

# Müllerian inhibiting substance contributes to sex-linked biases in the brain and behavior

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Many behavioral traits and most brain disorders are common to males and females but are more evident in one sex than the other. The control of these subtle sex-linked biases is largely unstudied and has been presumed to mirror that of the highly dimorphic reproductive nuclei. Sexual dimorphism in the reproductive tract is a product of Müllerian inhibiting substance (MIS), as well as the sex steroids. Males with a genetic deficiency in MIS signaling are sexually males, leading to the presumption that MIS is not a neural regulator. We challenge this presumption by reporting that most immature neurons in mice express the MIS-specific receptor (MISRII) and that male *Mis*<sup>-/-</sup> and *Misrii*<sup>-/-</sup> mice exhibit subtle feminization of their spinal motor neurons and of their exploratory behavior. Consequently, MIS may be a broad regulator of the subtle sex-linked biases in the nervous system.

anti-Müllerian hormone | exploratory behavior | motor neuron | sexual dimorphism

Many behavioral traits are more evident in one sex than the other, but they do not define the biological sex of an individual. Empathy, for example, has a female bias, but some of the greatest men are empathetic. Likewise, girls engage in less rough-and-tumble play than boys, but a boy who shuns rough-and-tumble play is still a boy. With traits such as these, the characteristics of individual males and females overlap, with the sex-linked bias existing only within the population as a whole. This subtle form of sexual dimorphism is pervasive throughout the nervous system and involves nuclei whose function is not directly related to reproduction. In this paper, we refer to this form of sexual dimorphism as sex-linked bias, to distinguish it from the sexual dimorphism of the primary reproductive tissues, which is binary in nature.

The nervous system also contains nuclei where the male and female forms do not overlap. Such nuclei control primary sexual function, with their dimorphic nature being generated by testosterone, or its active metabolites (1). The regulation of sex-linked bias in the brain has been widely assumed to mirror that of the sexually dimorphic nuclei. However, testosterone is only episodically present during male development, with third-trimester male fetuses and boys older than 1 year having minimal levels of testosterone (2–4). Extensive brain development occurs during these periods, with some regions having increased anatomical dimorphism during the period when testosterone is minimal (5, 6). Thus, although the sex steroids contribute to sex-linked biases, other factors must also be important. We present evidence here that Müllerian inhibiting substance (MIS) (synonym, anti-Müllerian hormone) is one of these factors.

MIS is a testicular hormone that triggers the regression of the uterine precursor (Müllerian ducts) in males around the ninth week of gestation (7). However, the level of MIS in the blood of males does not decline after the loss of the Müllerian ducts, remaining high until puberty, at which stage it falls by an order of magnitude (8–10). The low level of ovarian production of MIS, in contrast, begins only postnatally (8–10). High levels of plasma MIS are thus a unique feature of developing males. This hormonal MIS may affect the maturation of the fetal lung (11), but there is currently no explanation for why MIS is present in

male blood for such an extended period of development. We report here that most developing neurons express the MIS-specific receptor, MISRII, and that male mice with null mutations in either *Mis* or *Misrii* have partial feminization of their sex-linked biases, even though they are phenotypically and sexually male. This finding implicates MIS as a regulator of sex-linked bias.

## Results

**The Testes Is the Sole Embryonic Source of MIS.** The gonads have historically been considered to be the sole source of MIS, but we have recently shown that mature motor neurons in both sexes also produce it (12). We therefore determined whether production of MIS also occurred in the developing brain. In confirmation of the historic understanding, only trace levels of MIS mRNA were detected in the embryonic head, which by mass is predominantly the brain. The only proportion of the embryo that contained significant levels of MIS mRNA was the urogenital region (Fig. S1A). Similarly, MIS protein was detected by immunohistochemistry in the testes (Fig. S1B), but not in the developing brain (Fig. S1C). This finding contrasts with the mature brain, where motor (12) and various other neurons contain readily detectable levels of MIS.

