Lack of age-related clinical progression in PGC-1α-deficient mice – implications for mitochondrial encephalopathies

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Abstract

Impaired peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) function has been demonstrated in several neurodegenerative diseases, and murine whole-body knockouts of PGC-1α have been considered as models for Huntington's disease. Recent neuropathological studies, however, rather propose these animals to be morphological models of mitochondrial leukoencephalopathies, with special reminiscence of Kearns-Sayre syndrome. PGC-1α-deficient animals have already been subjected to behavioral assessments; however, the contradictory findings and the paucity of data assessing long-term progression necessitated further examinations. This study provides a comprehensive neurological phenotypic profiling of full-length-(FL-)PGC-1α-deficient mice in a broad age spectrum, with special focus on previously controversial findings, the issue of long-term phenotypic progression, the histopathological assessment of previously non-characterized tissues of potential clinicopathological relevance, and the gene expression profile of novel brain-specific isoforms of PGC-1α. Our findings demonstrate moderate hypomotility with signs of gait and trunk ataxia in addition to severe impairments in coordination and muscle strength in FL-PGC-1α-deficient mice, phenotypic features consistent of a mitochondrial disease. Intriguingly, however, these early alterations did not progress with age, the understanding of which may unveil mechanisms of potential therapeutic relevance, as discussed. The observed phenotype did not associate with retinal or spinal cord alterations, and was accompanied by mild myopathic changes. Based on these, FL-PGC-1α-deficient mice can be regarded not only as morphological but behavioral models of mitochondrial leukoencephalopathies, with an important temporal limitation that has now been clarified. The mechanisms capable of halting a potentially lethal phenotype are to be unveiled, as they may hold therapeutic value for mitochondrial diseases.

Keywords: behavior, isoforms, pathology, PGC-1α, phenotype, progression.

Highlights:

- FL-PGC-1α -/- mice have a symptomatology corresponding with mitochondrial encephalopathy.
- The behavioral alterations intriguingly do not progress with age.
- The alterations do not associate with retinal or spinal cord involvement.
- The histopathology of skeletal muscle demonstrates only mild myopathic changes.
- CNS-specific isoforms are the predominant PGC-1α mRNAs in the murine brain.
- The NT isoforms are overexpressed in FL-PGC-1α -/- cerebella.

Abbreviations:

AD, Alzheimer's disease
ALS, amyotrophic lateral sclerosis
CNS, central nervous system
COX, cythochrome oxidase
ERR, estrogen-related receptor
FL-PGC-1α, full-length PGC-1α
GFAP, glial fibrillary acidic protein
HE, hematoxylin and eosin
HD, Huntington's disease
KLB, Klüver-Barrera staining
KSS, Kearns-Sayre syndrome
LS, Leigh syndrome
MEF2C, myocyte-specific enhancer factor 2C
MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MERRF, myoclonic epilepsy with ragged-red fibers
MNGIE, mitochondrial neurogastrointestinal encephalopathy
NARP, neuropathy, ataxia, retinitis pigmentosa
NRF-1, and -2, nuclear respiratory factor 1 and 2
NT-PGC-1α, N-terminal fragment of PGC-1α
PPARγ, peroxisome proliferator-activated receptor-gamma
PD, Parkinson's disease
PGC-1α, PPARγ coactivator 1-alpha
RT-PCR, real-time polymerase chain reaction

1. Introduction

Impaired mitochondrial function has widely been linked to the development of various neurodegenerative disorders, including Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS) [1-4]. These disorders are characterized by a relatively selective loss of neurons in different central nervous system (CNS) areas, corresponding to the leading clinical manifestations, in most of them with specific motor impairments. In addition, alterations of either nuclear or mitochondria genome affecting mitochondrial functions at various levels are pathognomonic of a group of congenital multi-systemic diseases, collectively termed mitochondrial encephalopathies [5-8]. These diseases that include Kearns-Sayre syndrome (KSS), Leigh syndrome (LS), mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibers (MERRF), neuropathy, ataxia, retinitis pigmentosa (NARP), and mitochondrial neurogastrointestinal encephalopathy (MNGIE) generally present with a progressive symptomatology corresponding to the multi-systemic involvement of the brain, skeletal muscles, heart, and liver, among other less commonly affected organs [5-8]. The involvement of the brain manifests in different forms of mitochondrial encephalopathy in the different syndromes, commonly characterized by various extents of white matter vacuolation accompanied by reactive astrogliosis with or without apparent selective neurodegeneration in particular brain regions.

Several lines of evidence indicate that peroxisome proliferator-activated receptor-gamma (PPARγ) coactivator 1-alpha (PGC-1α), a nuclear-encoded coactivator of an armada of transcriptional factors that play extensive roles in the activation of adaptive mitochondrial responses, may contribute to the mitochondrial dysfunction in neurodegenerative disorders, including PD, AD, HD and ALS [1-5]. The coactivation of genes such as nuclear respiratory factor 1 and 2 (NRF-1, -2), estrogen-related receptors (ERRs), myocyte-specific enhancer factor

2C (MEF2C), and PPARs results in an enhanced expression of a wide spectrum of proteins involved in mitochondrial function and biogenesis [9], including mitochondrial replication and transcription, the import and assembly of respiratory complex subunits, a tissue-dependent induction of oxidative phosphorylation and thermoregulation, the enhancement of gluconeogenesis and fatty acid oxidation, as well as the increase of defense against oxidative stress [10, 11]. In line with these, experimental evidence suggest that pharmacological or transcriptional activation of PGC-1α may hold therapeutic value in neurodegenerative as well as mitochondrial diseases [11-13].

Despite its broad roles in mitochondrial functions, the absence of PGC-1α is compatible with life. The first pioneering publications with two independent murine PGC-1α knockout strains commonly described signs of striatal degeneration, proposing that these animals might model HD [14, 15], which corresponded with the findings of reduced striatal expression of PGC-1α and its target genes in the striatum of HD patients [16, 17] as well as in its transgenic *in vivo* [16-18] and *in vitro* models [16, 17] (comprehensively reviewed in [11]). Not doubting the potentially essential role of PGC-1α dysfunction in the pathogenesis of HD, the concept that PGC-1α-deficient animals themselves might model HD, however, has recently been questioned by serial findings of independent morphological and molecular biological analyses [19-21]. One of the two pioneering publications reported the development of a complete (*i.e.*, with no residual expression) PGC-1α whole-body knockout strain, exhibiting hyperactivity with no overt muscle phenotype [14]. Though PGC-1α has recently been demonstrated to have a number of previously unknown isoforms [22], to the current knowledge, this strain can still be regarded as a complete knockout of PGC-1α [23]. Contrastingly, the second pioneering publication reported the development of another whole-body knockout strain demonstrating hypomotility and weakness [15], a strain later turned out to express a functional N-terminal fragment of PGC-1α (NT-PGC-1α) but not the full-length protein (FL-PGC-1α) [24]. This was

