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## Elesclomol alleviates Menkes pathology and mortality by escorting Cu to cuproenzymes in mice

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### Abstract

Loss-of-function mutations in the copper (Cu) transporter ATP7A cause Menkes disease. Menkes is an infantile, fatal, hereditary copper-deficiency disorder that is characterized by progressive neurological injury culminating in death, typically by 3 years of age. Severe copper deficiency leads to multiple pathologies, including impaired energy generation caused by cytochrome c oxidase dysfunction in the mitochondria. Here we report that the small molecule elesclomol escorted copper to the mitochondria and increased cytochrome c oxidase levels in the brain. Through this mechanism, elesclomol prevented detrimental neurodegenerative changes and improved the survival of the mottled-brindled mouse—a murine model of severe Menkes disease. Thus, elesclomol holds promise for the treatment of Menkes and associated disorders of hereditary copper deficiency.

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**Author contributions:** L.M.G. established *mo-br* rodent colony, husbandry and breeding, drug formulations, and pharmacokinetics and toxicological (PK/Tox) experiments; developed interventional protocols, efficacy studies, and behavioral studies; and wrote the manuscript. A.S., T.C.S., S.S., M.Z., H.F.G., B.L., C.D.V., and E.N. assisted in husbandry and biological sample processing for PK/Tox, histology, and biochemical experiments. S.S. and M.Z. performed tissue, Cu, and biochemical studies. A.A. synthesized ES-Cu<sup>2+</sup> complex. V.S. and M.J.P. developed the *ATP7A*<sup>-/-</sup> B16 melanoma cell line and provided technical expertise. F.R.L. performed histopathology sectioning, generation of digital slide sets, and analysis. S.Y. and B.-E.K. established cardiac *Ctrl* KO rodent colony, husbandry and breeding, efficacy studies, histology, and biochemical studies. V.M.G. and J.C.S. developed the concept of ES drug therapy for hereditary Cu disorders, provided administrative and technical expertise, undertook data analysis, and helped write the manuscript.

**Competing interests:** L.M.G., J.C.S., S.S., and V.M.G. are inventors on the patent application PCT/US2019/041571 submitted by Texas A&M University entitled “Compositions for the Treatment of Copper Deficiency and Methods of Use.”

**Data and materials availability:** All data are available in the main text or the supplementary materials.

Copper (Cu) is an essential micronutrient required for numerous critical enzymes, including cytochrome c oxidase (CcO) of the adenosine 5'-triphosphate (ATP)-generating electron transport pathway found in mitochondria (1). Paradoxically, Cu possesses inherent toxicity, in part because of its ability to generate hydroxyl radicals in biological systems (2). Organisms have evolved highly complex systems of metallochaperones and transporters to safely distribute Cu (3, 4). Mutations that impair the function of any component of Cu transport can influence numerous cellular processes, affecting systems as diverse as energy production (5, 6), catecholamine biosynthesis, and connective tissue maturation—resulting in debilitating, often-fatal human diseases (7).

The coordinated efforts of the two major Cu membrane transporters, CTR1 and ATP7A, regulate intracellular Cu levels and directional transport across polarized epithelial layers, such as the intestinal enterocyte lining (8) and choroid plexus (9). CTR1 affects initial Cu entry, whereas ATP7A facilitates Cu egress from cells.

Mutations in the Cu-transporting adenosine triphosphatase (ATPase), ATP7A, result in Menkes disease—a fatal, X-linked infantile condition with no Food and Drug Administration (FDA)-approved treatment (10). Clinical presentations of Menkes include abnormal catecholamine ratios, characteristic kinky hair, hypopigmentation, connective tissue defects, and severe neurodegeneration (7,10,11). In the brain, this Cu deficit causes secondary CcO dysfunction, which leads to progressive neurological injury and death (12–14). Efforts to restore normal Cu levels and enzyme function by means of parenteral-Cu supplementation using hydrophilic complexes, such as copper histidine (HIS-Cu<sup>2+</sup>), do not always ameliorate severe neurological pathology in Menkes patients because of poor penetrance and low restoration of neuronal CcO function in the brain (14, 15). We previously reported that elesclomol (ES), a small, highly lipophilic Cu<sup>2+</sup>-binding molecule, restores mitochondrial function in the context of defective Cu transport in yeast and mammalian cell lines (16). A membrane traversing drug like ES, capable of Cu delivery to key cuproenzymes, such as CcO in brain mitochondria, could alleviate the neurodegenerative aspects of Menkes disease. This would be similar to hinokitiol, a lipophilic carrier that restores iron levels in the context of defective membrane transport both in vitro and in vivo (17,18).

## ES restores mitochondrial function in *Ctr1* knockout H9c2 cells and mice

We measured the oxygen consumption rate (OCR)—an indication of electron transport activity—and ATP levels in the Cu importer *Ctr1* knockout (KO) H9c2 rat cardiomyocytes. *Ctr1* KO H9c2 cells exhibited significant reduction in basal OCR and ATP levels. Preincubation with 1 nM of ES restored OCR to 103% (+34%) of wild-type (WT) (2.5 versus 3.7 pM min<sup>-1</sup> per microgram of protein;  $P < 0.001$ ) (fig. S1A). Similarly, ES treatment increased ATP levels compared with the *Ctr1* KO vehicle (0.74 versus 1.01,  $P < 0.001$ ) (fig. S1B).

