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Ferrous and ferric iron accumulates in the brain of aged Long–Evans Cinnamon rats, an animal model of Wilson's disease

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Abstract

The Long–Evans Cinnamon (LEC) rat, which accumulates excess copper (Cu) in its liver, is an animal model of Wilson's disease. We evaluated and compared the distributions of Cu, ferrous (Fe^{2+}), and ferric (Fe^{3+}) iron in four-brain regions, namely, in the cerebral cortex, cerebellum, substantia nigra (SN), and striatum of LEC and Long–Evans Agouti rats at 30 and 55 weeks. Cu levels were elevated in the striatum of LEC rats, and Fe^{2+} and Fe^{3+} were higher in the striatum and SN of LEC rats. Ratios of Fe^{2+} to Fe^{3+} were >1 in four regions, and were highest in the striatum and SN of LEC rats. Cu and iron levels were found to be augmented during aging, and we suggest that these accumulations may exert deleterious effects in aged LEC rats. This study is the first report that demonstrates regional differences of Fe^{2+} and Fe^{3+} accumulation in the brain of aged LEC rats. Further studies are required to elucidate the mechanisms of Cu and iron accumulations and of their effects.

Keywords: Long–Evans Cinnamon rat; Wilson's disease; Copper; Iron; Ferrous iron; Ferric iron

The Long–Evans Cinnamon (LEC) rat is an inbred mutant strain of Long–Evans rat and exhibits spontaneous hepatitis [17]. Approximately 40% of LEC rats die of fulminant hepatitis, and the 60% that survive develop chronic hepatitis and subsequently liver cancer [7]. LEC rats are an established animal model of Wilson's disease (WD), a hereditary disease caused by a copper (Cu) metabolism disorder [12,15,22]. The gene encoding Cu-binding ATPase, which is required for Cu efflux from hepatocytes, is located on chromosome 13 in humans and on chromosome 16 in rats, and is found to be defective in WD patients and LEC rats [1,16,23,24]. The defective expression of the WD gene results in the accumulation of Cu in the livers of WD patients and LEC rats, and causes several symptoms common to both [12,15,22].

LEC rats have a mutation in the atp7b gene, which is homologous to the human WD gene [1,16,23,24]. The resulting defect in the gene product causes defective Cu excretion from hepatocytes into bile and thus causes hepatic Cu accumulation [5,12,15,22]. In addition to this hepatic Cu accumulation as occurs in WD patients, LEC rats also show an age-dependent hepatic iron overload [8,9]. Furthermore, hepatic iron deprivation prevents spontaneous fulminant hepatitis and liver cancer in LEC rats [8]. It is well known that Cu and iron can efficiently produce reactive oxygen species (ROS) [10,19], and that ROS can induce several types of DNA damage, e.g., base alterations and DNA strand breaks [10]. Moreover, it has been reported that the amounts of 8-hydroxydeoxyguanosine in DNA, a marker of ROS-derived DNA damage, are elevated in the liver, kidney, and brain of LEC rats at 15 weeks of age, compared with 5- and 10-week-old animals, and that at 15 weeks

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these levels are 1.8-fold higher than those in control rats [25].

In this study, we measured Cu and iron levels in the liver and brain, and evaluated the regional distribution of these metals in the brain of LEC rats versus Long–Evans Agouti (LEA) rats. To investigate the role of iron, we measured levels of ferrous iron (Fe^{2+}), which is actively involved in the metabolism, and ferric iron (Fe^{3+}), which is stored in ferritin or metallothionein. In addition to examining the effects of aging on the distributions of Cu and iron, adult and aged animals were compared.

Male LEC (Charles River Japan, Inc., Atusgi & Hino Breeding Center) and LEA (a gift from Dr. Kozo Matsumoto at the University of Tokushima, Japan) rats (controls) were used in this study. The experiments were performed at two different ages, i.e., in the adult and in the old. The animals were selected at two ages, 30 and 55 weeks. LEC rats older than 60 weeks showed abruptly increased mortality. A few LEC rats survived to the age of 65 weeks, but they were severely moribund. Therefore, 30-week-old rats were chosen as adults and the animals at 55 weeks as the old. Concentrations of Cu, Fe^{2+}, and Fe^{3+} were measured in liver and in four regions of the brain (cerebral cortex, cerebellum, substantia nigra (SN), and striatum) ($N = 10$). The animal protocols adopted were approved by our Institutional Animal Care and Use Committee, and conformed to the animal welfare guidelines set out in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, 1985). Animals were anesthetized intraperitoneally with 1 cm^3/kg lumpen/ketamine solution. The portal vein was cannulated with a 21-gauge Teflon catheter, and to prevent hemoglobin iron contamination, each rat was perfused with 50 ml of 0.9% NaCl. The liver, cerebral cortex, cerebellum, SN, and striatum were then resected using a glass scissors, and the Cu concentrations in each tissue were determined using a flame ionization atomic absorption spectrophotometer with an air/acetylene flame (type 208; Hitachi Kohki Co., Tokyo, Japan), as described previously [12]. All glassware was acid-washed and dried to prevent iron and Cu contamination.

