Neuroendocrinology Letters Nos.1/2, Feb-Apr Vol.25, 2004
 ISSN 0172–780X **www.nel.edu**

ORIGINAL ARTICLE

Impaired somatostatin accumulation within the median eminence in mice with *mosaic* mutation

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Submitted: *November 12, 2003*
Accepted: *November 23, 2003*

Key words: ***mosaic* mutant mice; copper deficiency; somatostatin; median eminence**

Neuroendocrinol Lett 2004; ***25****(1/2):78–82 NEL251204A09*

Abstract

OBJECTIVES: The *mosaic* mutation (*Atp7a$^{mo\text{-}ms}$*) linked to X-chromosome is caused by changes in the Atp7a gene encoding CPx-type protein responsible for the ATP-dependent copper transport across cell membranes. *Mosaic* mutant males represent an animal model for Menkes disease in humans. Starting from the eighth day of life the *mosaic* males exhibit a progressive decrease in body weight with poor viability and progressive paresis of the hind limbs. In order to examine whether hypothalamic somatostatin metabolism may be different in normal and copper deficient mice, somatostatin accumulation at the level of median eminence in 14 days old normal and *mosaic* mutant males was compared.

METHODS: An electron microscopic immunocytochemical study on ultrathin brain slices was performed according to post-embedding immunogold procedure.

RESULTS: In non-mutant animals somatostatin has been detected in many synapses within median eminence. Gold particles moderately decorated synaptic vesicles and mitochondria. In *mosaic* mutant animals somatostatin expression within the median eminence was very low and only a few gold particles represented somatostatin. Particles were sporadically associated with synaptic vesicles, mitochondria or cytoskeleton elements. Moreover, pre- and post- synaptic parts of synapses were very often swollen.

CONCLUSION: Our data demonstrating that copper deficiency leads to the pathological changes within the median eminence ultrastructure and severe impairment of somatostatin expression suggest that this trace metal is an important element necessary for normal neurohormonal brain development.

Introduction

Copper and other trace metals like iron, zinc, manganese and molybdenum maintain living systems through catalytic and structural roles they play in proteins and other biomolecules. Since discovery that copper was necessary for hemoglobin formation in rats [1] many attention have been paid to reveal its specific role in metabolic processes within the cell. Copper is now recognized as an essential cofactor of enzymes catalyzing redox reactions and among over 30 enzymatic and non enzymatic copper-dependent proteins there are cytochrome c-oxidase, superoxide dismutase, ceruloplasmin, copper-thionein, protein-lysine 6-oxidase, dopamine-β-monooxygenase, dopamine β-hydroxylase, monophenol monooxygenase and peptide-α-amidating monooxygenase [2, 3]. The transport of copper ions across membrane barrier occurs via membrane-bound, copper transporting adenosine triphosphatases (Cu-ATPases) belonging to the P-type ATP-ase family [4, 5] that enable cellular egress of this metal and copper transporter CTR1, encoded by *Ctr1* gene, responsible for copper uptake [6, 7]. That copper transport via the membrane is carefully balanced has been revealed by the discovery of two human recessive genetic disorders associated with altered copper metabolism: Menkes syndrome and Wilson disease as well as by the finding that inactivation of the *Ctr1* gene in mice leads to early embryonic lethality in homozygous mutant embryos [7]. Isolation and cloning of the gene responsible for X-linked Menkes disease, have shown that defect in the ubiquitously expressed *ATP7A* gene is responsible for the sequestration of copper in the intestine and kidney what in a consequence leads to its deficiency in the remaining parts of the body [8, 9] while defect in *ATP7B* gene, whose expression is liver specific, is responsible for autosomal recessive Wilson disease [10, 11] characterized by the toxic accumulation of copper in liver and brain.