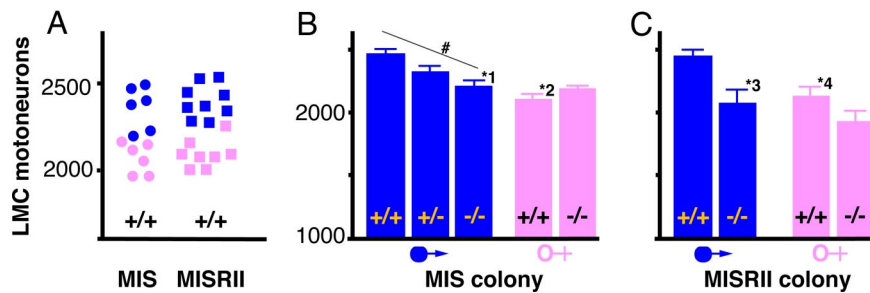
**MIS Regulates the Number of Spinal Motor Neurons.** The numbers of motor neurons that innervate the limbs have been extensively studied, but the possibility that they exhibit subtle sex-linked bias has only rarely been considered (13). Our recent observation that MIS promotes the survival of embryonic motor neurons in vitro (12) suggests that they should be. Simply, over one-half of the motor neurons present in the spinal cords of mouse embryos die between the 13th day of gestation and birth (14), with the extent of this cell death being controlled by multiple survival factors. MIS is produced in the embryonic testes from the 12th day of gestation onward (15), which should attenuate motor neuron loss in male fetuses, provided that MIS is a neuronal survival factor in vivo. Consistent with this prediction, the number of motor neurons in the lumbar lateral motor column (LMC) of wild-type male and female mice had almost no overlap in both the *Mis* and *Misrii* colonies (Fig. 1A). The magnitude of the male bias was 16–17% in both colonies. This sex-linked difference depended on MIS, because MIS-deficient (*Mis*<sup>-/-</sup>) males had female numbers of motor neurons (Fig. 1B), with heterozygous males (*Mis*<sup>+/-</sup>) being intermediate between wild-type males and females (Fig. 1B). The effect of *Mis* gene dose on motor neuron number was significant ( $P = 0.03$ ) by regression analysis.

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**Fig. 1.** The LMC of the lumbar spinal cord has MIS-dependent dimorphism. (A) Number of motor neurons in male (blue) and female (pink) wild-type mice derived from matings of either *Mis*<sup>+/-</sup> (circles) or *Misrii*<sup>+/-</sup> parents (squares). (B and C) Effect of *Mis* (B) or *Misrii* (C) genotype on the sexual dimorphism illustrated in A. The bars are the mean  $\pm$  standard error of the mean of 6–9 mice. \*, Significant difference (Student's *t*) from the wild-type males: 1,  $P = 0.002$ ; 2,  $P < 0.001$ ; 3,  $P = 0.013$ ; 4,  $P = 0.010$ . #, A significant effect ( $P = 0.030$ ) of gene dose by regression analysis.

MIS is a member of the TGF $\beta$  superfamily, which signals through a complex consisting of type I and type II receptors. MISRII is thought to be a unique and obligatory receptor for MIS, because null mutations of *Mis* and *Misrii* both lead to persistent Müllerian duct syndrome (retention of the uterine precursor in males) (7). MISRII mRNA in neurons is abundant compared with the mRNA for other growth factor receptors but is an order of magnitude less abundant than that present within the classical MIS targets (Müllerian ducts, testes) (12, 16). This observation raises the issue of whether MISRII is essential for MIS signaling in neurons. Consistent with this possibility, male *Misrii*<sup>-/-</sup> mice had feminized numbers of LMC motor neurons (Fig. 1C), a malformation that mirrored that seen in the *Mis*<sup>-/-</sup> male mice (Fig. 1B). Female *Mis*<sup>-/-</sup> and *Misrii*<sup>-/-</sup> mice both had wild-type numbers of LMC motor neurons (Fig. 1B and C), which was expected given that female embryos do not produce MIS (15, 17). Consequently, MISRII is a useful marker for MIS-sensitive neurons (see below).

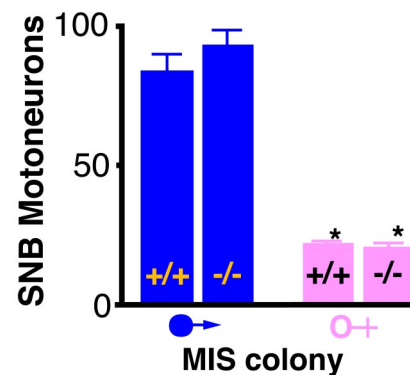
**Androgen-Dependent Dimorphism Is Independent of MIS.** The motor neurons involved in primary sexual function are highly dimorphic, under the control of testosterone acting through the androgen receptor (18, 19). Some LMC motor neurons express androgen receptors (18), creating the possibility that the effect of MIS on LMC neurons is secondary to perturbation of testosterone function. If this was occurring, then testosterone-dependent dimorphism should be diminished in the *Mis*<sup>-/-</sup> mice. We therefore examined the spinal bulbocavernosus motor nucleus, which is involved in the function of the penis (18, 19). In marked contrast to the LMC motor neurons, the numbers of motor neurons in the bulbocavernosus nucleus of male *Mis*<sup>-/-</sup> mice were similar to those of a wild-type male and were overtly different from the female number (Fig. 2).