then followed by reports on muscle-specific complete PGC-1α knockouts with myopathic signs and weakness [25], as well as on complete whole-body and brain-specific PGC-1α knockouts failing to recapitulate hyperactive behavior [20, 26] but demonstrating impaired coordination [20] and ataxia proposed to be of cerebellar type [27]. Notably, the increasing behavioral data from the complete knockout phenotypes tend to delineate a phenotype resembling a relatively compensated mitochondrial disease. Correspondingly, recent neuropathological analyses on FL-PGC-1α -/- mice reported wide-spread, locally dramatic vacuolation of the white matter, predominantly affecting the thalamus, basal ganglia, internal capsule, pontomedullary brainstem as well as the cerebellum, accompanied by reactive astrogliosis in the brainstem and cerebellar nuclei [19]. In fact, this pathology is highly reminiscent of that seen in a human mitochondrial spongiform leukoencephalopathy, with particular resemblance to KSS [21]. Notably, no morphological correlates of striatal axonal or neuronal degeneration were apparent in FL-PGC-1α -/- animals [19] even at 75 weeks of age [21], which corresponds with earlier findings on young (postnatal day 10) FL-PGC-1α -/- animals via immunohistochemistry of striatal neurofilament [28] and has recently been confirmed by Lucas et al. demonstrating no significant loss of medium-sized spiny neurons via molecular biological methods in complete PGC-1α -/- mice [20]. Of note, the same group reported their observation of a relatively stable phenotype in complete PGC-1α -/- mice, with no robust progression between 4 and 12 weeks of age [20].

All these above detailed contradictory findings on PGC-1α-deficient mice and the recent report of a relatively stable phenotype in young PGC-1α -/- animals prompted us to perform a comprehensive profiling of motor phenotype of FL-PGC-1α -/- mice bred in our institute through a wide spectrum of age, with a special focus on the issue of phenotypic progression. The manuscript also addresses the expression profile of novel CNS-specific isoforms of PGC-1α in FL-PGC-1α -/- mice (containing exon B4) [29], a feature previously not characterized in

this strain. As a supplement of prior published neuropathological findings on adult [19] and aged [21] FL-PGC-1α -/- brains, additional neuropathological work-up has been performed to better understand the potential background of the observed phenotypic alterations, including histopathological analysis of the retina, spinal cord and skeletal muscle.

2. Materials and Methods

2.1. Behavioral analysis

2.1.1. Animals

The study enrolled male FL-PGC-1α -/- (n = 20-24) and C57Bl/6J wild-type (n = 22–26) [15]. The animals were housed in cages (maximum 4 per cage) in standard conditions with 12-12 h light-dark cycle and *ad libitum* access to standard pellet food and water. The experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local Animal Care Committee. The study animals were randomized from a population ranging through a wide spectrum of age (10–90 weeks, overall means: 50.7 w (±2.6 w) FL-PGC-1α -/- vs. 50.5 w (±2.5 w) wild-type) to yield age-matched representative wide-age-range populations for the different methods applied. On assessing phenotypic progression, subgroups of the young and elderly halves of the above cohorts have been created (overall means: 30.1 w (±2.4 w) and 67.4 w (±2.5 w) FL-PGC-1α -/- vs 30.6 w (±2.5 w) and 66.2 w (±2.2 w) wild-type for the young and elderly subgroups, respectively). The animals were placed in a transport room localized in the immediate vicinity of the examination room 12 hours prior to the behavioral examinations for adaptation to the examination conditions. The animals were examined during the same time of the day to minimize the influence of diurnal rhythm. The examination setups were cleaned with a solution containing 30% ethanol and were let dry completely after each session to avoid bias from olfactory cues. Standard light and temperature conditions were provided throughout the study.

2.1.2. Inverted screen test

For the assessment of the skeletal muscle strength, we performed an inverted screen test along the principles described in [30], with slight modifications. Briefly, the examined animal was placed onto the top of a grid, which was then gently rotated by 180° (inverted) and fixed in a position 40 cm above a soft bedding of wooden chips abundantly applied (10 cm). The measurement was started immediately when the grid reached the inverted position and the latency to fall from the grid was recorded. The performance was measured in three consecutive sessions with 1-1 min relaxation time. The best performance was regarded as a correlate of muscle strength to rule out the influence of accidental falls due to potentially affected coordination.

2.1.3. Open-field test

For the assessment of spontaneous locomotor activity, a black box setup with an open field of 48*48 cm and a side wall height of 36 cm was used, with the measurements performed along the principles previously described in [31]. Briefly, the examined animal was placed onto the middle of the field and let freely and spontaneously explore for 30 min. The movement pattern was recorded by built-in infrared led beams associated with the Conducta 1.0 software (Conducta 1.0 System, Experimetria Ltd., Hungary). The total distance ambulated (cm) and the total time spent with rearing (s) were parameters used for the analyses. The measurements were performed under indirect dim light (2.5 lux).

2.1.4. Rotarod test

For the assessment of motor coordination, a computer-assisted rotating rod with a diameter of 3 cm and with an anti-slip ribbed surface located 25 cm above the bottom of the setup was used, with the measurements performed along the principles previously described in [32]. Briefly, the animals were trained on the rod on the two preceding days to learn the movement pattern

skill necessary to stay on the top of the rotating rod for longer periods. On the first training day a rotation speed of 5 rpm, whereas on the second day a speed of 10 rpm was applied for 5 min for three consecutive sessions with 30 min relaxation intervals. On the recording day, three 5 min sessions with a gradually accelerating rod from 1 rpm to 30 rpm were used in a similar manner. The latency to fall from the rod was recorded by built-in infrared led beams associated with the TSE RotaRod Advanced software (TSE Systems, Bad Homburg, Germany). The mean performance was used for the analyses.

2.1.5. Gait analysis

For the assessment of gait pattern, a setup with a 6.5-cm-wide catwalk with a 40-cm-long recording area transparent from below, equipped with a video recording system using the Hypercam 1.6 software was applied (Hyperionics Technology, Murrysville, PA, USA). The measurements were performed along the principles described in [27], with modifications including the applied setup, the observer-controlled evaluation and the introduction of a novel standardized outcome parameter. The length of the recording area and the distance of the camera were adjusted and optimized to minimize spherical aberration. The catwalk was opaque from above and the sides to exclude visual bias. The examined animal was placed onto the catwalk and let freely and spontaneously explore the catwalk while recording. No visual or olfactory cues or any specific motivation were applied. The recording was stopped when the examiner managed to record 6 successful runs on-line. A run was regarded successful if the mouse moved through the recording area with an even speed, did not stop or turn before reaching the opposite end of the corridor, and did not make zigzag movements. The acquisitions were followed by an off-line re-evaluation of the runs, and eventually 4-6 successful runs per animal with an average of 14 strides could be used for further analysis. The video records were then subjected to a conversion to graphical images with colored dots indicating an anatomically standard region of each paw print and corresponding colored lines indicating the axis of

movement for each limb. The stride length and the base of support values were measured by ImageJ software (separately for the forelimbs and the hindlimbs, in both cases using the average of both sides). To rule out the potential bias arising from even subtle differences in size between the animals, a standardized measure (gait index) was introduced and defined as the ratio of the stride length and base of support. The average gait indices for both the forelimbs and the hindlimbs were used for the analyses.