The *mosaic* mutation (*Atp7a*$^{mo\text{-}ms}$) linked to X-chromosome arose spontaneously in the outbred mouse colony of the Department of Genetics and Evolution, Jagiellonian University, Cracow, Poland [12, 13] and is caused by changes in the Atp7a gene encoding CPx-type protein [5] responsible for the ATP-dependent copper transport across cell membranes. Comparison between mouse *Atp7a* and human *ATP7A* genes revealed a significant homology and mouse *mosaic* mutant males represent an animal model for Menkes disease in humans [14, 15]. Several clinical features characteristic for copper deficiency have been observed in mosaic mutant mice, including defect in pigmentation and hair structure [13], short life-span for *mosaic* male hemizygous and female homozygous (they die at about the 16 postnatal day) as well as decreased copper level in brain, liver and heart and increased accumulation in the small intestine and kidneys [16, 17]. Moreover, it has been reported that starting from the eighth day of life the *mosaic* mutant males exhibit a progressive decrease in body weight with poor viability and progressive paresis of the hind limbs [18, 13].

Observed impaired body growth suggests that growth axis activity might be also changed in these animals. So far there are no studies on discrete brain structures morphology in *mosaic* mutant males as well as no data concerning somatostatin synthesis and localization in their brains. Isolated from hypothalamic extracts tetrapeptide somatostatin [19] was shown to inhibit the release of growth GH from the anterior pituitary. Immunocytochemical studies combined with retrograde neuronal tracing have shown that projecting to the median eminence somatostatin-containing nerve fibers originate in somatostatinergic perikarya in the hypothalamic periventricular nucleus (PeN) as well as ventromedial hypothalamic nucleus (VMH) [20, 21, 22] and are part of the parvocellular system [23] inhibiting hormone secretion from the anterior pituitary gland.

In order to examine whether hypothalamic somatostatin metabolism may be different in normal and copper deficient mice, the purpose of this study was to compare somatostatin accumulation at the level of median eminence in 14 days old normal and *mosaic* mutant males.

Material and Methods

Animals were bred in The Department of Genetics and Evolution of the Jagiellonian University, Cracow. Hemizygous mutant males *Ms*/– were obtained from crosses between heterozygous females Ms/+ and normal males +/–. All experimental (n = 5) and control (n=5) animals were of 14 days of age. The mice were kept under controlled light (LD 12:12) and temperature (22 C) conditions with free access to standard Murigran diet (Motycz, Poland) and tape water.

For ultrastructural and immunocytochemical studies mice were sacrificed by transcardiac perfusion with 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1M PBS. Then, blocks of brain tissue containing median eminence were carefully excised, washed for 30 min in PBS and soaked for 1h with 1% OsO_4. Next, samples were subsequently dehydrated in an ethanol gradient and finally embedded in Epon. Ultrathin sections were processed according to the post-embedding immunogold procedure. The sections were mounted on formvar-coated nickel grids, incubated for 10 min in 10% hydrogen peroxide then rinsed for 15 min in water and PBS an finally blocked for 10 min in 3% BSA in PBS.

The rabbit anti-somatostain antibody (ICN, Biomedicals, Inc., Aurora, Ohio, USA) was diluted 1:50 in PBS and kept for 1h at 37C. After that antibody was applied to tissue slices for overnight incubation at 4C, and next day grids were washed for 30 min in PBS and exposed to donkey anti-rabbit IgG conjugated with 18 nm colloidal gold particles (Jackson Immuno Research Laboratories West Grove, DA, USA) diluted 1:50 in PBS. After 1h incubation at 37C grids were washed for 15 with PB and rewashed in redistilled water for 15 min. The samples were air dried, stained for 15 min with 4,7% water solution of uranyl and finally for 5