Loss of CTR1 results in severe cellular Cu deficiency in mice. *Ctr1*<sup>-/-</sup> mice demonstrate lethality in utero (19). The cardiac-specific *Ctr1* KO mouse (20) exhibits growth retardation, severe hypertrophic cardiomyopathy due to cardiac muscle CcO dysfunction, and death

around postnatal day (PND) 12. To assess the ability of ES to escort  $\text{Cu}^{2+}$  through CTR1-deficient cardiac cellular membranes, we administered subcutaneous ES at  $10 \text{ mg kg}^{-1}$ . WT mice tolerated ES injections well with favorable tolerability and pharmacokinetics (table S1 and fig. S2). We observed a 100% 26-day survival rate in ES-treated mice whereas those receiving vehicle died between PND 8 and 12 (Fig. 1, A and B). Growth of ES mice was restored to WT pattern with no pronounced deviation from either WT vehicle or WT ES-treated groups (Fig. 1C and fig. S3A).

ES mice demonstrated normalization of total body, heart, and spleen weights at PND 10 (Fig. 1, D and E, and fig. S3, B to D). Cardiac histopathology at PND 10 of vehicle-treated KO mice showed pronounced hypertrophy characterized by increased cell area compared with WT tissue samples ( $186$  versus  $87 \text{ mm}^2$ ,  $P < 0.01$ ) (fig. S3, E and F). ES treatment ameliorated severe cardiac pathology with a partial reduction in hypertrophy ( $119 \text{ mm}^2$ ,  $P < 0.01$ ) (fig. S3, E and F). Cardiac [Cu] increased with ES treatment from a vehicle KO level of 34 to 55% ( $1.6$  versus  $2.6 \mu\text{g g}^{-1}$ ,  $P = 0.04$ ) (Fig. 1F). The 21% increase in cardiac [Cu] resulted in a 28% increase in COX1, the Cu-containing subunit of CcO (1) (Fig. 1, G and H). SOD1, a cuproenzyme highly resistant to Cu depletion (21), remained unchanged (fig. S4).

### ES alone does not rescue mottled-brindled mice

The mottled-brindled (*mo-br*) mouse phenotypically recapitulates Menkes disease (22). *Mo-br* mice possess a 6-base pair (bp) deletion in exon 11 of the mouse *Atp7A* gene, which results in an in-frame deletion of Leu<sup>799</sup> and Ala<sup>800</sup>. This deletion results in little residual Cu transporting function with severe disease phenotypes, including hypopigmentation, kinky whiskers, growth delay, neurological abnormalities, seizures, and death around PND 14. As with most Menkes patients, the *mo-br* mouse shows little response to treatment with hydrophilic Cu complexes alone (23–25).

In preliminary studies, we administered ES at  $10 \text{ mg kg}^{-1}$  of body weight. Unlike the cardiac *Ctrl* KO mice, this pilot study demonstrated no enhanced survival among treated *mo-br* males. This was likely because *mo-br* mice were too deficient in systemic Cu to benefit from ES alone, whereas the cardiac *Ctrl* KO mice possess an elevated serum Cu pool capable of ES-mediated redistribution to deficient cardiomyocytes (20). We hypothesized that, by preloading ES with  $\text{Cu}^{2+}$ , we would address the systemic Cu deficiency and ATP7A-mediated defective transport.

### ES-Cu<sup>2+</sup> complex rescues *mo-br* mice

Menkes disease is characterized by severe neurodegeneration, and therefore any successful treatment must involve a drug that facilitates Cu delivery across the blood-brain barrier or the blood-cerebral spinal fluid barrier (9,10,14). Brain pharmacokinetic studies on PND 7 mice demonstrated high levels of ES in the brain at  $201.3 \pm 41.7 \text{ ng mg}^{-1}$  (fig. S5A), whereas adult exposure, though still significant, was lower at  $7.8 \pm 5.0 \text{ ng mg}^{-1}$  (fig. S5B).

We next administered ES-Cu<sup>2+</sup> at  $3.625 \text{ mg kg}^{-1}$  per dose by subcutaneous injection on PND 7 and 10. The total dose of Cu approximated the total amount of Cu ( $4 \mu\text{g}$ ) in a 4-g WT mouse (26). Additional *mo-br* cohorts included vehicle, ES only, and HIS-Cu<sup>2+</sup>, formulated

with an equivalent dose of Cu compared to the ES-Cu<sup>2+</sup> cohort (Table 1). We selected HIS-Cu<sup>2+</sup> as a control because of the drug's investigational status for Menkes disease (14). ES-Cu<sup>2+</sup> was well tolerated and exhibited favorable pharmacokinetics (tables S2 to S4 and figs. S6 and S7).

As reported, *mo-br* mice exhibited hypopigmentation with death around PND 14 (Fig. 2, A to C). Within 24 hours, we observed pigment production in ES-Cu<sup>2+</sup>-treated mice in the immediate vicinity of the injection site (Fig. 2B and fig. S8A). Pigment production indicated increased activity of the secretory pathway cuproenzyme tyrosinase. This was in agreement with the in vitro assessment of an ATP7A KO B16 melanoma cell line, which showed that 1 nM of ES was able to partially rescue tyrosinase activity (fig. S8B). Because tyrosinase requires the action of ATP7A for Cu import into the Golgi complex (27), our findings were unexpected and suggest that ES-Cu<sup>2+</sup> was delivering Cu to cuproenzymes metalated in the Golgi. We also observed that whisker appearance improved from bushy, highly kinked clumps to near-normal by PND 70 in only the ES-Cu<sup>2+</sup> cohort (fig. S8C), which indicated improved sulfhydryl oxidase activity (28).

