should be noted that overexpression of the wt protein also led to reduced swimming. The reason for this is not clear. Obviously, voluntary-free swimming is a complex phenotype affected by several factors. Upon wt seipin expression, we did not observe a decrease in the TG content of motor neuron or astrocytoma cells or of the developing zebrafish brain, implying that the phenotype may be mechanistically different from the seipin N88S situation. On the other hand, wt seipin overexpression has been reported to result in reduced TG storage in HeLa cells (15) and in mouse adipocytes (20). Therefore, it is also possible that an excess of the wt protein mimics the effects of the N88S mutation in some zebrafish cell types and tissues not analyzed in the present study.

The N88S mutation in seipin alters its subcellular distribution, leading to localization in cytoplasmic inclusions that are devoid of aggressive markers vimentin, ubiquitin and Hsp70 (8). This aggregation was originally thought to underlie the pathology of seipinopathy, but this view was recently challenged by a report, suggesting that the inclusions might actually be cell protective (16). This study showed that HeLa cells harboring seipin N88S inclusions exhibited lower levels of ER stress and apoptotic markers when compared with cells that exhibited a reticular seipin N88S pattern. Based on the co-localization of seipin N88S and the Z variant of α1-antitrypsin in the inclusions, they were identified as ER-derived protective organelles. However, the ultrastructure of these organelles as judged by electron microscopy resembled lipid droplets (1,16). Indeed, we found that upon oleic acid loading, seipin N88S redistributed from the ER to punctuate inclusions that were lipid droplets. This relocation was

Figure 2. Seipin N88S is segregated from the ER upon oleic acid loading. U251MG cells were transfected with GFP-fused wt seipin or seipin N88S for 1 day in the absence (no load) or presence of 0.1 mM oleic acid overnight (OA load), fixed and stained with anti-calreticulin antibodies. Co-localization of seipin and calreticulin was analyzed from confocal micrographs representing a single focal plane. Bars: fraction of seipin signal co-localizing with calreticulin ± SEM. Number of cells 50–81, *P < 0.05. Scale bars, 20 μm.
paralleled by reduction in the ER stress induced by seipin N88S expression. With the wt protein, such a relocalization did not occur in response to oleic acid loading. This is interesting considering that oleic acid failed to rescue the reduced swimming of wt seipin overexpressing fish. It is tempting to speculate that as the (excess) wt protein is not distributed to lipid droplets, induction of their formation is not helpful in protecting against the harmful effects.

When the protein-folding capacity of the ER is exceeded or perturbed, the cells induce an unfolded protein response that aims at restoring ER functions (21). This stress response involves increased phospholipid synthesis and, subsequently, expansion of the ER membrane (22,23). Activation of lipogenesis also leads to increased TG synthesis and lipid droplet formation, especially in the presence of oleic acid (24,25). Moreover, in yeast, induction of secretory stress switches the channeling of lipid precursors to TG and other neutral lipids at the expense of phospholipids (26). We observed that ER stress caused by not only seipin N88S expression but also tunicamycin was alleviated when TG storage was induced by exogenous oleic acid. Furthermore, prevention of lipid droplet lipolysis by ATGL silencing was protective against both seipin N88S and tunicamycin-induced ER stress. This is in accordance with a recent study reporting protection from ER stress in hepatic cells derived from ATGL knockout mouse (27). In this work, the protective effect was proposed to result from the lack of toxic metabolites deriving from lipid droplet hydrolysis. We suggest that additionally, the presence of lipid droplets as such may be protective: lipid droplets offer large hydrophobic surfaces that may help to reduce the concentration of un/misfolded proteins in the ER and prevent their aggregation in anticipation of proper folding or degradation. Indeed, seipin N88S mutant redistributed to the surface of lipid droplets upon induction of TG storage. Moreover, wt seipin also localized to lipid droplets in a fraction of oleic acid-loaded cells treated with tunicamycin (our unpublished observations). It is conceivable that perturbed N-glycosylation leads to misfolding that allows the protein to partition to the surface of lipid droplets. This may be particularly characteristic of membrane proteins with several hydrophobic segments such as seipin (1).