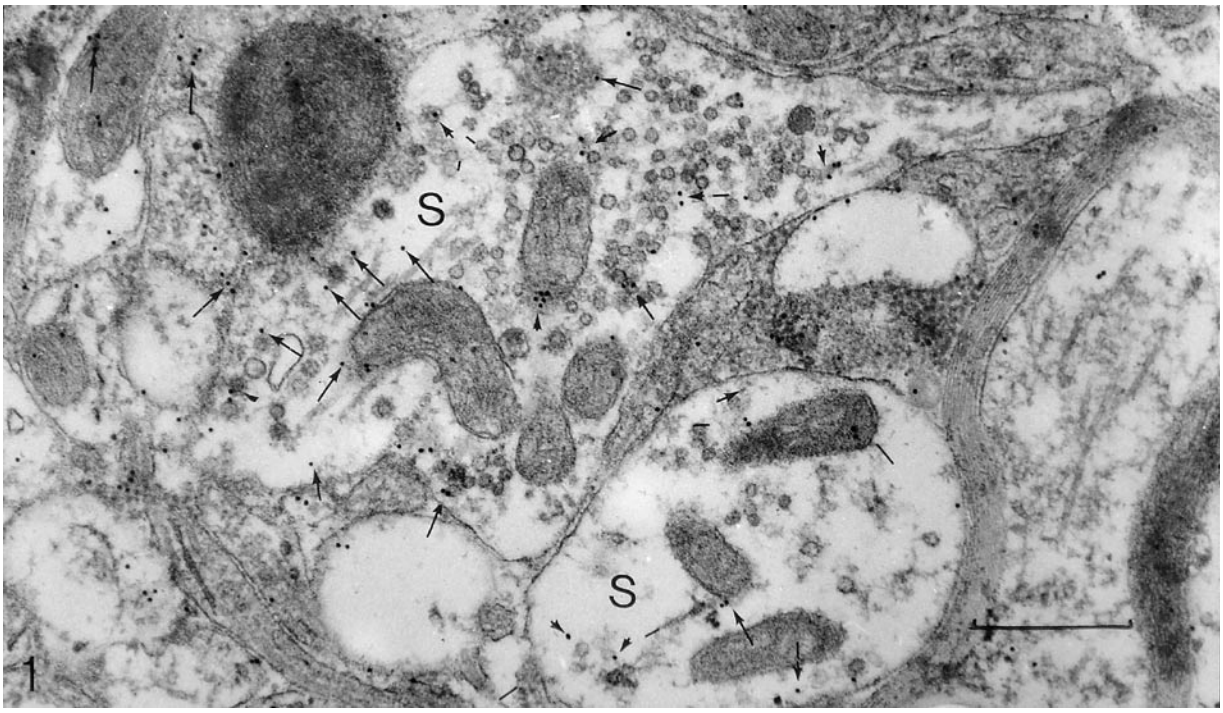

Fig.1. Ultrastructure of the median eminence in normal 14 days old, non-mutant male mouse. Picture presents synapses (S) containing somatostain-like gold particles moderately decorating synaptic vesicles, mitochondria and synaptic membranes. Bar = 500nm