*Mo-br* vehicle, ES only, and HIS-Cu<sup>2+</sup> cohorts developed seizures beginning around PND 11 (movie S1). Seizures increased in severity with subsequent death of all individuals in these groups between PND 14 and 21. We only observed a negligible survival advantage for HIS-Cu<sup>2+</sup> over vehicle alone (Table 1). ES-Cu<sup>2+</sup>-treated *mo-br* adult mice did not have seizures and exhibited similar body size to that of their WT siblings (Fig. 2, D and E, and movie S2). ES-Cu<sup>2+</sup> increased the survival of *mo-br* mice and successfully rescued 82% of animals at 10 weeks of age with a median survival of 203 days ( $P < 0.01$ , log-rank test) (Fig. 2F, fig. S8D, and Table 1). After treatment, ES-Cu<sup>2+</sup> *mo-br* mice experienced accelerated growth and near normal body weight by week 10 (Fig. 2G and fig. S8E). Histological examination of the livers of WT vehicle and both ES-Cu<sup>2+</sup>-treated WT and *mo-br* mice demonstrated no pathological changes associated with drug exposure (fig. S9).

## Neuromotor assessment of ES-Cu<sup>2+</sup> rescue

Ten-week-old *mo-br* mice were evaluated by phenotype and neuromotor functional tests. ES-Cu<sup>2+</sup>-treated *mo-br* mice exhibited hypo-pigmentation but no other gross abnormalities on observation (Fig. 2, D and E). On a forelimb grip-strength test using a Chatillon force apparatus, ES-Cu<sup>2+</sup>-treated *mo-br* mice possessed 73% grip strength compared with WT animals (0.94 versus 1.29 N,  $P < 0.01$ ) (Fig. 3A). On the accelerating rotarod, a test of endurance and motor coordination, ES-Cu<sup>2+</sup> *mo-br* mice exhibited average latency-to-fall time of 222 s compared with 379 s ( $P < 0.01$ ) for WT (Fig. 3B). On the gait treadmill, overall ataxic indices for ES-Cu<sup>2+</sup> *mo-br* mice were statistically insignificant for both pelvic and shoulder girdles compared with WT (Fig. 3C, table S5, and movies S3 and S4). In the open field, *mo-br* mice demonstrated 35% decreased movement time, 24% increased rest time, and traveled 60% of total distance compared with WT ( $P < 0.01$ ) (Fig. 3, D to F).

## Brain histology and biochemical markers of ES-Cu<sup>2+</sup> therapy

Brain sections of vehicle and HIS-Cu<sup>2+</sup>-treated mice at 2 weeks of age showed marked, diffuse neurodegeneration of cortical and hippocampal neurons (Fig. 4A and fig. S10A). In the hippocampus, necrotic pyramidal neurons represented 10.5% of 3200 counted cells in vehicle and 4.9% in HIS-Cu<sup>2+</sup> *mo-br* mice. ES-Cu<sup>2+</sup> preserved cortical and hippocampal neurons, with hippocampal regions showing no signs of necrosis as characterized by pyknotic nuclei (Fig. 4A). The Purkinje neuron layer in the cerebellum was also preserved. (fig. S10B). At 10 weeks of age, brain structures were preserved with no distinct differences between WT and ES-Cu<sup>2+</sup>-treated *mo-br* mice (Fig. 4B and fig. S11, A to C).

Two-week-old mice treated with ES-Cu<sup>2+</sup> showed normalized serum [Cu] (Fig. 5A) with increased brain [Cu] from the vehicle baseline of 22% of WT to 41% (0.45 versus 0.82 mg g<sup>-1</sup>,  $P < 0.01$ ) compared with 24% with HIS-Cu<sup>2+</sup> (0.48 mg g<sup>-1</sup>) (Fig. 5B). ES-Cu<sup>2+</sup> proved superior to HIS-Cu<sup>2+</sup> at delivering Cu to brain tissue ( $P < 0.01$ ). Necropsy at 2 weeks showed significant decrease in total brain mass in *mo-br* animals treated with vehicle and HIS-Cu<sup>2+</sup> (-16 and -12%) (Fig. 5C and tables S6 and S7). ES-Cu<sup>2+</sup>-normalized brain mass was comparable to that of WT littermates (<2% difference between WT and *mo-br* ES-Cu<sup>2+</sup> cohorts) (Fig. 5C).

Mitochondrial COX1 levels exhibited a 14% improvement with ES-Cu<sup>2+</sup> intervention (30 versus 16%,  $P = 0.03$ ) (Fig. 5, D and E). We did not observe any significant correction in COX1 levels in vehicle or HIS-Cu<sup>2+</sup>-treated mice (17%,  $P = 0.99$ ) (Fig. 5, D and E). Though overall brain [Cu] in the ES-Cu<sup>2+</sup> cohort was about half of that in the WT cohort (0.82 versus 1.95 μg g<sup>-1</sup>), the mitochondria-specific delivery mechanism of ES-Cu<sup>2+</sup> could explain the degree of COX1 metalation. We also observed significant improvements in [Cu] and COX1 levels in the hearts of ES-Cu<sup>2+</sup>-treated *mo-br* mice both at PND 14 and 70 (fig. S12). SOD1 levels remained unchanged (fig. S13).