2.2. RT-PCR analysis

Two FL-PGC-1α -/- and two wild-type 45-week-old mice were sacrificed and decapitated, and the striata, the overlying cortices and the cerebellum were dissected immediately on ice and placed to –80°C until use. Total RNA was isolated from the specimens using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. 1 μg of total RNA was reverse-transcribed using random hexamer primers. Real-time polymerase chain reaction (RT-PCR) was performed on CFX 96 Real Time System (Bio-Rad, USA) to detect changes in mRNA expression, using various primer pairs at a final volume of 20 μl. Thermal cycling conditions for long products were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s, and 72°C for 1 min. Target gene expression was normalized to the endogenous control gene 18S. The relative mRNA level was calculated by the $2^{-\Delta\Delta Ct}$ method. The relative expressions are presented in relation to the expression level of FL-PGC-1α mRNA transcribed from the reference promoter in the striatum of wild-type animals to allow comparison. The following primers were used: reference forward: 5'-TGAGTCTGTATGGAGTGACATCGAGTG-3'; CNS-specific forward: 5'-AATTGGAGCCCCATGGATGAAGG-3'; FL reverse: 5'- TTGGGGTCATTTGGTGAC-3'; NT-268 reverse (for wild-type mice): 5'-GGTCACTGGAAGATATGGC-3'; NT-254 reverse (for FL-PGC-1α -/- mice): 5'-TATCTTCTTGAGCATGTTGCG-3'.

2.3. Histopathological work-up

The brains of two newborn mice were removed on ice and halved at the midline immediately after decapitation. Spinal cord segments from cervical, thoracic and lumbar regions of 8 wild-type and 7 FL-PGC-1α -/- male mice aged 70-75 weeks were prepared on ice immediately after dissection. Eyes of 8 and 8 wild-type and 8 and 7 FL-PGC-1α -/- male mice aging 30 and 70-75 weeks, respectively, were dissected immediately after decapitation. Quadriceps muscles were dissected from 4 wild-type and 3 FL-PGC-1α -/- male mice aging 50 weeks. Half brains, spinal cord segments and the eyes were fixed in 4% paraformaldehyde overnight and kept in 15% glycerol in 4°C until embedding in paraffin. 3-μm-thick sections were stained with hematoxylin and eosin (HE) and Klüver-Barrera (KLB; Luxol and Fast red) stainings, and the anti-glial fibrillary acidic protein (GFAP; 1:3000, Dako, Glostrup, Denmark) polyclonal antibody was used for the visualization of astroglial reaction in the newborn brains. The DAKO EnVision detection kit, peroxidase/DAB, rabbit/mouse (Dako) was used for visualization of antibody reactions. The quadriceps specimens were snap-freezed in isopentane cooled with liquid nitrogen immediately after dissection, and stored in –80°C until embedding. In addition to HE staining, we performed enzyme histochemistry for cytochrome *c* oxidase (COX).

2.4. Statistical analyses

The analyses of the obtained parameters were performed by the use of SPSS 22.0 program. The distribution of each dataset was assessed by normality analysis by the use of Shapiro-Wilk test. The comparison of continuous variables with normal distribution was performed by the parametric Student's unpaired t-test using also the Levene's test for the assessment of the equality of variances, whereas variables showing non-normal distribution were compared by the Mann-Whitney U test. In case of correlation analyses, variables with normal distribution were subjected to Pearson's correlation, whereas Spearman's rank correlation was performed in case of non-normal distribution. To control the analyses for potential confounding effect of age and to estimate the effect sizes of variables with significant between-group differences,

binary logistic regression analyses were performed. Multiple comparisons of pre-defined subgroups of age were performed via the parametric one-way analysis of variance (ANOVA) or the non-parametric one-way Kruskal-Wallis test based on the normality of data, followed by corresponding pairwise *post hoc* analyses with p values corrected for multiple comparisons by the method of Bonferroni. Data are presented as mean (± standard error of mean) or median [intequartile range] for variables with normal and non-normal distribution, respectively. A p value < 0.05 was regarded as significant.

3. Results

3.1. Behavioral analysis

FL-PGC-1α -/- animals demonstrated remarkably poorer performance on the inverted screen compared to their wild-type counterparts with significantly shorter latency to fall from the grid ($p = 0.001$; 33.5 s [15.25 s – 84.25 s] vs 99.5 s [51.0 s – 231.75 s]; Fig. 1A). The worse performance of FL-PGC-1α -/- animals was not attributable to an increased body weight, contrastingly, FL-PGC-1α -/- animals showed a tendency towards decreased body weight compared to controls ($p = 0.076$; 27.70 g [26.17 g – 28.63 g] vs 28.31 g [27.12 g – 31.13 g]). Notably, while the between-group difference of body weights *per se* was not statistically significant due to the substantial overlap within the wide age range (21-34 g), the logistic regression analysis identified body weight as a significant predictor of genotype ($p = 0.030$, Wald = 4.712) with an Exp(B) = 1.141 (95% confidence interval (CI) = 1.034–1.934) when the analysis was controlled for age as a confounder, representing a 14.1% increase in the odds of being wild-type with each gram elevation of bodyweight. Proposing that body weight may influence the measurement of grip strength in a setup such as the inverted screen, the estimation of the effect size in this case was controlled for both age and body weight as potentially confounding covariates in a binary logistic regression model, which detected the latency to fall performance as a significant predictor of the genotype ($p = 0.010$, Wald = 6.644) with an Exp(B)

= 1.011 (95% CI = 1.003–1.020), calling for a 1.1% increase in the odds for an animal to be wild-type with every additional second spent on the grid. Unexpectedly, the decreased performance in the inverted screen did not show any clear association with age (Table 1); likewise, no significant difference could be observed between the young and elderly subgroups in either group ($p > 0.05$ for all *post hoc* analyses).

In addition to signs of decreased strength, FL-PGC-1α -/- animals showed significantly reduced ambulatory behavior manifesting in modestly decreased ambulation distance ($p < 0.001$; 6341.0 cm (±472.9 cm) vs 9026.7 cm (±509.5 cm); Fig. 1B) and markedly reduced time spent with rearing ($p < 0.0001$; 116.8 s [46.7 s – 150.0 s] vs 210.4 s [160.0 s – 237.3 s]; Fig. 1C). The binary logistic regression analyses detected both of these parameters as significant predictors of genotype, with every additional meter distance ambulated increasing the odds of being wild-type with 5.1% ($p = 0.002$, Wald = 9.429, Exp(B) = 1.051 (95% CI = 1.018–1.084)) and every additional second spent with rearing increasing the odds of being wild-type with 3.5% ($p < 0.001$, Wald = 11.919, Exp(B) = 1.035 (95% CI = 1.015–1.055)) during the 30 min recording period, when controlling for age. These parameters, however, also did not demonstrate any progression with age (Table 1) and no significant differences could be observed between young and elderly subgroups in either group ($p > 0.05$ for all *post hoc* analyses).

