Iron homeostasis in the brain: complete iron regulatory protein 2 deficiency without symptomatic neurodegeneration in the mouse

Bruno Galy[1], Sabine M. Hölter[2], Thomas Klopstock[3,5], Dunja Ferring[1], Lore Becker[3,5], Sylvia Kaden[6], Wolfgang Wurst[2,4], Hermann-Josef Gröne[6], and Matthias W. Hentze[1].

[1]European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany.
GSF-National Research Center for Environment and Health, [2]Institute of Developmental Genetics and [3]Institute of Experimental Genetics, Ingolstädter Landstrasse 1, 85764 Neuherberg, Germany.
[4]Max-Planck-Institute of Psychiatry, Kraepelinstrasse 2-10, 80804 München, Germany.
[5]Department of Neurology, Ludwig-Maximilians-Universität, Marchioninistrasse 15, 81377 München, Germany.
[6]Abteilung Zelluläre und Molekulare Pathologie, Deutsches Krebsforschungszentrum Heidelberg, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.

Corresponding author: Tel: +496221387501, Fax: +496221387518, E-mail: hentze@embl.de

Key words: Iron metabolism, IRE, iron regulatory protein, neurodegenerative disease, conditional mouse mutants.

Abstract

Alterations in brain iron metabolism can cause neurodegeneration. Iron regulatory protein (IREB)-2 deficiency in the mouse was reported to cause cerebral iron deposits with ataxia, tremor and bradykinesia. We demonstrate that mice completely lacking IREB2 display only a mild neurological phenotype and no histological or ultrastructural defects, although they exhibit molecular abnormalities in the regulation of IRE-containing target genes. Our data demonstrate that IREB2 deficiency *per se* does not cause major neurodegeneration.

Text

Abnormalities in brain iron metabolism cause or contribute to severe human disorders, including retarded development[1], restless legs syndrome[2] and different forms of hereditary and acquired neurodegeneration[3]. It is therefore critical to unravel the mechanisms controlling brain iron metabolism, which are presently not well understood. Strikingly, aged adult mice with a targeted deletion of the iron regulatory protein-2 (*Ireb2*) gene were reported to suffer from a neurodegenerative movement disorder characterized by ataxia, bradykinesia and tremor[4]. Histopathologically, these clinical signs were accompanied by iron deposits in white matter tracts and nuclei throughout the brain, suggesting that deficiency for a key regulator of cellular iron homeostasis[5], IREB2, causes neurodegeneration by altering brain iron metabolism. Molecularly, increased ferritin expression was noted in several areas of white and gray matter, preceding axonal degeneration with, importantly, a loss of Purkinje cells[4]. It was hypothesized that iron sequestration within overexpressed ferritin shells might lead to functional iron deficiency, or that ferritin could transport and release iron in distal axons and presynaptic terminals, resulting in oxidative damage[6].

To study kinetic parameters and/or cell type-autonomous functions of IREB2, we generated mouse lines for conditional ablation of *Ireb2*[7,8]. First, we derived a line with complete and constitutive IREB2 deficiency by Cre-mediated gene disruption in the germline[8]. The generation of a complete IREB2 null-phenotype has been thoroughly documented[8] and is also shown in Fig.1a. We analysed 13-14 month-old mice, i.e. at an age when the severe neurodegenerative phenotype reported by LaVaute et al[4] was fully

manifest. In agreement with their reports[4,9], our aged *Ireb2*$^{-/-}$ mice show increased ferritin and decreased transferrin receptor 1 (TfR1) expression in the brain, with a more pronounced decrease of TfR1 expression in the cerebellum (**Fig.1a**). Altered ferritin and TfR1 expression in the brain occurs without a detectable increase in total non-heme iron content (measured as described previously[10], whole brain: 197±42 mg/g of dry tissue for *Ireb2*$^{+/+}$, n=8, compared with 188±35 mg/g for *Ireb2*$^{-/-}$, n=8; cerebellum: 168±23 mg/g for *Ireb2*$^{+/+}$, n=8, compared with 139±30 mg/g for *Ireb2*$^{-/-}$, n=8). Moreover, serial sectioning and DAB-enhanced Perl's staining of the brain revealed no iron deposits (not shown). Thus, the changes in ferritin and TfR1 expression in *Ireb2*$^{-/-}$ brains can be explained by the primary lack of IREB2 rather than by a secondary response to increased cellular iron levels.