At 10 weeks, COX1 levels in ES-Cu<sup>2+</sup> mice were 42% of those in WT mice (Fig. 5, F and G). Serum [Cu] reverted to the earlier-established *mo-br* baseline of 28% of WT (0.48 versus 1.73 μg mL<sup>-1</sup>) (Fig. 5H). In the brain, Cu levels declined from 41 to 34% of WT (1.57 versus 4.64 μg g<sup>-1</sup>) (Fig. 5I), but brain weights remained indistinguishable (0.448 versus 0.432 g,  $P = 0.23$ ) (Fig. 5J and table S6).

## Discussion

Although deficiencies in Cu transport and processing adversely affect numerous important biological pathways, the most profound pathological changes occur as a result of perturbation of the electron transport chain (5, 6). Specifically, CcO requires Cu for assembly and catalytic activity of two subunits, COX1 and COX2 (1). CcO dysfunction secondary to Cu deficiency results in cardiomyopathy, neurodegeneration, and premature death (13,14). ES at relatively low dose stopped early mortality and conferred near-normal cardiac and brain histology while improving COX1 abundance in Cu-deficient cardiac-specific *Ctrl* KO and *mo-br* mice.

In cardiac *Ctrl* KO mice, affected animals possess elevated serum [Cu] (20), which allows for in vivo ES-Cu<sup>2+</sup> complex formation and redistribution of Cu across CTR1-deficient cardiomyocyte membranes to mitochondria for metalation of CcO without supplementation of exogenous Cu. ES treatment completely reversed the delayed growth and slowed disease progression in these mice. ES-treated mice exhibited improved survival at PND 26. Heart COX1 levels improved from 34% baseline to 66% (+28%) with a corresponding normalization of heart weight and reduced cardiomyocyte area, despite only modest improvement in total tissue Cu levels.

HIS-Cu<sup>2+</sup> clinical trials for Menkes disease have shown mixed results. Although there were some improvements in survival and clinical markers, these improvements were primarily observed in a subset of patients with mutations that display residual ATP7A activity (14, 25). The *mo-br* mouse, possessing little residual transporter functionality (22), only marginally benefits from HIS-Cu<sup>2+</sup> therapy. In contrast, ES-Cu<sup>2+</sup> significantly improves total brain tissue [Cu] levels compared with HIS-Cu<sup>2+</sup>, with improved outcomes for survival, restoration of growth, preservation of neurological structures, tissue Cu delivery, and COX1 abundance. Two doses of ES-Cu<sup>2+</sup>, equaling ~4 mg of Cu by PND 10, rescued *mo-br* males and improved median survival from 14 to 203 days.

The 14% improvement in brain COX1 level in 2-week-old mice sufficiently preserved key neurological structures, such as cortical and hippocampal neurons. Preservation of brain structures and COX1 persisted past the 2-week assessment. At 10 weeks of age, the ES-Cu<sup>2+</sup>-treated *mo-br* mice demonstrated normal brain structures and increased COX1 metalation with only small defects in gross neuromotor function, as determined by open field, rotarod, grip strength, and gait treadmill experiments.

Our in vivo results indicate that the mechanism of ES-mediated Cu relocalization may not be limited to the mitochondria. Morphological changes in fur pigmentation and whisker structure indicate improvement in the secretory pathway cuproenzymes tyrosinase and sulfhydryl oxidase, enzymes whose metalation requires ATP7A activity in the Golgi complex. Partial improvement in other secretory pathway cuproenzymes, such as dopamine- $\mu$ -hydroxylase and lysyl oxidase, could further explain the beneficial effects given the profound deficiency of most Cu-utilizing systems associated with Menkes disease.

Administration of ES or ES-Cu<sup>2+</sup> complex in a 1:1 stoichiometry and appropriate formulation exhibited good pharmacokinetic properties, low toxicity, and efficacy. Our results indicated that ES corrects defective CTR1 and ATP7A membrane-Cu transport with beneficial, targeted improvement of mitochondrial CcO metalation, owing to the ES-Cu<sup>2+</sup> complex's mechanism of selective mitochondrial Cu release. The membrane-permeating properties of ES, coupled with its directed release mechanism, make this agent a good candidate for drug repurposing as a Cu courier for disorders affecting metalation of CcO. Our results in cardiac *Ctrl* KO and *mo-br* mice indicate that ES or ES-Cu<sup>2+</sup> hold promise as a potential, efficacious therapeutic agent for the treatment of hereditary Cu-deficiency disorders.