In addition to seipin, other proteins implicated in neurodegenerative diseases have recently been linked to the regulation of neutral lipid storage. For instance alpha-synuclein, a protein...
associated with Parkinson’s disease, may function in regulating the TG content in the brain (28,29). Moreover, spartin/SPG20, a protein mutated in a motor neuron disease called Troyer’s syndrome, is found on lipid droplets and has a role in their turnover (30). Interestingly, spartin was also shown to have a function in adipose tissue, as its loss of function led to a decreased size but increased number of adipocytes in female mice (31). It appears that neutral lipid deposition and lipid droplet formation indeed have a role in neuronal functions, and it may be particularly important under stress conditions.

In summary, this study provides the first evidence that expression of seipin N88S that leads to seipinopathy decreases the cellular and nervous tissue TG content. Furthermore, restoration of TG storage leads to reduced cell stress and improves the motility defect observed in a zebrafish seipinopathy model. Our data suggest that TG storage in lipid droplets helps cells to cope with ER stress induced by protein misfolding, possibly by sequestering poorly folded membrane proteins from the ER. It can be envisioned that cells with an intrinsically low neutral lipid content, such as motor neurons, are particularly vulnerable to ER stress induced by protein misfolding. Thus, rescuing TG levels may provide a novel therapeutic strategy in motor neuron diseases such as seipinopathy.
**MATERIALS AND METHODS**

**Antibodies and reagents**

Anti-seipin rabbit polyclonal antibodies were obtained from Abnova, anti-CHOP rabbit polyclonal and mouse monoclonal antibodies from Santa Cruz, anti-calreticulin rabbit polyclonal antibodies from Thermo Scientific and anti-ATGL rabbit polyclonal antibodies from Cell Signaling Technology. Alexa 568-conjugated anti-IgG secondary antibodies, BODIPY 493/503 dye and BODIPY 558/568 C12 fatty acid were obtained from Invitrogen. Oleic acid, fatty acid-free bovine serum albumin (BSA), oil red O and tunicamycin were obtained from Sigma-Aldrich and cell culture media and reagents were obtained from Sigma-Aldrich and Lonza.

**Complementary DNA constructs and siRNAs**

To create fluorescent fusion constructs, complementary DNAs (cDNAs) encoding human wt (GenBank accession no. BC012140) and N88S seipin (3) (courtesy of H. Yang,
University of New South Wales, Sydney, Australia) were subcloned into pEGFP-C1 vectors (Clontech). For red fluorescent fusion constructs, the EGFP cassette was replaced with sequences encoding monomeric RFP (R. Tsien, University of California, San Diego, USA). Control plasmids were empty pCDNA3.1 plasmid (mock) (Invitrogen); pEGFP encoding soluble GFP (Clontech) and GFP-F encoding farnesylated GFP that directs GFP into membranes (Clontech). For mRNA synthesis, the cytomegalovirus promoter of the plasmids was replaced by T7 promoter. Stealth pre-designed siRNAs (Invitrogen) against human seipin were YACAGCCGUUCUUUUUG CAGGUUAA (sense) and UUAACCUGCAAGAGAAGCG GUGUC (antisense) and against murine ATGL/PNLPA2 GAA GAUAUCCGGUGGAUGAtt (sense) and UCAUCCACCAG AUACUUCag (antisense)(siRNA 1); and CCACUUUAG CUCCAAGGAAUt (sense) and AUCCUUGAGCUAAAGU GGga (antisense) (siRNA 2). Control siRNA against firefly luciferase2 (GL2) has been described (32).

Cell culture and transfection

U251MG astrocytoma cells were grown in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 1,glutamine and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). NSC-34 motor neuron cells (CELLutions Biosystems) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% FBS, 1,glutamine and antibiotics as above. Oleic acid was supplied as a BSA complex (stock was 1 mM oleic acid, 10:1 molar ratio to BSA in serum-free DMEM) and used at indicated concentrations, diluted to standard growth medium. Tunicamycin was used at 2 μM each), 10% glycerol, 2 mM ethylene glycol tetra-acetic acid, 1% NP-40 and 1% SDS with protease inhibitors as above. For the determination of TG content, lipids were extracted and analyzed by high-performance thin layer chromatography as described (33). For anti-CHOP immunoblotting, the cells were lysed in 1% Triton X-100, 0.1% Sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate and 1 mM EDTA in TBS (pH 8.0) and supplemented with protease inhibitors (chymostatin, leupeptin, antipain and pepstatin, at 25 μg/ml each), 1μM sodium orthovanadate, 10 μM sodium fluoride and 10 μM MG132. Zebrafish tissues were lysed manually with a pestle in 137 m M NaCl, 20 mM Tris–HCl (pH 8.0), 10% glycerol, 2 mM ethylene glycol tetra-acetic acid, 1% NP-40 and 1% SDS with protease inhibitors as above. For anti-seipin immunoblots, the cells were lysed in 1% Triton X-100 and 1% SDS in PBS or, alternatively, in 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 1% SDS with protease inhibitors as above. Protein determinations were performed according to Lowry or by using a Bio-Rad protein assay, and comparable amounts of protein were used for immunoblotting. Western blotting was performed as described (34). Immunoblots were quantified by densitometric scanning.