Clinically, *Ireb2*$^{-/-}$ mice were described to exhibit an unsteady, wide-based gait with a gradual development of ataxia, tremor, bradykinesia, and postural abnormalities[4]. At 1 year of age, the described IREB2-deficient mouse line displayed poor grooming, piloerection, tremor, eye encrustment with purulent exsudate, unsteady backward ambulation, and a flexed position of the hindlimbs with the abdomen resting on the floor[4]. Upon testing, these mice performed poorly in the hanging wire test[4]. Our IREB2-deficient mice do not suffer from any of these problems: they display no overt movement or postural abnormalities, nor eye purulence. Although the apparent lack of a neurological phenotype would not have called for detailed neurological and behavioural testing, the earlier reports motivated such analyses. 13-14 month-old mice were analysed using the modified hole board test, a paradigm that combines the characteristics of an open field and of a hole board test[11]. This test shows that IREB2 deficiency does not

impair horizontal locomotor activity (**Fig.1b,c**), as evidenced by the number of line crossings, total distance, mean and maximum velocity and meander parameters (**supplementary table1** online). In fact, *Ireb2*$^{-/-}$ mice even display a mild tendency for increased horizontal locomotion (**Fig.1b** and **supplementary table1** online). While iron regulatory protein-1 (ACO1) (rather than IREB2) deficiency has been noted in substantia nigra autopsy samples of RLS patients[12], it will be interesting to explore the increased horizontal locomotion of *Ireb2*$^{-/-}$ mice in the context of the motor hyperactivity observed in RLS patients.

Amongst many parameters tested, *Ireb2*$^{-/-}$ mice display a slight but significant decrease in self-grooming activity, which may indicate a trend towards reduced vertical locomotor activity (further evidenced by a tendency towards reduced rearing). The tendency for increased horizontal locomotion could thus also represent a shift of the behavioral repertoire towards horizontal exploration due to mildly impaired vertical exploratory abilities. As rearing and self grooming require balance and motor coordination, our mice were analysed using the grip strength and rotarod tests that, in contrast to the hanging wire test, offer the possibility to discriminate muscular strength from balance and/or motor coordination deficits. The lack of IREB2 does not affect forelimb grip strength (**Fig.1d**), but causes a two-fold reduction in the mean latency to fall off the accelerating rotarod (**Fig.1e** and **supplementary Fig.1** online). These data suggest that IREB2 deficiency may be associated with a discrete impairment of balance and/or motor coordination that does not weaken horizontal locomotion and becomes apparent only with specific challenge tests. In summary, our *Ireb2*$^{-/-}$ mouse line exhibits no overt signs

of neurodegeneration and largely unaltered clinical performance, in contrast to the reported marked neurodegeneration[4,6,9].

In the light of the altered ferritin and TfR1 expression of our *Ireb2*$^{-/-}$ mice, we also followed-up with further histological investigations. Various stains including TUNEL staining and silver staining for cellular degeneration revealed no abnormality in *Ireb2*$^{-/-}$ brains (not shown). No changes in astrocyte organization were detected by glial fibrillary acidic protein immunohistochemistry; the expression of myelin-associated glycoprotein and amyloid-β precursor protein were unchanged (not shown). Importantly, calbindin staining of cerebellar Purkinje cells showed intact dendritic trees and axons and no cell loss (**Fig.2a-d**). At the ultrastructural level, electron microscopy revealed intact cells with preserved nuclear structure, regular cytoplasm and organelles, and specifically mitochondriopathy was not apparent. Lipofuscin-like bodies are observed in forebrain neurons and in Purkinje cells of the cerebellum (**Fig.2e-h**). These structures, which accumulate in senescence and whose formation can be promoted by iron, can be involved in cellular degeneration[13]. However, their occurrence was globally similar in aged *Ireb2*$^{-/-}$ and wildtype brains. These data show that neither altered ferritin and Tfr1 expression nor the complete lack of IREB2 *per se* cause neurodegeneration. Therefore, the phenotype of the *Ireb2*$^{-/-}$ mice reported by LaVaute et al[4] likely involves additional or alternative explanations, including genetic, environmental and technical differences.