## Supplementary Material

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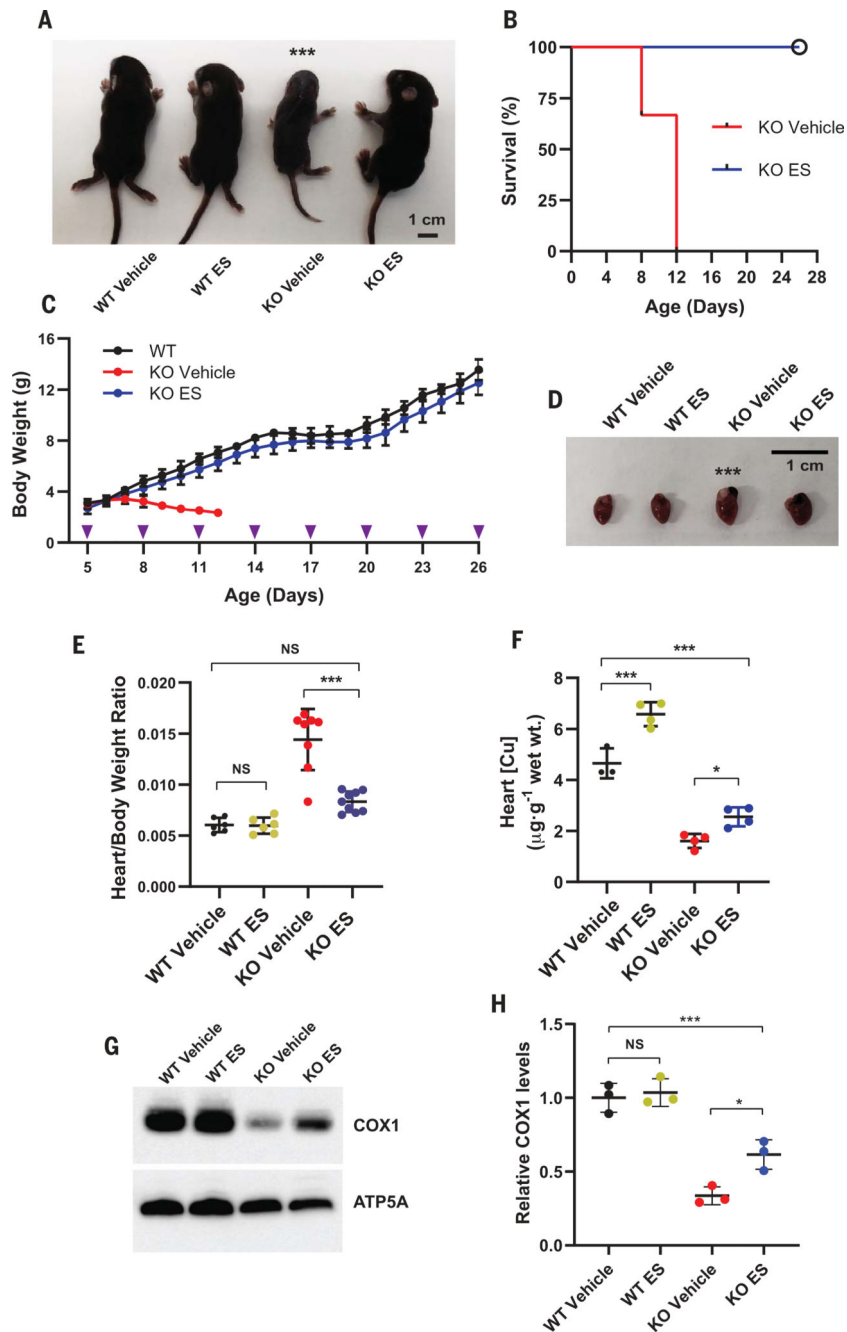
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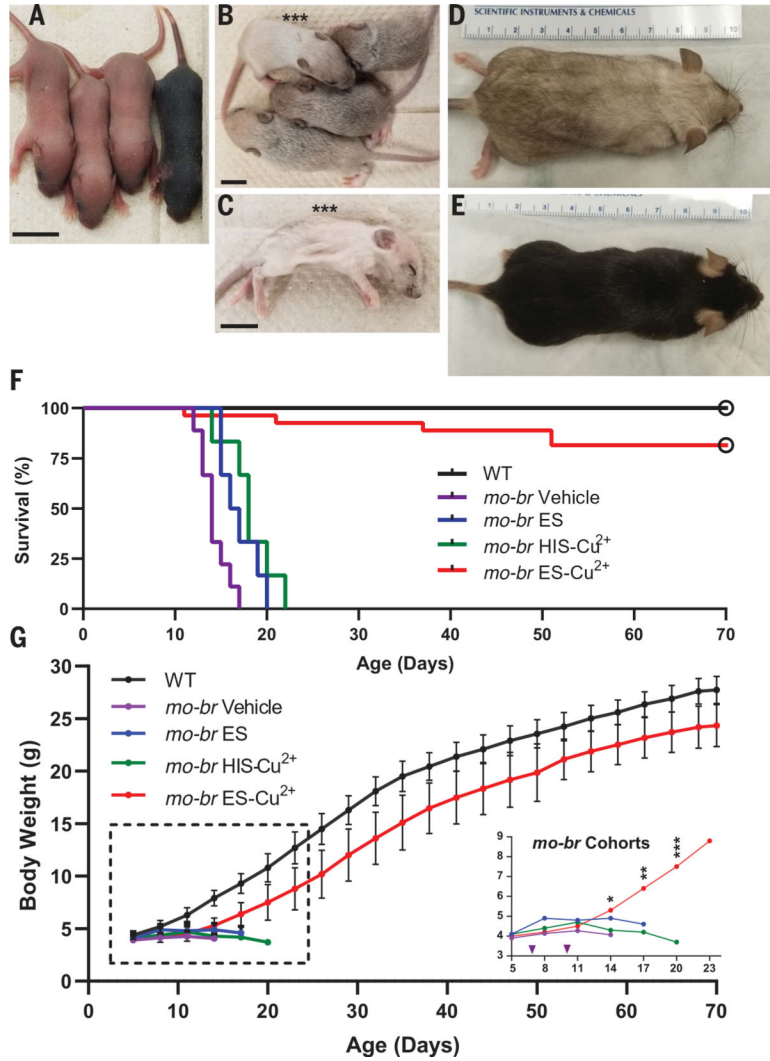


