The effect of copper supplementation on the concentration of copper in the brain of the brindled mouse

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The brindled mutant mouse is a useful model to study Menkes kinky-hair syndrome. The metabolic dysfunctions in both human and rodent are related to insufficient levels of bioavailable copper. Recently, copper supplementation therapy has been able both to prevent the appearance of various neuropathological changes and to prolong the life of these mutant mice. The optimum conditions for supplementation have been shown to be two intraperitoneal injections on postnatal days 7 and 10. The present study reports on the brain copper concentrations before, during and after the intraperitoneal copper therapy. The results demonstrate that postnatal days 7 and 10 correspond to two important epochs in copper homeostasis. The supplementation therapy seems to provide sufficient bioavailable copper to respond to the needs of the animal at these crucial time points. The results are discussed in terms of their importance to the human copper disorder.

In 1962, Menkes *et al.* described a sex-linked recessive neurological disorder in infants that was shown to be the result of a disturbed copper homeostasis (Danks *et al.*, 1972; Camakaris *et al.*, 1979) and an impaired copper absorption in the gastrointestinal tract, further complicated by an abnormal copper distribution in various tissues (Danks *et al.*, 1972; Danks, 1977; Horn *et al.*, 1978). The concentration of the copper in the brain of an affected foetus has been shown to be 0.17 p.p.m., which is approx. 40% of the normal value (Nooijen *et al.*, 1981). This abnormal copper distribution is thought to be due to the presence of increased amounts of a cysteine-rich 10000-dalton copper-binding protein (metallothionein) (Labadie *et al.*, 1981).

Hunt (1974, 1976) has described an X-chromosome-linked mottled (*Mo*) mutant of the house mouse (*Mus musculus*), termed the 'brindled' mouse, as an animal analogue of this human disorder. The male hemizygote (*Mo^{br}/Y*) with this disorder usually does not survive past day 15, whereas the female heterozygotes (*Mo^{br}/+*) survive and are fertile. Experiments with this mouse model have greatly benefited the study of Menkes' disease (Prins & Van den Hamer, 1979, 1980; Camakaris *et al.*, 1979; Port & Hunt, 1979).

Both infants and mutant mice show a similar marked neuronal degeneration (Aguilar *et al.*, 1976; Menkes *et al.*, 1962; Iwata *et al.*, 1979; Martin,

1978), which is considered to be secondary to the copper deficiency (Nagara *et al.*, 1980a; Yajima & Suzuki, 1979a,b,c). Administration of copper salts to overcome this deficiency, either orally or parenterally, have been disappointing (Dekaban, 1974; Grover & Scrutton, 1975; Walker-Smith *et al.*, 1973; Daish *et al.*, 1978; Nooijen *et al.*, 1981).

By contrast, studies with brindled mice by one of us (K.S.) have shown that intraperitoneal injections of cupric chloride on certain days provides a noticeable clinical improvement and a significant decrease in the extent of neuronal degeneration (Nagara *et al.*, 1980b). Recent studies by Nagara *et al.* (1981) have shown that two intraperitoneal injections on postnatal days 7 and 10 (5–10 µg of copper/g body wt.) were most effective clinically. We now report on the brain copper concentrations of brindled mutant mice after the copper supplementation regimen described by Nagara *et al.* (1981).

Experimental

The mice used in the present study were originally obtained from Dr. Cyril L. Moore; thereafter the mutant mice have been maintained in our laboratory by mating heterozygous females with normal males. All animals were housed in a constant-temperature environment (68–72°F) on a 12h-light/12h dark cycle. Tap water and a commercial stock diet (Ralston Purina mouse chow) were provided *ad*

libitum. The concentration of copper in the chow ranged from 8 to 14 p.p.m., with an average of 10 p.p.m.

All of the mice from each litter received intraperitoneal injections ($10\mu\text{g}$ of copper/g body wt., in 0.9% NaCl) in the morning of postnatal days 7 and 10. Heterozygous females, hemizygous males and normal littermates were killed by cervical dislocation, at times corresponding to postnatal days 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 13, 15, 90, or 120. The brain was sectioned in the following manner: the cerebellum was taken in total, care being taken not to include any of the spinal cord; the cortex was shaved off by using a scalpel (this section also included the head of the caudate); the hippocampus was removed with the thalamic and hypothalamic areas. The sections were then placed in tared 30 ml acid-washed Pyrex beakers. The tissues were dried at 150°C for 72 h and then weighed and wet-digested with fuming HNO_3 (3–5 ml), first at $90\text{--}100^\circ\text{C}$ until clear and then at $150\text{--}160^\circ\text{C}$. The residual liquid films were dissolved in, and adjusted to 5.0 ml with, 0.01 M-HCl. Copper was analysed with a Perkin-Elmer atomic-absorption spectrometer (Series 360) with an HGA 2100 graphite furnace (Norwalk, CT, U.S.A.).