Fe^{2+} and Fe^{3+} levels were measured using the Ferrozine-ascorbate method. Fe^{2+} was determined using a Fe^{2+} chelator kit (Ferrozine, Hoffmann-La Roche). Brain samples were homogenized in 1.0 ml of buffer (sodium acetate, pH 4.5, 38% guanidine hydrochloride) and 50:1 of 39 mM Ferrozine. Homogenates were divided into two portions. Fe^{2+} was determined in the first portion, and 10 mg of granulated ascorbic acid was added to the second portion to reduce Fe^{3+} to Fe^{2+}. These homogenates were then incubated for 20 min at 37 °C and centrifuged at 15,000 rpm for 30 min, and the absorbances of the supernatants obtained were read at 578 nm and compared with those of standards [18].

All measurements were performed by researchers, unaware of sample histories. Nonparametric analyses were used to compare the values obtained. Wilcoxon signed-rank test and Kruskal–Wallis one-way ANOVA by ranks were performed. p values of <0.05 were considered significant.

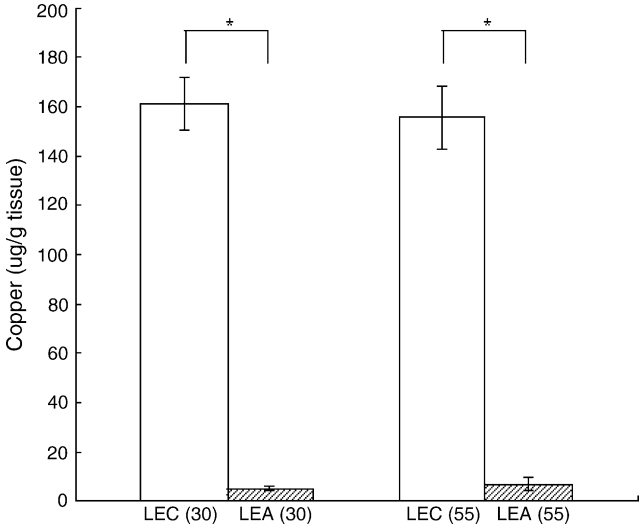

Fig. 1. Measurement of hepatic copper concentrations. Copper contents were measured in the livers of Long–Evans Cinnamon (LEC) and Long–Evans Agouti (LEA) rats of 30 (LEC30, LEA30) and 55 (LEC55, LEA55) weeks old. Values represent the means ± S.E.M. ($N = 10$). Asterisks denote statistical significance ($p < 0.05$). *Y*-axis values are μg/g of tissue.

The concentrations of hepatic Cu in the LEC rats were much higher than those of LEA rats (Fig. 1). No difference was observed between adult [30-week-old] and aged [55-week-old] LEC rats.

In the striatum, the Cu concentrations of LEC rats at both 30 and 55 weeks were higher than those of LEA rats (Fig. 2). SN Cu accumulations were more prominent in LEC rats than in LEA rats, but this was without significance. Cerebral cortex and cerebellum Cu levels were similar in LEC and LEA rats. With the advancement of age, striatal Cu levels became

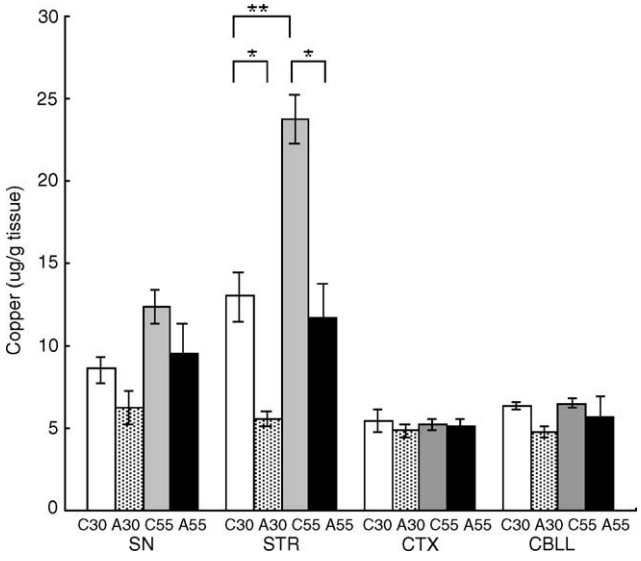

Fig. 2. Regional copper levels in the brain. Copper concentrations were measured in four regions of the brain, namely, substantia nigra (SN), striatum (STR), cerebral cortex (CTX), and cerebellum (CBLL). Values represent means ± S.E.M. ($N = 10$). Asterisks denote statistical significance at the $p < 0.05$ level. *Y*-axis values are μg/g of tissue.