**Fig. 1. Effects of ES treatment in cardiac *Ctrl* KO mice.**

(A) Gross appearance of mice at PND 10. Asterisks (\*\*\*) indicate moribund KO vehicle mouse. (B) Kaplan-Meier survival curve. (C) Growth curves. WT and WT ES mice exhibited identical growth curves (WT ES omitted for clarity; see fig. S3A). Cohorts consisted of WT vehicle ( $n = 3$  mice), WT ES ( $n = 7$ ), KO vehicle ( $n = 3$ ), and KO ES ( $n = 9$ ). (D) Gross appearance of hearts at PND 10. Asterisks (\*\*\*) indicate hypertrophied KO vehicle mouse heart. (E) Heart-to-body weight ratio. Cohorts consisted of  $n = 6, 6, 8,$  and  $9$  animals, respectively. (F) Heart [Cu] levels. Cohorts consisted of  $n = 4$  per treatment. (G)

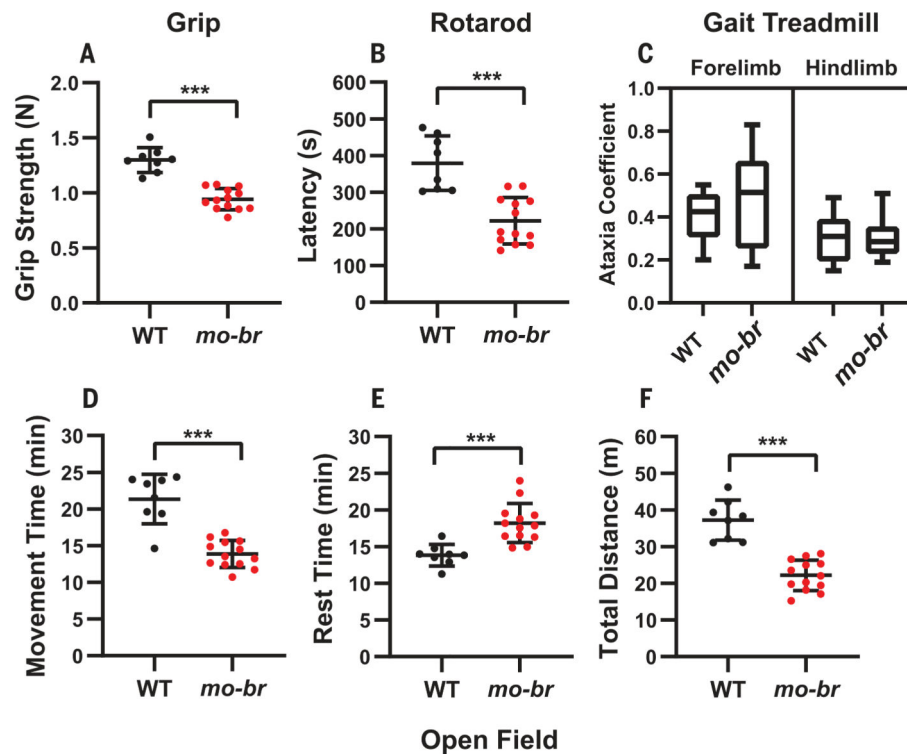


Heart COX1 levels. **(H)** Quantification of relative COX1 levels. Data are reported as means  $\pm$  SD with statistical significance assessed by one-way analysis of variance (ANOVA) with Tukey's post hoc test or Welch one-way ANOVA with Tukey's post hoc test. NS, not significant; \* $P < 0.05$ ; \*\*\* $P < 0.001$ . Western blot images in (G) were analyzed with ImageJ software.



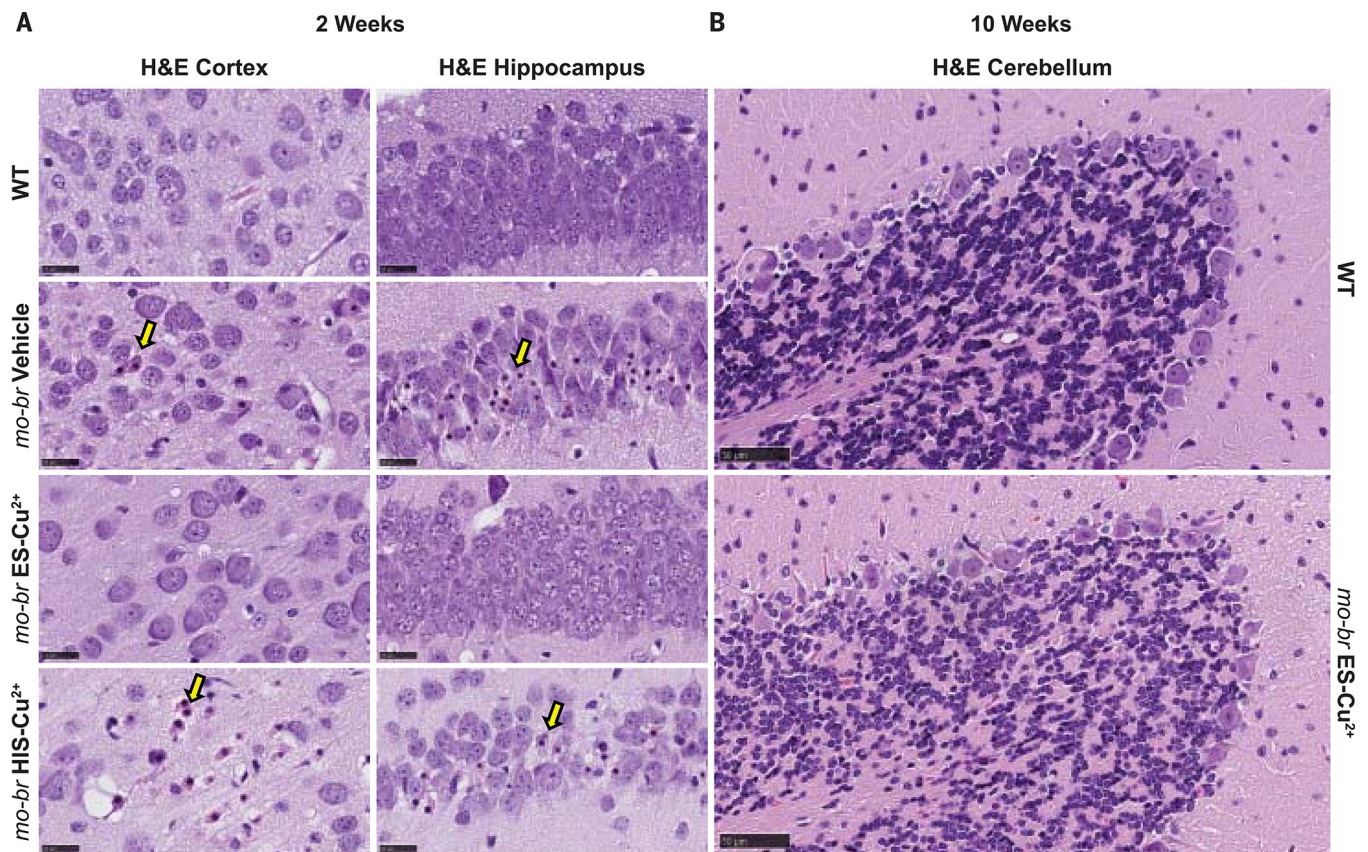
**Fig. 2. Effects of ES-Cu<sup>2+</sup> treatment in *mo-br* mice.**