Results

The influence of intraperitoneal copper administration at postnatal days 7 and 10 on the cerebral

copper concentrations are shown in Fig. 1. Each datum point represents the mean and s.d. for six mice. Although the terminal body weights of the male hemizygotes were consistently lower than the mottled heterozygous female or non-mutant normal mice, the brain weights were similar at each datum point tested.

Fig. 1 demonstrates that a severe copper deficiency exists throughout life in the brain of the hemizygous male. The level of copper in the brain of the female heterozygotes, who survived to be fertile, was similar to their littermate non-mutant controls. The significant rise in copper levels on postnatal days 4 and 5 in the non-mutants did not occur in either the heterozygous females or the hemizygous males. Instead, for only the heterozygous females, there was a more gradual increase to a similar final level on postnatal days 8–9.

An initial intraperitoneal injection of copper on postnatal day 7 produced a dramatic increase in the brain copper levels in the hemizygous males, which levelled off to an intermediate value by postnatal day 9 or 10. A second injection on day 10 produced another more dramatic increase, before decreasing to a final level that was significantly lower than that in the non-mutants. Even as young adults, the brindled hemizygous males had consistently lower brain copper levels than the female heterozygotes. The male hemizygotes survived, however, even though their brain copper levels at 5 months were significantly lower than those of controls. Although

Fig. 1. Changes in brain copper concentrations with age in treated and untreated brindled mutant mice. The copper concentration is expressed as μg of copper per g dry weight of brain tissue. * indicates a P value of less than 0.01 versus the heterozygous females and/or the non-mutant controls; ** indicates a P value of less than 0.01 versus non-mutant controls; both were determined by using Student's t test. Each datum point represents the mean $\pm$ s.d. ($n = 6$). ●, Controls; ▲, heterozygous females; □, hemizygous males; ---, copper-treated.

active and apparently healthy, these males never showed normal hair pigmentation and did not mate and produce offspring.

Discussion

The brindled mutant mouse is useful not only because it provides an excellent experimental model of human Menkes kinky-hair syndrome, but also because it offers an opportunity to examine normal copper metabolism.

The changes in brain copper concentrations in these mice fall into two phases after birth. In non-mutants there is an initial steep rise in copper concentration. This initial phase is followed by a slower, but steady, increase to adult levels at age 5 months. The initial increase seen in the present study begins about postnatal day 3 or 4 and levels off between days 7 and 10. Although this increase occurred in each group, the concentration of copper in the brain in the hemizygous mutant males began at a significantly ($P < 0.01$) lower level, rose more slowly, and reached a plateau at a significantly lower final concentration at about day 9. Although the non-mutant littermates continued to accumulate copper, the hemizygous males' abilities to do the same were apparently compromised, and the levels continued to be low until death.

We would speculate that the copper supplementation at postnatal days 7 and 10 is overloading the abnormal metallothionein copper-binding proteins, and thereby allowing essential amounts of copper to become more 'bioavailable'. Postnatal day 7 may be important because it coincides in non-mutants with the first significant rise in brain copper levels since birth. The day-10 supplementation may only provide an added boost to maintain levels during a crucial developmental period of the brain.

The general increase of brain copper levels after the copper-supplementation therapy described by us is in agreement with the results of others (Hunt, 1976; Mann *et al.*, 1979). However, clinically our results with only two injections are more effective than repeated injections (Hunt, 1976) or injections of larger amounts (Mann *et al.*, 1979), suggesting the importance of the time of the copper supplementation rather than the dosage. In agreement with the results reported by Mann *et al.* (1979), the increased copper levels in hemizygous males after injection were also transient in our study, and adult copper-treated hemizygous males revealed significantly lowered brain copper levels when compared with either normals or treated heterozygous females.

Because the brindled mouse and kinky-hair syndrome in humans are considered to be due to the same metabolic defect (Prins & Van den Hamer, 1979, 1980), it might be possible to define similar critical periods of copper requirement in humans during

development. When this is done, it may be possible to outline an effective therapeutic process.

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