On assessment of motor coordination, the average performance of FL-PGC-1α -/- mice in the accelerating rotarod was remarkably lower than that of the wild-type mice ($p < 0.0001$; 27.3 s [10.9 s – 74.8 s] vs 137.3 s [95.1 s – 168.8 s]; Fig. 1D). Age-controlled logistic regression analysis revealed the average latency to fall from the rod as a significant predictor of genotype ($p < 0.001$, Wald = 10.982) with an Exp(B) = 1.030 (95% CI = 1.012–1.048), which calls for a 3.0% increase in the odds for an animal to be wild-type with every additional second spent on the rod. Though this feature did show significant negative association with age among wild-type animals, it did not show any progression with age among FL-PGC-1α -/- mice (Table 1),

and likewise, significant difference could only be observed between young and elderly wild-type ($p = 0.041$) but not FL-PGC-1α -/- animals ($p > 0.05$).

The gait analysis of the animals revealed significantly decreased gait indices corresponding to widened bases of support in FL-PGC-1α -/- mice, a feature also consistent with ataxia. The differences were comparable as regards the forelimbs ($p = 0.003$; 3.44 (±0.14) vs 4.08 (±0.14)) and in the hindlimbs ($p = 0.018$; 1.76 (±0.09) vs 2.05 (±0.08); Fig. 1E-F), with age-controlled logistic regression analyses revealing 16.0% ($p = 0.008$, Wald = 7.057, Exp(B) = 1.160 (95% CI = 1.040–1.294)) and 21.7% ($p = 0.024$, Wald = 5.079, Exp(B) = 1.217 (95% CI = 1.026–1.444)) increases in the odds of being wild-type with every 0.1 arbitrary unit elevation in forelimb and hindlimb gait indices, respectively. Neither the hindlimb nor the forelimb gait index showed any association with age (Table 1), and the subgroup analyses between young and elderly animals did not show difference either ($p > 0.05$ for all *post hoc* analyses).

3. 2. Histopathological work-up

Histopathological analysis of different spinal cord segments revealed no apparent involvement of the spinal white matter tracts in the vacuolar change previously found to be prominent in certain CNS regions of FL-PGC-1α -/- mice (Fig. 2A-B). Likewise, no vacuolar alterations were observed in the brains of newborn FL-PGC-1α -/- mice (Fig. 2C-D), and no signs of reactive astrogliosis were apparent in the newborn brainstem (data not shown), a feature characteristic of adult FL-PGC-1α -/- animals. Histology of the eyes demonstrated no signs of retinal degeneration in FL-PGC-1α -/- mice at any age; in particular, the outer nuclear layer showed no signs of neuronal degeneration (*i.e.*, that of retinitis pigmentosa), a feature characteristic of certain mitochondrial cytopathies, particularly the KSS. On histological and enzymehistochemical analysis of the quadriceps muscles, both the wild-type and the FL-PGC-1α -/-mice showed fibers with enhanced subsarcolemmal accumulation of mitochondria (data not shown); however, we did not observe complete COX-deficient fibers in the COX staining

(Fig. 2E-F; inlet in lower right quadrant). In the FL-PGC-1α -/- mice, we observed mild caliber changes together with enhanced internalization of nuclei. There was a lack of highly atrophic fibers or grouping of atrophic fibers (Fig. 2E). In sum, we interpreted these findings as mild myopathic changes in FL-PGC-1α -/- mice.

3. 3. RT-PCR analysis

FL-PGC-1α -/- animals demonstrated diminished expression of FL-PGC-1α isoforms transcribed from either the reference promoter or from the CNS-specific promoter (Fig. 3), demonstrating that the nomenclature of FL-PGC-1α -/- in this strain is appropriate, even in light of the recently discovered CNS-specific isoforms of PGC-1α [29]. The expression levels of CNS-specific isoforms were remarkably higher in all examined brain regions as compared with that of the isoforms transcribed from the reference promoter; this was true for both the FL and the NT isoforms in the wild-type and the NT-254 isoform in FL-PGC-1α -/- animals. The highest levels of expression were consistently detected in the cerebellum for both the FL- and the NT isoforms, independently of the promoter of transcription. The expression of the NT-254 isoform was comparable in FL-PGC-1α -/- mice to that detected in wild-type animals in the striatum and the cortex; however, it was remarkably upregulated in the cerebellum of FL-PGC-1α -/- mice compared to wild-type counterparts (Fig. 3).

4. Discussion

Recent neuropathological analyses on FL-PGC-1α-deficient mice revealed a pathology highly reminiscent of KSS [19] even at the ultrastructural level [21], suggesting that PGC-1α-deficient murine strains might serve as models for mitochondrial diseases. However, based predominantly on vacuolation and astrogliosis reported in the striatum, former studies on the complete PGC-1α as well as the FL-PGC-1α whole-body knockout strains had proposed that PGC-1α-deficient mice could be regarded as models of HD [14, 15]. This well corresponded

with the findings of impaired striatal expression of PGC-1α and many of its target genes in the striatum of HD patients, transgenic animals and in cell cultures (reviewed in [11]). Furthermore, this concept was claimed to be supported phenotypically by the finding of hyperactivity – without apparent weakness – in the complete PGC-1α -/- strain, which was considered to be typical of HD [14]. Since then, the vast majority of scientific reports dealing with PGC-1α associates its deficiency with striatal neurodegeneration and HD features. However, a number of contradictions accompany these observations, and increasing amount of studies tend to question this concept. First, it should be noted that repeated and comprehensive neuropathological analyses of FL-PGC-1α-deficient mice demonstrated no direct or indirect signs of striatal neurodegeneration in the striatum (including also the complete lack of astroglial reaction even at the oldest age group analyzed in this study) [19, 21], which findings were supported by an independent study on the complete PGC-1α-deficient strain using molecular biological techniques [20], and by another group by the use of neurofilament immunohistochemistry [28]. Based on our recent ultrastructural and immunohistochemical analysis, vacuolation in the striatum (and also in several other regions) indeed appears to be of myelinic (most probably oligodendroglial) origin, alterations which are characteristic of mitochondrial and other metabolic diseases, and which cannot be regarded as signs of neurodegeneration [21]. Secondly, the translatability of hyperactivity (*i.e.*, increased locomotion) observed in the complete PGC-1α -/- strain to hyperkinetic movement disorders (*i.e.*, chorea and ballism) characteristic of human HD is questionable. Indeed, though early studies with isolated local intrastriatal lesions by excitotoxins such as quinolinic or kainic acid lead to either spontaneous or amphetamine-induced increased locomotor behavior [33, 34], systemic toxin-induced or transgenic murine models of HD generally present with a phenotype characterized by markedly decreased rather than increased locomotor performance [31, 32, 35, 36]. Contradictory enough, the other pioneering study examining the FL-PGC-1α-deficient

strain reported hypomotility and weakness as opposed to hyperactivity observed in the complete knockout [14, 15], a difference that is hard to explain merely by the different genetic engineering background. Notably, a muscle-specific variant of the proposedly hyperactive complete PGC-1α -/- strain exhibited decreased locomotion and muscle strength, similarly to the FL-PGC-1α -/- strain [37]. Furthermore, a more recent studies on the complete whole-body as well as the brain-specific PGC-1α -/- strains did not reveal signs of increased locomotion [20, 26], but observed decreased exploratory behavior by means of a diminished ability to rear [20], further increasing the number of contradictions in PGC-1α-related literature. It is also of note, that though there was a study examining signs phenotypic progression at an early age of development [20], no studies have been reported dealing with phenotypic progression on longer scales.