**Most Neurons Express MIS Receptors.** All of the classical neuronal survival factors affect multiple types of neurons. We therefore examined whether MIS was a specific regulator of limb-innervating motor neurons or a broad regulator of the nervous system. Consistent with the latter, the levels of mRNA for the MIS-specific receptor in the embryonic brain and spinal cord were similar. The cellular lineages that produce MISRII were identified by using genetically modified mice that produce lacZ in MISRII-expressing cells (MISRII-Cre-lacZ) (Fig. S2). Most neurons in the brain, spinal cord, and peripheral nervous system of adult MISRII-Cre-lacZ mice were lacZ-positive, whereas the glia, blood vessels, and fibroblasts were lacZ-negative (Fig. 3A–C). Once lacZ is induced in a cell in the MISRII-Cre-lacZ mice, all daughter cells will express it (Fig. S2). The immediate precursor of neurons (neuroepithelia) undergo a series of stem cell-like divisions, which give rise to postmitotic neurons during the early stages of the lineage and various glia in the terminal divisions. The absence of lacZ in the glia

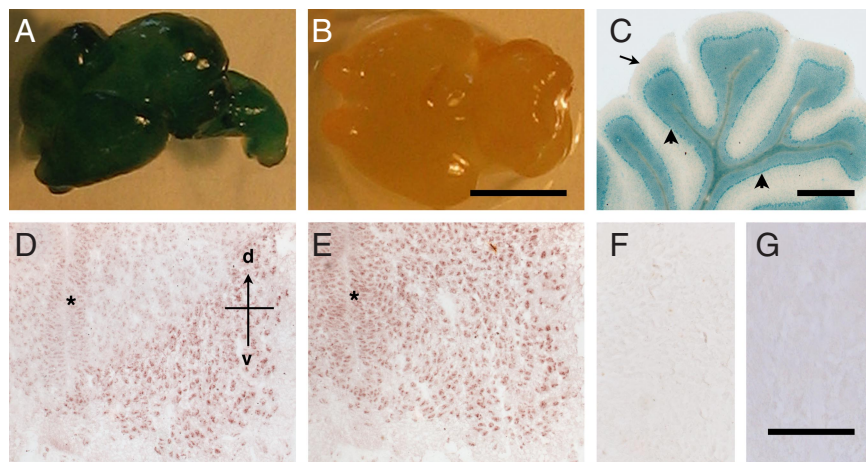
thus indicates that postmitotic neurons are the only cells within the neuronal lineage to express significant levels of MISRII. This contention was verified by examining the brains of MISRII-Cre-lacZ embryos. The neuroepithelium of the spinal cord and brain were lacZ-negative, whereas lacZ-positive neurons were abundant in the brains of embryonic day 12 (E12) MISRII-Cre-lacZ embryos. This finding indicates that lacZ reporter is activated within 24 h of neurons becoming postmitotic and beginning to differentiate.

Sections of embryonic brains were then stained with an antibody to MISRII to determine whether the expression of MISRII in neurons is continuous during development and whether all developing neurons expressed MISRII at a similar level. The antibody is specific to MISRII as it immunoprecipitates a single protein from the spinal cord (12) and does not stain sections of *Misrii*<sup>-/-</sup> brains (Fig. 3G). The anti-MISRII antibody stained most neurons in the developing brain and spinal cord but with differing intensities. The motor neurons in the spinal cord, for example, were more intensely stained than adjacent interneurons, although this may merely be because motor neurons are comparatively large and are the most mature neurons in the developing brain. This differential staining of motor and interneurons persisted throughout development and is also evident in the mature spinal cord (12). The staining of neurons in the ventral spinal cord preceded that of the dorsal cord, with dorsal spinal neurons having only trace levels of MISRII protein at E12 (Fig. 3D). This dorsal–ventral difference was lost by E14 (Fig. 3E).

**Exploratory Behavior Is MIS Dependent.** We then examined whether the expression of MISRII in the brain leads to a male bias in a



**Fig. 2.** The dimorphism of the spinal bulbocavernosus (SNB) motor neurons is normal in mice that lack the *Mis* gene. The bars are the mean  $\pm$  standard error of the mean of 4–6 mice. \*, Both female groups are significantly different from both of the male groups,  $P < 0.001$ .