(A) *Mo-br* hemizygous males and WT littermate at PND 5 before intervention. (B) Pigmentation changes in *mo-br* males administered ES-Cu<sup>2+</sup> compared with vehicle (\*\*\*) on PND 12. (C) Moribund *mo-br* vehicle (\*\*\*) mouse on PND 14. (D and E) *Mo-br* ES-Cu<sup>2+</sup> (D) and WT littermate (E) at PND 70. (F) Kaplan-Meier survival curve. All WT mice survived experimental protocol. (G) Growth curves of indicated groups: WT and WT ES-Cu<sup>2+</sup> mice exhibited near identical growth curves (WT ES-Cu<sup>2+</sup> omitted for clarity; see fig. S8E). Cohorts consisted of WT vehicle ( $n = 15$ ), WT ES-Cu<sup>2+</sup> ( $n = 13$ ), *mo-br* vehicle ( $n = 9$ ), *mo-br* ES ( $n = 6$ ), *mo-br* HIS-Cu<sup>2+</sup> ( $n = 6$ ), and *mo-br* ES-Cu<sup>2+</sup> ( $n = 27$ ). Inset shows a close-up of the curves in the region indicated by the dashed-line box, including the curves of *mo-br* cohorts only. Data are reported as means  $\pm$  SD with statistical significance assessed by one-way ANOVA test or Welch one-way ANOVA with Tukey's post hoc test. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Scale bars in (A) to (C), 1 cm.



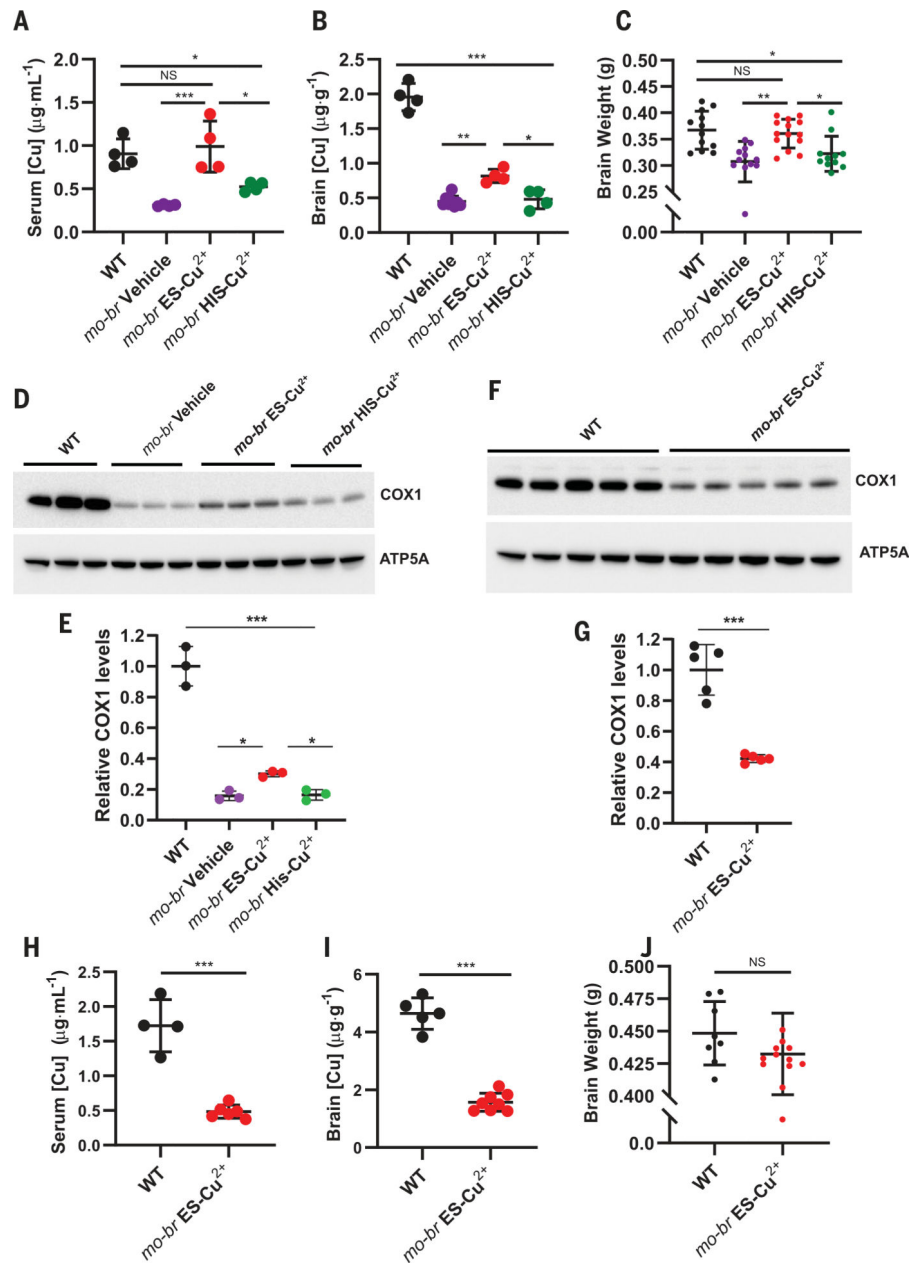
**Fig. 3. Neuromotor tests of 10-week-old mice.**