All these above detailed contradictions and the paucity of long-term phenotypic progression studies prompted us to examine the FL-PGC-1α -/- population bred in our institute with a battery of methods profiling motor phenotype, with special attention to the issue of phenotypic progression via enrolling age-matched male mice from a wide range of age. The motor phenotype was profiled on the axes of muscle strength, spontaneous locomotion and ataxia/motor coordination. The inverted screen test demonstrated consistently and markedly shorter latency to fall for FL-PGC-1α -/- mice compared to wild-type counterparts, a feature indicative of decreased muscle strength. The shorter latency to fall was not related to increased body weight as it could have been proposed; quite contrary, the FL-PGC-1α -/- mice had a consistent tendency of decreased body mass, which covariate was indeed a significant predictor of the genotype when controlling for the age of the animals. This observation corresponds with studies describing tendencies of decreased body weight in complete PGC-1α -/- mice [14, 27, 38], but contradicts with a prior study on FL-PGC-1α -/- mice reporting decreased body weight only in the first weeks of life, and increased body weight values at later ages [15]. Based on the

findings that muscle-specific PGC-1α -/- mice exhibit decreased body weight [25] and that brain-specific PGC-1α -/- animals exhibit similar body weight to their controls [26], this lean phenotype could as well be attributable to myopathy (as demonstrated by prior biochemical and gene expression analyses [14, 15, 25, 38]) and subsequent muscle atrophy corresponding to a wasting condition due to chronic mitochondrial disease. However, this hypothesis could not be fully confirmed by current histopathological analyses of quadriceps muscles, demonstrating only mild myopathic changes in the PGC-1α-deficient animals without overt signs of mitochondrial muscle disease and/or neurogenic atrophy, corresponding with prior reports on younger animals [14, 15, 25, 38]. Although we observed a certain amount of subsarcolemmal mitochondria in both the wild-type and FL-PGC-1α -/- mice, there were no COX-negative fibers; therefore, we could not define these as unequivocal histopathological evidence for a mitochondrial myopathy. These altogether also suggest that the weak phenotype observed in FL-PGC-1α -/- mice may either be predominantly attributed to the CNS alterations or at most influenced by a functional muscle disorder with only mild pathological correlates. Along these lines, the robustly decreased total time of rearing, as observed in the open-field paradigm, may also be suggestive of a weakening of the axial muscles in FL-PGC-1α -/- mice; however, it may also be accounted for by trunk ataxia due to the apparent cerebellar involvement [19, 21, 27]. The predominant involvement of the central lesions in the decreased ability to rear is supported by similar findings on brain-specific complete PGC-1α -/- mice [20]. The remarkably decreased performance on the rotarod, a test traditionally applied to measure motor coordination (*i.e.*, ataxia), can be explained in particular by neuronal degeneration reported in the brainstem and the cerebellum, as well as the robust myelin vacuolation in the basal ganglia [19, 27]. A similar decrease in rotarod performance has also been described in the CNS-specific conditional knockout variant of the complete PGC-1α -/- strain [20], which also calls for a dominant role of CNS alterations underlying the observed movement disorder. An impaired coordination in

FL-PGC-1α -/- mice was also represented by significantly decreased gait indices observed in our gait analysis, manifesting in widened bases of support accompanied by relatively preserved stride lengths in both the fore- and the hindlimbs, a feature consistent with ataxia. In accordance with these, a recent study on the complete PGC-1α -/- strain assessing the gait pattern of 24-week-old mice in an automated catwalk system revealed multiple alterations in gait pattern also characteristic of ataxia, which phenotype was linked to the degeneration and loss of cerebellar Purkinje cells [40]. All these correspond well to the concept proposing PGC-1α-deficient strains as models for mitochondrial encephalopathy, especially since the neuropathological alterations described by our group on FL-PGC-1α -/- mice were remarkably reminiscent of that seen in KSS [19, 21], a mitochondrial disease where the overt loss of Purkinje neurons is virtually pathognomonic [7, 21, 39, 40]. The significance of this

The observation that the mRNA expression levels of both the FL and the NT isoforms of PGC-1α either transcribed from the reference or the CNS-specific promoter are the highest in the cerebellum in wild-type C57Bl6/J mice, an observation recapitulating and extending prior findings in rats [41], together with the findings of multiple degenerative alterations reported in this region in PGC-1α-deficient mice [19, 21, 27], place the cerebellum in the potential center of relevance in the phenotype associated with PGC-1α deficiency. This concept gains further support by our observation of a remarkable upregulation in the expression of the residual NT-254 fragment in the cerebellum but not in the other examined CNS regions of FL-PGC-1α -/- mice, suggesting the existence of a functional feedback mechanism confined to this brain region, which may result in a compensatory elevation of PGC-1α in case of compromised mitochondrial function. A similar cerebellum-predominant upregulation of PGC-1α isoforms has also been observed in our prior report in transgenic HD mice [18]. The higher levels of expression of the CNS-specific isoforms in the wild-type mice are consistent with that observed previously in human brains [29].

The observed clinical phenotype of FL-PGC-1α -/- mice altogether with impaired muscle strength, decreased locomotion, disturbed coordination and ataxic gait, especially in light of the decreased body mass, is consistent with a phenotype expected in mice with a chronic systemic mitochondrial disease. Supplementing prior neuropathological analyses that revealed widespread white-matter vacuolation and signs of neuronal degeneration in the brainstem and the cerebellum of PGC-1α-deficient mice [19, 21, 27], our current histopathological analysis revealed that the observed phenotypic alterations were not associated with signs of retinal involvement, a characteristic feature of KSS. This is consistent with our findings of preserved spatial memory by the use of visual cues in FL-PGC-1α -/- mice (unpublished observation), suggesting that the observed phenotypic changes may not be influenced by visual deficits. Furthermore, we also did not observe apparent myelinopathy or neurodegeneration in the spinal cord of FL-PGC-1α -/- mice, excluding morphological evidence of a potential involvement of the spinal cord in the observed phenotype, with particular regards to ataxia. The identification of a potential model of mitochondrial encephalopathies is of significance because the majority of genetically engineered animals targeted at nuclear genes involved in disease-causing mutations resulted in early postnatal or embryonic lethality, whereas most viable strains have no neuropathology, rendering the modeling of this group of diseases rather difficult (reviewed in detail in [19]). Importantly, some of these disease-causing genes with reported knockouts are under the regulation of PGC-1α, including POLG-1 [42], ANT-1 [43], and MFN1 [44] and 2 [45]. Of note, knockouts of several other genes not yet identified as subjects of disease-causing mutations are also considered as animal models of mitochondrial diseases, which include the PGC-1α-regulated genes Tfam and SOD2 [46]. Fascinatingly, conditional knockout of Tfam has been dedicated as a model of KSS cardiomyopathy [47]; whereas SOD2-deficiency has been reported to resemble Leigh's encephalopathy [48]. Notably, PGC-1α-deficient animals nicely recapitulate key features of Tfam-deficient hearts [38] as well as SOD2-deficient brains