Cell fractionation

The protocol was adopted from (35), with minor modifications. Briefly, seipin N88S-transfected U251MG cells grown in the presence or absence of 0.1 mM oleic acid (confluent 75 cm 2 bottle per fractionation) were collected, and a post-nuclear supernatant prepared in fractionation buffer (50 mM Tris–HCl, pH 7.5; 150 mM sodium chloride and 5 mM EDTA with protease inhibitors). The supernatant (900 μl) was mixed with an equal volume of 2.5 mM sucrose, loaded at the bottom of the centrifuge tube and overlaid with a 360 μl of 30, 25, 20, 15, and 10 and 5% sucrose (w/v in fractionation buffer). The gradient was then centrifuged in a SW60Ti rotor (Beckmann Coulter Inc.) at 40 000 rpm (164 000 g) for 4 h at 4°C. A total of eight fractions (495 μl) were collected from the top and stored at −20°C prior to analysis of proteins (immunoblotting) or neutral lipids (lipid extraction followed by high-performance thin layer chromatography).

Microscopy and image analysis

Cells were fixed with 4% paraformaldehyde for 20 min at room temperature, quenched with 50 mM NHCl4 for 10 min and permeabilized with 0.1% Triton X-100 for 4 min at room temperature. Primary and secondary antibodies were diluted in 5% FBS and incubated at 37°C for 1 h or 30 min, respectively. For the detection of lipid droplets, the cells were incubated for 3 h with 5 μM BODIPY-fatty acid prior to fixation, or stained with BODIPY 493/503 dye or oil red O post fixation. The cells were imaged with a TCS SP2 confocal microscope (Leica), an IX70 inverted microscope (Olympus) equipped with a Polychrome IV monochromator (TILL Photonics) or an AX70 microscope (Olympus) with a digital camera. The lipid droplet area was analyzed with ImageJ software, and the co-localization with Image Pro Plus 5.1 software (co-localization coefficient m 2 function). Zebrafish larvae were imaged with a Leica MZFL III stereo-microscope with a digital camera or Zeiss Axio Examiner.AI with an MRe 5 digital camera and AxioVision software.

Zebrafish maintenance and microinjections

Zebrafish of the Turku line were maintained as described (36). When applied, oleic acid was administered as a BSA complex (stock was 1 mM oleic acid, 10:1 molar ratio to BSA in fish water) at 0.2 μM from 2 dpf onwards. For mRNA injections, the mRNAs were produced by using an mMESSAGE mMACHINE T7 kit (Invitrogen) according to manufacturer’s instructions and injected into the yolk at the two-cell stage (0.5–15 ng/embryo). To analyze motor neuron morphology, mRNAs were introduced at the two-cell stage (~1 ng/embryo) in gata6SAGFP10A transgenic embryos as described (37). Gata6SAGFP10A transgenic fish carry a gene-trap cassette encoding GFP linked to a gata6 splice acceptor at chromosome 15, approximately 81.4 kb downstream of dsclamb. GFP expression in this transgenic fish is observed.
in secondary motor neurons from 2 dpf onwards (see Supplementary Material, Figure S4).

Zebrafish swimming experiments
Zebrafish on 48-well plates were tracked using a CCD camera and analyzed by using the Ethovision 3.1 software (Noldus Information Technologies). Swimming was tracked for 10 min/fish/day.

Statistical analyses
The results are expressed as the mean of individual values ± SEM. Statistical significance was determined by a two-tailed Student’s t-test using Microsoft Excel software.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement
None declared.

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