Mice were assessed by grip strength, rotarod, gait treadmill, and open field at 10 weeks of age. (A) Forelimb grip strength. (B) Rotarod. (C) Gait treadmill. DigiGait-generated ataxia coefficients of shoulder and pelvic girdles were statistically insignificant. (D to F) Open field: movement time (D), rest time (E), and total distance (F). Cohorts consisted of WT ( $n = 8$ ) and *mo-br* ( $n = 13$ ). Data are reported as means  $\pm$  SD with statistical significance assessed by unpaired  $t$  test. \*\*\* $P < 0.001$ .



**Fig. 4. Neuropathology of 2- and 10-week-old mice.**

(A) Hematoxylin and eosin (H&E)-stained sections of cortex and hippocampus from PND 14 WT and *mo-br* mice, administered vehicle, ES-Cu<sup>2+</sup>, or HIS-Cu<sup>2+</sup>. Somatomotor cortical neurons in *mo-br* vehicle and HIS-Cu<sup>2+</sup> cohorts exhibit marked, diffuse neurodegenerative changes, characterized by numerous pyknotic nuclei with eosinophilic cytoplasm (yellow arrows). In the hippocampus, the pyramidal neuron layer of region CA1 demonstrates degenerative changes including necrotic neurons in vehicle and HIS-Cu<sup>2+</sup> *mo-br* mice (yellow arrows). (B) Cerebellar peduncles from PND 70 WT and *mo-br* ES-Cu<sup>2+</sup> mice revealed preservation of continuous Purkinje neuron layer. Scale bars in (A), 25  $\mu$ m. Scale bars in (B), 50  $\mu$ m.



**Fig. 5. ES-Cu<sup>2+</sup> rescues biochemical phenotypes in 2- and 10-week-old *mo-br* mice.**

(A) Serum [Cu] at PND 14 (all cohorts,  $n = 4$ ). (B) Brain [Cu] at PND 14 (all cohorts,  $n = 4$ ). (C) Brain weights at PND 14 (WT,  $n = 12$ ; *mo-br* Vehicle,  $n = 12$ ; *mo-br* ES-Cu<sup>2+</sup>,  $n = 14$ ; *mo-br* HIS-Cu<sup>2+</sup>,  $n = 11$ ). (D and E) Brain COX1 at PND 14 (all cohorts,  $n = 3$ ). (F and G) Brain COX1 at PND 70 (all cohorts,  $n = 5$ ). (H) Serum [Cu] at PND 70 (WT,  $n = 4$ ; *mo-br* ES-Cu<sup>2+</sup>,  $n = 6$ ). (I) Brain [Cu] at PND 70 (WT,  $n = 5$ ; *mo-br* ES-Cu<sup>2+</sup>,  $n = 8$ ). (J) Brain weights at PND 70 (WT,  $n = 8$ ; *mo-br* ES-Cu<sup>2+</sup>,  $n = 13$ ). Data are reported as means  $\pm$  SD with statistical significance assessed by one-way ANOVA or Welch one-way ANOVA with Tukey's post hoc test. NS, not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Western blot images in (D) and (F) were analyzed with ImageJ software.

Table 1.

**Treatment regime and 10-week survival in the *mo-br* mouse.**

Dashes indicate a value was not applicable.

Cohort	Treatment					Survival		
	<i>n</i>	Exposure (mg kg <sup>-1</sup> )	Total (µg)	ES/HIS (µg)	Cu <sup>2+</sup> (µg)	Median (days)	Viable mice 10 weeks	Percent (%)
WT vehicle	15	–	–	–	–	–	15	100
WT ES-Cu <sup>2+</sup>	13	7.25	29	25.02	3.98	–	13	100
<i>mo-br</i> vehicle	9	–	–	–	–	14	0	0
<i>mo-br</i> ES	6	6.25	25.02	25.02	–	16.5	0	0
<i>mo-br</i> ES-Cu <sup>2+</sup>	27	7.25	29	25.02	3.98	203	22	81.5
<i>mo-br</i> HIS-Cu <sup>2+</sup>	6	5.83	23.3	19.35	3.98	18	0	0