[19], in line with the decreased expression of Tfam [38] and SOD2 [10] in these animals. Based on all these, we believe that PGC-1α-deficient mice with a reasonable pathogenic factor and a highly reminiscent phenotype can be a useful animal model for mitochondrial disease, with particular regards to KSS. Though FL-PGC-1α-deficient mice have been not associated with large-scale mtDNA deletions typical of KSS [23], their phenotype is associated with perturbed mtDNA replication (with a decreased mtDNA amount) [38] as well as a globally increased oxidative stress [10], which together may be speculated to be predominantly accounted for the observed CNS phenotype.

In addition to the supplementation and in part confirmation of findings of prior studies on FL-PGC-1α -/- mice, our results demonstrate previously unrevealed commonalities with features of the complete PGC-1α -/- strain, such as impaired motor coordination/ataxia and decreased body weight, suggesting that the two whole-body knockout strains might not differ from each other as much as it was originally proposed in terms of the general locomotor phenotype. As a second main novelty, however, our results demonstrate that the observed phenotypic alterations, which are present even in the earliest ages examined, unexpectedly did not progress further with time. Though a similar observation has already been reported with complete PGC-1α -/- mice (both whole-body and brain-specific conditional knockouts), the oldest animals in that study were 12 weeks old [20], which is consistent with the age of the youngest animals examined in our study cohort. To our knowledge, the examination of PGC-1α-deficient animals up to 90 weeks of age is unprecedented. The explanation for this non-progressive nature of phenotype after a certain age is currently unrevealed, and is presumably attributable to the activation of salvage pathways that can compensate for the loss of function of PGC-1α protein. It can also be proposed that PGC-1α itself is only essential for early postnatal development, and not for embryonal or later postnatal (*i.e.*, adult) life. This is consistent with a thermoregulatory deficit observed only in PGC-1α-deficient younglings but

not in adult mice [15], the elevated mortality of puppies ([14], corresponding to our unpublished observation with this strain) accompanied by a normal number of newborns in the litter [14, 15] and an apparently unaffected life expectancy thereafter, and is also in accordance with the lack of neuropathological alterations in newborn brains confirmed by our current study. These all nicely correspond with the findings of a comprehensive study demonstrating largely diminished PGC-1α expression in the brain of adult rats at the protein level, with the highest expression reached during late embryonal and early postnatal development [41]. Of note, this early postnatal period also corresponds with the peak of mammalian myelinogenesis, which is consistent with an impaired myelin production in FL-PGC-1α -/- mice as early as postnatal day 10 [28], and may also explain the lack of myelin vacuolation in the newborn mice. It would be fascinating to see whether this long-term non-progressive nature of motor phenotype is present in complete PGC-1α -/- animals (either whole-body or tissue-specific knockouts), which could exclude the potential compensatory role of downstream factors coactivated by the NT isoforms readily expressed by FL-PGC-1α -/- animals examined in our study. The revelation of such compensatory mechanisms that are able to withhold a robust early onset pathology as seen in PGC-1α-deficient mice might be of therapeutic relevance in disorders where mitochondrial function is compromised, especially in those where the involvement of PGC-1α dysfunction has already been established.

5. Conclusion

We conclude that FL-PGC1α -/- animals and the applied behavioral assessment techniques can be suitable for pharmacobehavioral studies with a therapeutic aim in mitochondrial encephalopathies, as they exhibit several clinical and neuropathological phenotypic hallmarks of such diseases. This is of importance because of the paucity of viable animal models that exhibit both morphological and behavioral symptoms of a mitochondrial disease. An important limitation revealed, however, in our study is that the potential treatments should be initiated in

the earliest age possible, as motor alterations that have already been developed do not progress any further. The mechanisms underlying this compensation are to be revealed, as they may provide information of therapeutic relevance in this currently intractable group of neurological diseases.

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Fig. 1.