Genetic background can profoundly affect mouse phenotypes[14]. Our mice were generated using the 129P2/OlaHsd ES cell line and were backcrossed to C57BL/6J mice. B6N3F2 animals were used in this study. LaVaute et al[4] report their mice to be on a C57BL/6 +

Sv129 background, but they do not state the respective substrains and proportions. In the study by LaVaute et al[4], the *Ireb2* locus was targeted with a gene replacement vector bearing a PGK-Neo insertion. We ensured that no selectable marker is retained in our IREB2-deficient line[8]. We consider this to be important, because retained selectable markers can affect the expression of neighbouring genes[15]. Interestingly, the murine *Ireb2* locus is close to the *Psma4* gene encoding a subunit of the 20S proteasome whose dysfunction is associated with movement disorders, as well as a cluster of subunits of the nicotinic acetylcholine receptor (Chrna3, Chrna5, Chrnb4). Finally, environmental factors need to be taken into account[16], although we consider gene-environment interactions as an unlikely explanation for marked phenotypic differences with severe histopathological correlates. Smith et al report that the lack of one *Aco*1 allele aggravates the phenotype of their *Ireb2*$^{-/-}$ mice[9]. This result supports our conclusion that iron regulatory protein-1 (ACO1) and iron regulatory protein-2 (IREB2) are partially redundant and can compensate for each other[10]. It also represents a genetic argument that IREB2 deficiency contributes to the phenotype of the mice described by LaVaute et al[4].

In conclusion, a novel mouse line with total and constitutive IREB2 deficiency displays the expected altered ferritin and TfR1 expression in the brain but only a mild clinical phenotype and no pathological signs of neurodegeneration at more than one year of age. Our study shows that IREB2 deficiency *per se* is not sufficient to produce a severe neurodegenerative disorder in the mouse.

Acknowledgments

We thank the staff of the EMBL animal house and Cornelia Schneider for their excellent technical assistance. This work was supported by the NGFN (grants No. 01GR0430 and No. 01GR0434) and Eumorphia (grant No EU QLG2-CT-2002-00930), and by funds from the Gottfried Wilhem Leibniz Prize to M.W.H.

Legends to Figures.

Figure 1: IREB2**-deficient mice display altered ferritin and TfR1 expression, but largely normal locomotion**

a) Western blot analysis of brain extracts from three *Ireb2*$^{+/+}$ and three *Ireb2*$^{-/-}$ mice. Mice lacking IREB2 (upper panel) display ferritin upregulation and decreased TfR1 expression in the CNS (middle panels), with a more pronounced TfR1 downregulation in the cerebellum (Cb) compared to whole brain. β-actin was used as a standard for equal loading (lower panel). The performance of 13-14 month-old mice was analysed quantitatively using the modified hole board test[9] (**b,c**). Representative activity patterns reveal that IREB2 deficiency does not impair horizontal locomotor activity. *Ireb2*$^{-/-}$ mice (**c**) even display a tendency for increased horizontal locomotion and decreased avoidance behaviour compared to wildtype (**b**). Detailed results of the modified hole board tests are given in **supplementary table 1** online. (**d**) *Ireb2*$^{-/-}$ mice have normal forelimb grip strength (10 trials per mouse within 1 min), but exhibit decreased mean latency (n=4

trials) to fall off the accelerating rotarod (**e**). See online material for experimental procedures and statistical analyses. Values are presented as average +SEM (*Ireb2*$^{+/+}$ n=10, *Ireb2*$^{-/-}$ n=11).

Figure 2: **Similar brain morphologies of wildtype and** IREB2**-deficient mice at the histological and ultrastructural level**

(**a-d**) Sagittal sections of the brain were stained with an anti-calbindin antibody. (**a,b**) The number of Purkinje cells is unchanged in cerebellar folia sections of *Ireb2*$^{-/-}$ brains. The molecular (M) and granular cell layers (G) and Purkinje cells (P) are indicated (original magnification 10X). (**c,d**) Confocal microscopic analysis of *Ireb2*$^{-/-}$ Purkinje cells reveals intact cellular extensions. Electron micrographs of cerebellar Purkinje cells (**e,f**) and neurons of the forebrain (**g,h**) show intact ultrastructure with well preserved mitochondria and endoplasmic reticulum. Lipofuscin-like bodies present in both samples in globally equal numbers are indicated with arrows.