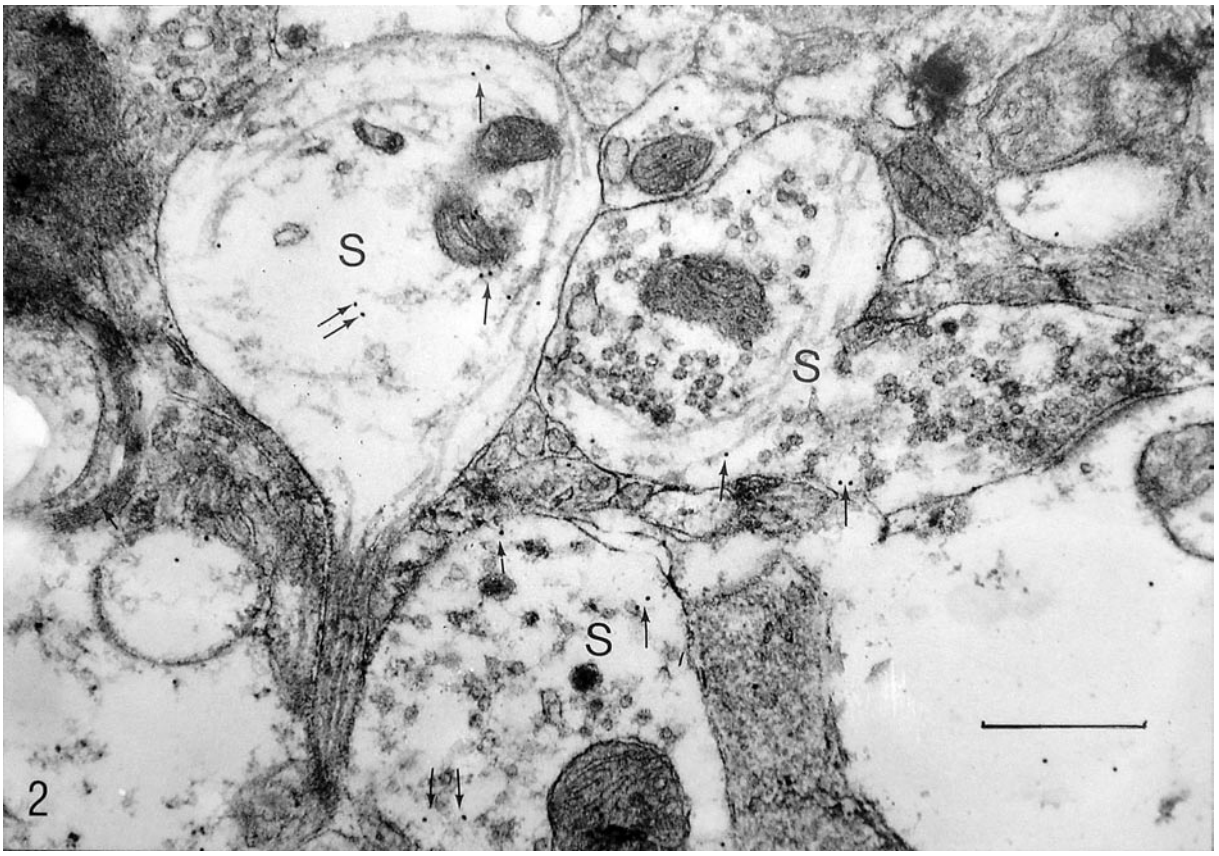

Fig. 2. Ultrastructure of the median eminence in 14 days old, *mosaic* mutant male mouse. In copper deficient mutant male only a few somatostatin-like gold particles are present in morphologically changed, swollen synapses. Bar = 500nm.

min with lead citrate. Grids incubated with 3% BSA in PBS instead of rabbit anti-somatostain antibody served as a method control The sections were examined and photographed in JEOL 1200 EX electron microscopy.

Results and Discussion

Presented results demonstrate a significant differences in the immunohistochemical distribution of somatostatin in median eminence of *mosaic* and normal mouse brain. As shown in Fig 1, in non-mutant control animals somatostatin has been detected in many synapses within this region. Gold particles (18nm) moderately decorated synaptic vesicles and mitochondria, thus showing that in non-mutant mice somatostatin synthesis occurs in a normal way. But in *mosaic* mutant animals with genetically impaired copper absorption, somatostatin expression within the median eminence was very low. As presented in Fig.2 only a few gold particles represented somatostatin in this structure and they were sporadically associated with synaptic vesicles, mitochondria or cytoskeleton elements. Moreover, pre- and post- synaptic parts of synapses were very often swollen.

Our data demonstrating that copper deficiency leads to the pathological changes within the median eminence ultrastructure and severe impairment of somatostatin expression suggest that this trace metal is also an important element involved in neurohormonal brain development. With its deficit, median eminence – a crucial hypothalamic structure responsible for the neurohormones release into the portal circulation – appears to be significantly less functional, what in consequence may lead to disorder in neurohormonal activity. Somatostatin is one of the neurohormones released from the median eminence into the hypophyseal portal circulation, then carried to the anterior pituitary where it inhibits GH, TSH as well as prolactin release [24] and together with GH-releasing hormone (GHRH) acts in concert to regulate pulsatile GH secretion [25, 26]. Since growth hormone is the key hormone responsible for the body growth and metabolism regulation [27] changes in its synthesis and release pattern evoked by altered hypothalamic somatostatin activity may in consequence lead to the impairment of body growth in *mosaic* mutant male mice. Studies on effects of perinatal copper deficiency in 1 month old Sprague-Dawley rat offspring have shown that low copper treatment resulted not only in an impaired body growth and 10-fold lower liver copper levels [28] but also caused a significant changes in copper and brain catecholamines content in discrete brain structures. Moreover, copper deficiency impairs the developmental expression of PKC isoforms thus severely affects brain development as well as is known for its crucial role in catalyzing α-amidation of several neuropeptides [29, 30]. Our results show that copper is also necessary for brain somatostatin activity development but detailed studies are needed to explain the precise levels (neurons development, neuropeptide synthesis, processing, transport, release) at which copper might be significantly involved.

Acknowledgments

The authors wish to express their thanks to Dr Tomasz Blasiak (Institute of Zoology, Jagiellonian University, Cracow) for his skillful help in mice brain preparation.

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