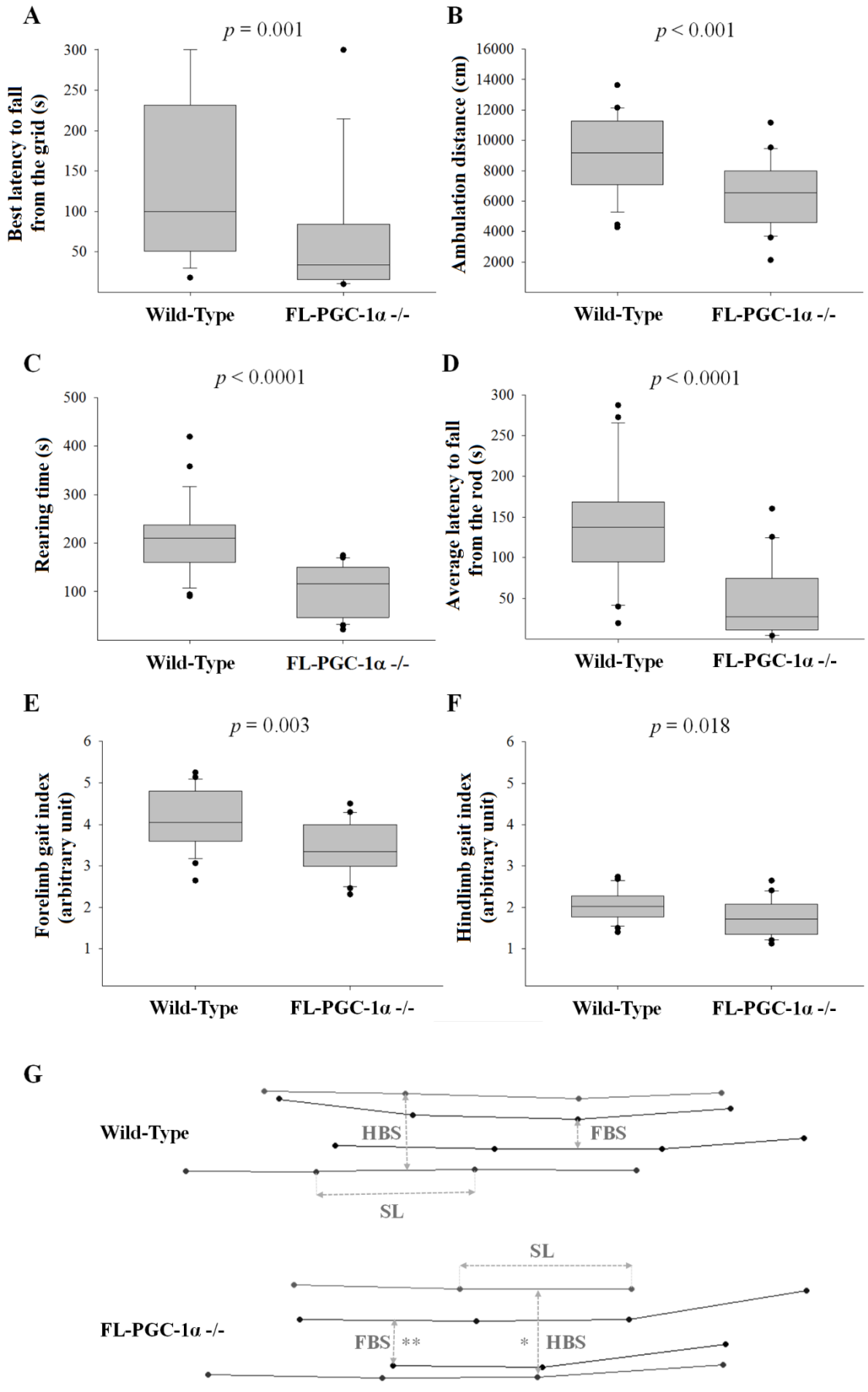
A
p = 0.001
Best latency to fall from the grid (s)
300
250
200
150
100
50
Wild-Type
FL-PGC-1α -/-
B
p < 0.001
Ambulation distance (cm)
16000
14000
12000
10000
8000
6000
4000
2000
Wild-Type
FL-PGC-1α -/-
C
p < 0.0001
Rearing time (s)
500
400
300
200
100
Wild-Type
FL-PGC-1α -/-
D
p < 0.0001
Average latency to fall from the rod (s)
300
250
200
150
100
50
Wild-Type
FL-PGC-1α -/-
E
p = 0.003
Forelimb gait index (arbitrary unit)
6
5
4
3
2
1
Wild-Type
FL-PGC-1α -/-
F
p = 0.018
Hindlimb gait index (arbitrary unit)
6
5
4
3
2
1
Wild-Type
FL-PGC-1α -/-
G
Wild-Type
HBS
FBS
SL
SL
FL-PGC-1α -/-
FBS
**
*
HBS

Fig. 2.

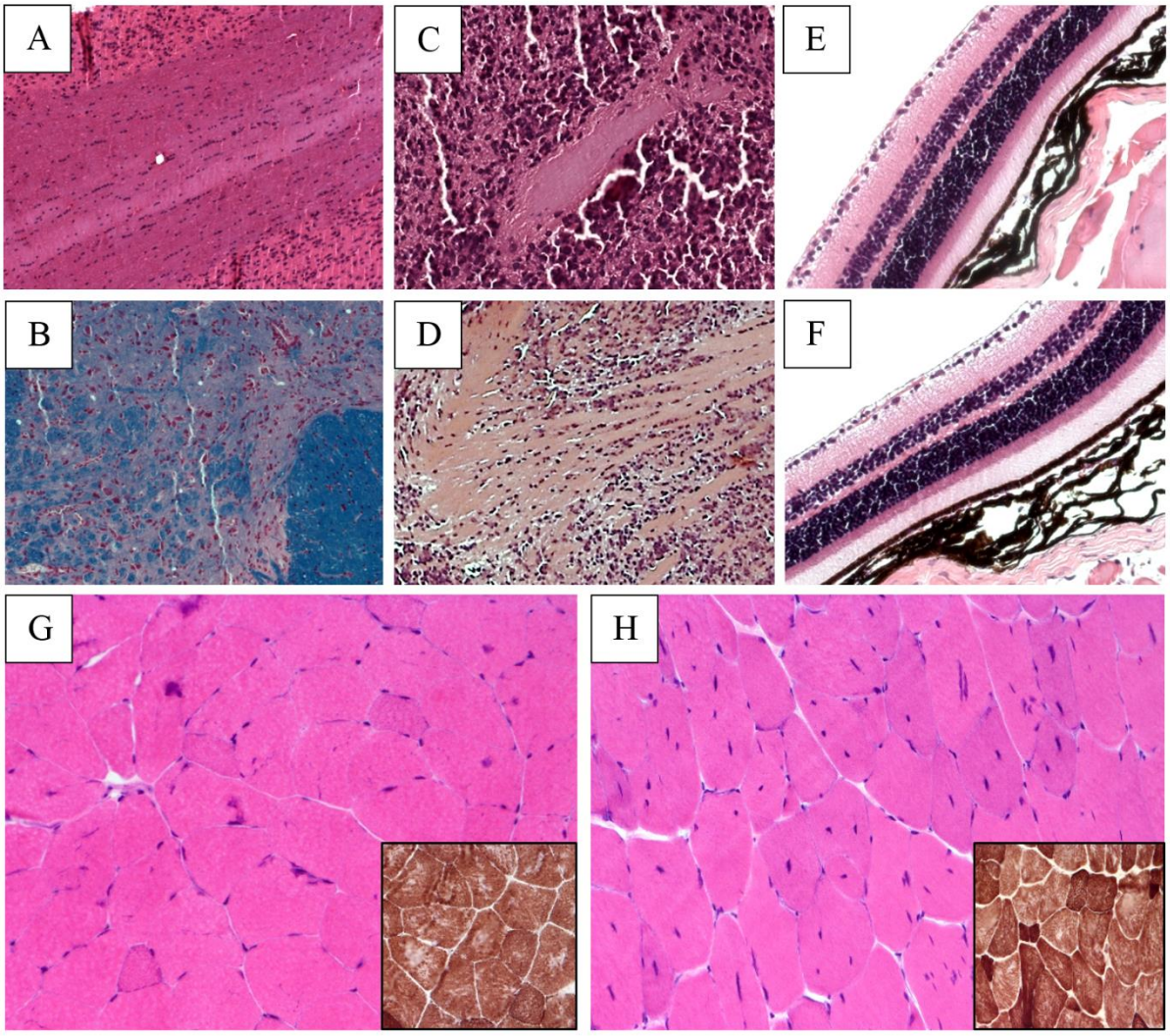

Fig. 3.