References

1 Grantham-McGregor, S. & Cornelius A. *J. Nutr.* **131**, 649S-668S (2001).

2 Hogl, B. & Poewe W. *Curr. Opin. Neurol.* **18**, 405-410 (2005).

3 Zecca, L., Youdim, M.B., Riederer, P., Connor, J.R. & Crichton, R.R. *Nat. Rev. Neurosci.* **5**, 863-873 (2004).

4 LaVaute T., *et al. Nat. Genet.* **27**, 209-214 (2001).

5 Pantopoulos K. *Ann. N.Y. Acad. Sci.* **1012**, 1-13 (2004).

6 Rouault, T.A. *Nat. Genet.* **28**, 299-300 (2001).

7 Galy, B., Ferring, D., Benesova, M., Benes, V. & Hentze, M.W. *RNA* **10**, 1019-1025 (2004).

8 Galy, B., Ferring, D. & Hentze, M.W. *Genesis* **43**, 181-188 (2005).

9 Smith SR., *et al. Ann. N.Y. Acad. Sci.* **1012**, 65-83 (2004).

10 Galy B., *et al. Blood* **106**, 2580-2589 (2005).

11 Ohl, F., Holsboer, F. & Landgraf, R. *Behav. Res. Methods Instrum. Comput.* **33**, 392-397 (2001).

12 Connor, J.R., *et al. Neurology* **62**, 1563-1567, (2004).

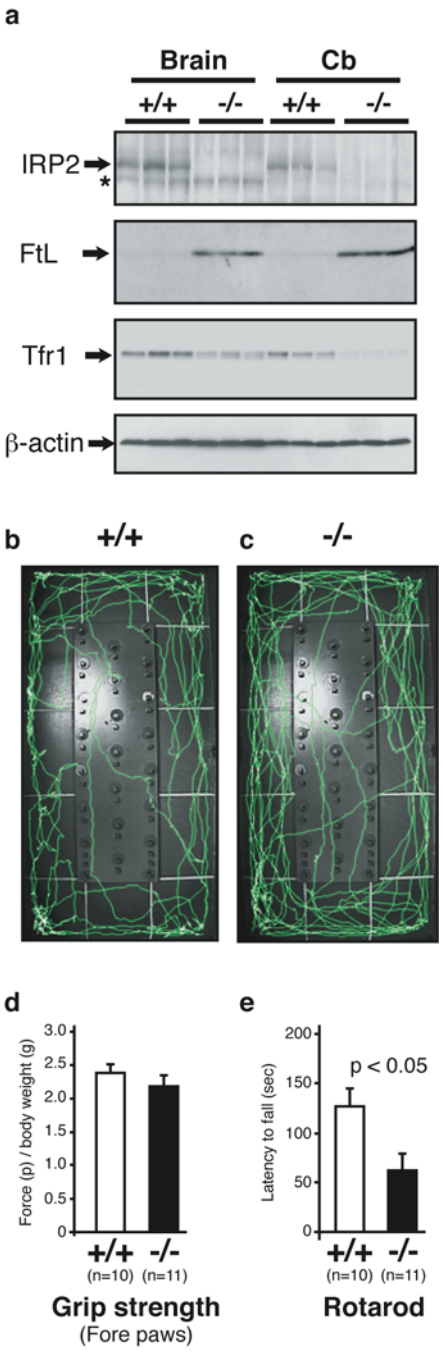
13 Brunk, U.T. & Terman A. *Free Radic. Biol. Med.* **33**, 611-619 (2002).

14 Wahlsten, D. *et al. J. Neurobiol.* **54**, 283-311 (2003).

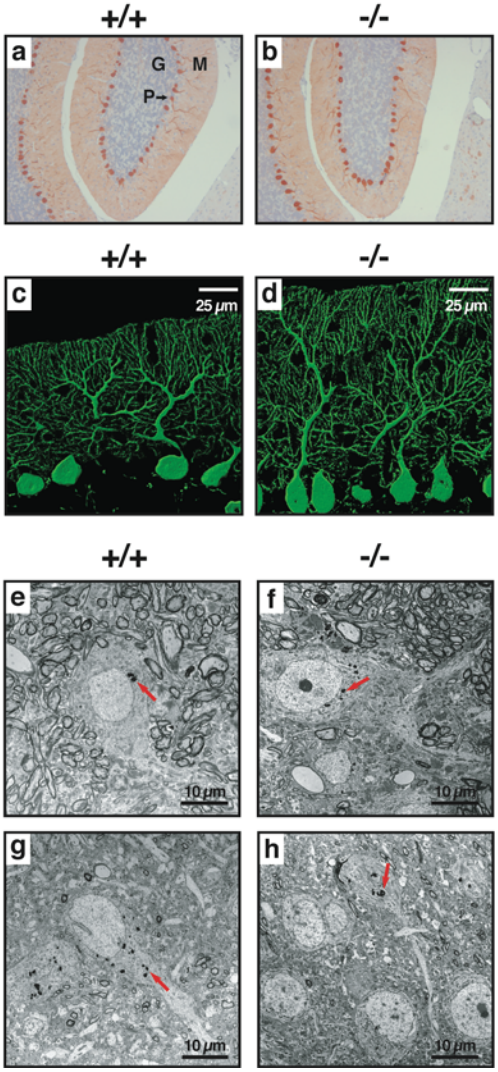
15 Olson, E.N., Arnold, H.H., Rigby, P.W. & Wold, B.J. *Cell* **85**, 1-4 (1996).

16 Bothe, G.W., Bolivar, V.J., Vedder, M.J. & Geistfeld, J.G. *Genes Brain. Behav.* **3**, 149-157 (2004).

Galy et al., Fig.1



Galy et al., Fig.2



Supplementary Table 1: **Phenotyping of wildtype- versus IREB2-deficient mice using the modified hole board test.**

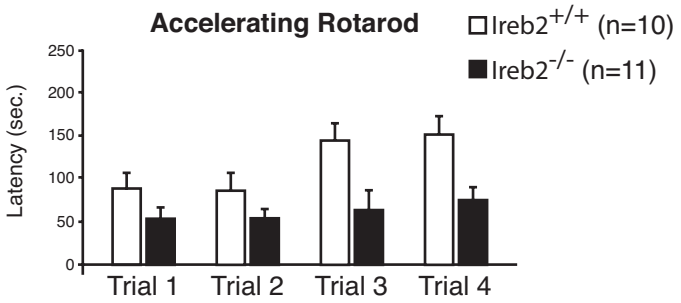
		Ireb2$^{+/+}$ (n=10)	*Ireb2*$^{-/-}$ (n=11)	
		Mean	Mean	*p*
Horizontal locomotor activity				
Line crossings	No.	63.1 ±4.4	76.1 ±10.1	*0.259*
	Latency (sec)	11.6 ±2.2	12.1 ±1.7	*0.756*
Total distance travelled (cm)		1604 ±93	2124 ±247	*0.071*
Mean velocity (cm/sec)		13.9 ±0.6	15.1 ±0.7	*0.178*
Maximum velocity (cm/sec)		57.5 ±3.0	64.9 ±2.4	
Turn angle (degrees)		1003 ±51	1293 ±141	*0.073*
Mean turn angle (degrees)		30.9 ±1.3	28.0 ±3.1	*0.084*
Angular velocity (degrees/sec)		211 ±8	206 ±20	*0.173*
Absolute meander (degrees/cm)		22.9 ±1.1	20.5 ±2.2	*0.084*
General exploration				
Rearings in the box	No.	21.2 ±3.7	13.18 ±2.53	*0.092*
	Latency (sec)	30.4 ±7.4	54.8 ±11.3	*0.061*
Directed exploration				
Holes explored	No.	31.1 ±6.9	55.1 ±9.2	*0.052*
	Latency (sec)	27.5 ±8.7	21.3 ±4.1	*0.972*
Object exploration				
Familiar object	No.	5.4 ±0.7	3.8 ±0.6	*0.100*
	Latency (sec)	53.6 ±8.5	60.3 ±17.1	*0.557*
	Duration (%)	0.90 ±0.11	0.95 ±0.25	*0.511*
Unfamiliar object	No	4.4 ±1.0	5.0 ±1.0	*0.683*
	Latency (sec)	81.4 ±22.5	71.7 ±24.7	*0.314*
	Duration (%)	1.02 ±0.23	1.64 ±0.41	*0.223*
Physiological arousal				
Grooming	No	3.9 ±0.6	2.2 ±0.5	***0.041***