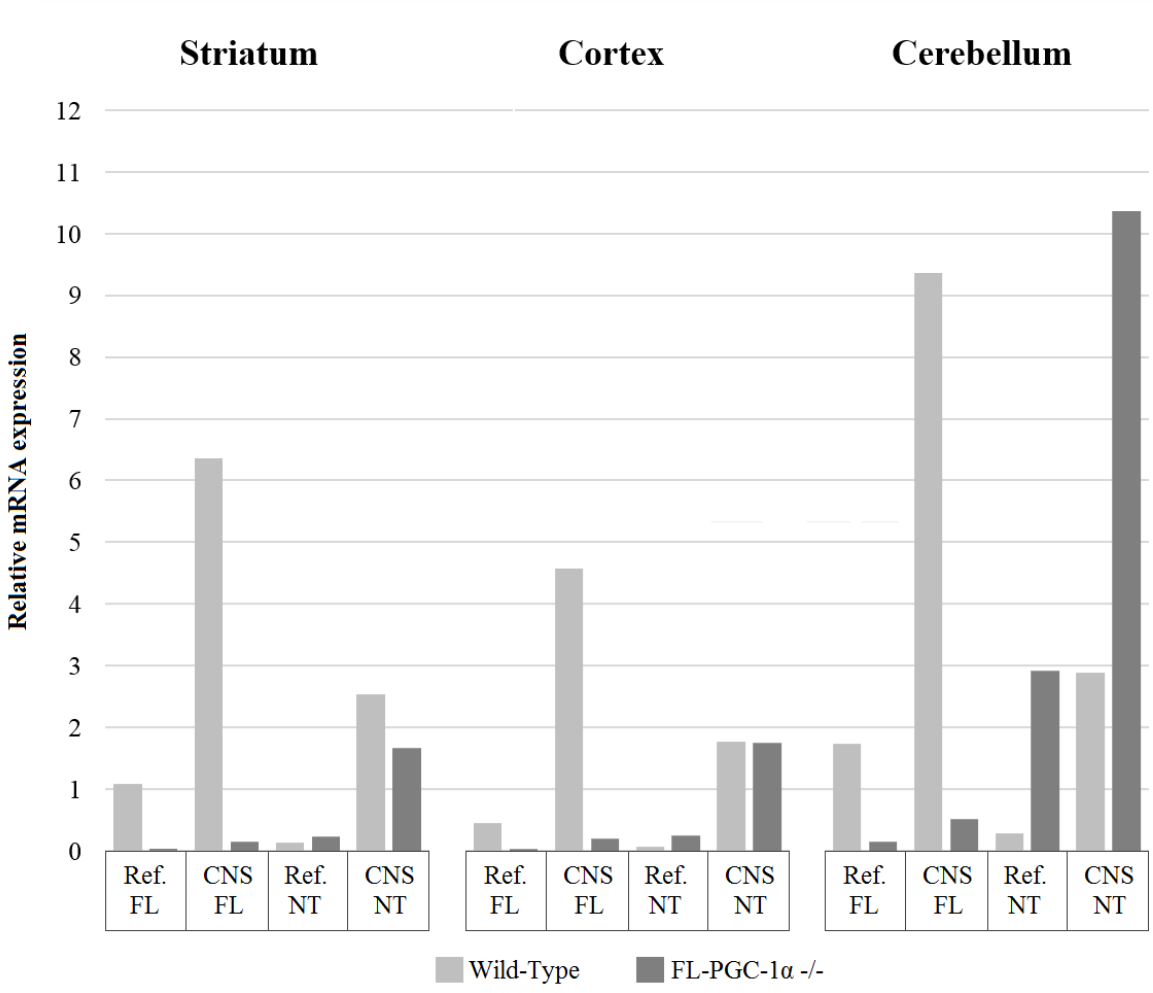
Striatum
Cortex
Cerebellum
Relative mRNA expression
12
11
10
9
8
7
6
5
4
3
2
1
0
Ref. FL
CNS FL
Ref. NT
CNS NT
Wild-Type
FL-PGC-1α -/-

Table 1.

A	Latency to fall from the grid in light of age	
	WT	**FL-PGC-1α -/-**
Rho	-0.093	0.365
p	**0.652**	**0.079**
n	26	24

B	Ambulation distance in light of age	
	WT	**FL-PGC-1α -/-**
Rho	0.111	0.388
p	**0.606**	**0.074**
n	24	22

C	Rearing time in light of age	
	WT	**FL-PGC-1α -/-**
Rho	0.264	0.328
p	**0.213**	**0.136**
n	24	22

D	Latency to fall from the rod in light of age	
	WT	**FL-PGC-1α -/-**
Rho	-0.560**	-0.126
p	**0.007**	**0.598**
n	22	20

E	Forelimb gait index in light of age	
	WT	**FL-PGC-1α -/-**
Rho	-0.089	- 0.012
p	**0.686**	**0.958**
n	23	21

F	Hindlimb gait index in light of age	
	WT	**FL-PGC-1α -/-**
Rho	-0.022	0.263
p	**0.921**	**0.249**
n	23	21

Figure and table captions

Fig. 1. Phenotypic profile.

FL-PGC-1α -/- mice exhibit remarkable reduction in muscle strength (A) with asthenia, decreased spontaneous locomotion (B) and rearing (C), which were accompanied by impaired motor coordination (D) with decreased gait indices (E and F) consistent with widened bases of support as visualized by the representative gait traces (G), indicative of ataxia. Abbreviations: WT, wild-type; SL, stride length; FBS, forelimb base of support; HBS, hindlimb base of support.

Fig. 2. Histopathological work-up.

Neuropathology of FL-PGC-1α -/- mice does not associate with vacuolar change in the white matter of the spinal cord (A, longitudinal section, HE; B, cross-section, KLB) as compared with prominent myelin vacuolation demonstrated previously in the brain. Newborn FL-PGC-1α -/- mice are free of myelin vacuolation in areas robustly affected in adult and aged FL-PGC-1α -/- mice (C, retroflex fascicle, HE; D, internal capsule, HE). No signs of retinal degeneration are apparent in FL-PGC-1α -/- mice (F, HE) as compared with wild-type counterparts (E, HE). Compared to wild-type animals (G), the quadriceps muscle of FL-PGC-1α -/- mice exhibits mild myopathic changes with caliber discrepancy and enhanced internalization of nuclei (H, HE); however, without the presence of COX-negative (inlet in the lower right quadrant) or highly atrophic fibers, we could not interpret these as unequivocal evidence for a mitochondrial myopathy.

Fig. 3. PGC-1α isoform analysis.

RT-PCR analysis demonstrates diminished FL-PGC-1α mRNA expression both from the reference promoter and the novel CNS-specific promoter. The highest expression of PGC-1α isoforms in wild-type mice associates with the cerebellum. The expression levels of CNS-specific isoforms are higher in all brain regions as compared with those transcribed from the reference promoter. The NT-PGC-1α isoforms are dramatically upregulated in the cerebellum of FL-PGC-1α -/- mice, possibly representing a functional feedback mechanism confined to this region.

Table 1. Progression analyses.

Correlation analyses demonstrate no statistically significant negative association with age in any of the assessed behavioral parameters in FL-PGC-1α -/- mice (A-F). These data correspond with that obtained from multiple comparison analyses between young and elderly subgroups, indicating the lack of evident phenotypic progression in FL-PGC-1α -/- mice.