	Latency (sec)	81 ±8	114 ±10	***0.020***
	Duration (%)	6.6 ±2.3	4.2 ±0.9	*0.282*
Defecation	No	1.2 ±0.5	1.5 ±0.5	*0.724*
	Latency (sec)	203 ±37	163 ±42	*0.756*
Social affinity				
Group contact	No	0	0	
Anxiety				
Entries on board	No	4.1 ±1.6	8.3 ±2.3	*0.149*
	Latency (sec)	122 ±35	89 ±32	*0.468*
	Duration (%)	4.93 ±1.73	9.85 ±2.13	*0.114*
Rearing on board	No	0.1 ±0.1	0.0	
Risk assessment				
Stretched body posture	No	0.70 ±0.30	0.18 ±0.12	*0.136*
	Latency (sec)	184 ±47	247 ±35	*0.511*

IREB2-deficient mice versus wildtype littermates were phenotyped at 13-14 months of age. The modified Hole Board test was carried out in an adapted version of the procedures described by Ohl et al.*. The test apparatus consisted of a test arena (100 cm X 50 cm), in the middle of which a board (60 cm X 20 cm X 2 cm) with 23 holes (1.5 cm X 0.5 cm) staggered in three lines with all holes covered by movable lids was placed, thus presenting the central area of the test arena as an open field. The area around the board was divided into 12 similarly sized quadrants by lines taped onto the floor of the box. Both box and board were made of dark grey PVC. All lids were closed before the start of the trial. For each trial, an unfamiliar object and a copy of a familiar object (remained for 48h in the home cage, removed 24h before testing) were placed into the test arena with a distance of 2 cm between them. Illumination levels were approximately 150 lux in the corners and 200 lux in the middle of the test arena. For testing, each animal was placed individually into the test arena in the same corner next to the partition, facing the board diagonally. The two objects were placed in the corner quadrant diametrical to

the starting point. During the 5 min trial, the animal's behavior was recorded by a trained observer with a hand-held computer. Data were analysed by using the Observer 4.1 Software (Noldus, Wagningen). Additionally, a camera was mounted 1.2 m above the center of the test arena, and the animal's track was videotaped and its locomotor path analysed with a video-tracking system (Ethovision 2.3, Noldus, Wageningen). After each trial, the test arena was cleaned carefully with a disinfectant. The number of animals in each group (n) is indicated. Data were statistically analysed by individual t-tests or Mann-Whitney tests for each parameter using SPSS software (SPSS Inc., Chicago, USA). The chosen level of significance was $p<0.05$. Data are presented as mean ± SEM.

* Ohl, F., Holsboer, F. & Landgraf, R. *Behav. Res. Methods Instrum. Comput.* **33**, 392-397 (2001).



Supplementary figure 1: Assessment of balance and motor coordination in wildtype- versus IREB2-deficient mice using the accelerating rotarod.

To measure the performance on the accelerating (4-40 rpm within a period of 300s) rotarod (TSE, Bad Homburg, Germany), the animals were placed perpendicular to the axis of rotation with the head facing the direction of the rotation and the latency to fall off the rod was recorded. Each animal was given 4 trials with 20 min intervals. The data were analysed by fitting a linear mixed-effects model (fixed factors: *genotype, sex, weight, trial number* and the respective interactions; random factor: *animals*; dependent factor: *latency*). This analysis shows that wildtype and *Ireb2*$^{-/-}$ mice perform differently in the course of the trial, with a significant interaction between genotype and trial number ($p<0.05$). Comparing the single trials shows significantly reduced latencies in trials 3 and 4 ($p<0.05$).