**Fig. 3.** Localization of MISRII in the nervous system. (A–C) MISRII-Cre-lacZ lineage tracing. (A) Whole brain of a MISRII-Cre-lacZ E16 embryo. (B) Littermate control (*Misrii*<sup>+/+</sup>, ROSA26-lacZ Cre). (C) Section of the cerebellum of a MISRII-Cre-lacZ adult mouse showing lacZ stain in neurons (arrowheads) but not the white matter (arrow). (D–G) Immunohistochemical detection of MISRII. Sections of lumbar spinal cord of E12 (D) and E14 (E) embryos stained with an anti-MISRII antibody. The asterisk indicates the neuroepithelium, with the dorsal–ventral (d–v) axis indicated. (F) Section of E14 spinal cord stained with control IgG. (G) Section of the cerebellum from a *Misrii*<sup>-/-</sup> mouse stained with the anti-MISRII antibody. (Scale bars: B, 10 mm; C, 1 mm; F, 100  $\mu$ m.)

behavioral trait. Male mice have larger territories and a greater tendency to explore than females. When mice were placed in a nocturnal open-field chamber, the males and female traversed the floor to similar extents (Fig. 4A), but the wild-type males reared twice as often as the wild-type females, in an attempt to move beyond the confines of the chamber (Fig. 4B). In marked contrast to the wild-type males, the behavior of *Misrii*<sup>-/-</sup> males was indistinguishable from that of the *Misrii*<sup>+/+</sup> and *Misrii*<sup>-/-</sup> females (Fig. 4A and B). This is consistent with the male bias in this behavior being generated by MIS. In other tests, such as running on a rotarod, *Misrii*<sup>+/+</sup> and *Misrii*<sup>-/-</sup> male mice were indistinguishable (Fig. 4C). This is also consistent with MIS regulating the subtle sex-linked differences in the brain, while being entirely dispensable for acquisition of the nondimorphic features of the brain.

## Discussion

**MIS Contributes to Sex-Linked Biases in the Brain.** Sex-linked biases are pervasive throughout the nervous system, as evidenced by quantitative anatomical, pharmacological, fMRI, and behavioral studies (6, 20, 21). The current report provides evidence that the testicular hormone MIS contributes to these sex-linked biases.

The natural expression of MIS in the current study was sufficient to increase the number of neurons in the LMC in male mice to beyond the female range (Fig. 1) and to produce male exploratory behavior that was overtly different than that of female mice (Fig. 4). The clarity of these sex-linked differences reflects the fact that the mice had minimal genetic variation and had been raised in controlled conditions. In human populations and outbred colonies, the size of motor nuclei varies greatly between individuals (22). In such populations, a 16% male–female difference (Fig. 1) would present as a subtle male bias, with the male and female populations having extensive overlap.

**MIS May Contribute to Variation in the Male Population.** The expression of MIS varies between individual males, which may contribute to diversity within the male population. In 1-year-old boys, for instance, the plasma concentration of MIS ranges from 10 to 444 ng/ml ( $n = 40$ ; girl range, 1–4 ng/ml) (ref. 8; see also ref. 23). The effect of MIS on neurons is dose dependent, both in vitro (12) and in vivo (Fig. 1B). Hence, MIS would be expected to produce a population of males that contains individuals who

are little different than females for a particular trait and other individuals who exhibit an extreme male phenotype for that trait.

The incidences and/or severities of most brain disorders exhibit a sex-linked bias (24). This observation suggests that the sex-linked biases in the brain are able to either attenuate or amplify the consequences of underlying brain pathology. If so, then individuals with a high expression of MIS may be predisposed to develop certain conditions with a male bias (e.g., attention-deficit hyperactivity disorder, autism, motor neuron disease) and to be relatively protected against some female-bias conditions (e.g., anorexia).

**MIS May Be a Broad Regulator of Neurons.** The neural basis of the sex-linked difference in the exploratory behavior of mice is unknown. However, the initiation of movement is controlled in the brain proper, indicating that the observed MIS-dependent bias in exploratory behavior is unlikely to be secondary to the effect of MIS on spinal motor neurons. The MIS receptor is broadly present in brain neurons (Fig. 3) (25), thus providing multiple theoretical pathways by which MIS could regulate exploratory and other behaviors. Furthermore, the physiological actions of neural regulators are often context dependent, variably regulating neuronal number, pattern of synaptic connection, neurotransmitter type, and neurotransmitter expression in a neuron-specific and stage-specific manner (26). Thus, although the current data implicate MIS as a regulator of neural networks, the definition of the breadth of its action will require detailed investigations of many different types of neurons.

**The Neural Actions of MIS Are Cryptic.** *Mis*<sup>-/-</sup> and *Misrii*<sup>-/-</sup> boys and male mice have been extensively studied with respect to the persistence of their Müllerian ducts. This fact raises the issue of why MIS has not previously been recognized as a regulator of the nervous system. Two issues may be relevant here.

First, the role of MIS in the brain appears to relate to sex-linked biases, rather than to binary sexual dimorphism. Sex-linked biases are evident only at the level of the population and would not be apparent unless groups of MIS-deficient individuals were compared with their peers. In humans, *Mis*<sup>-/-</sup> and *Misrii*<sup>-/-</sup> males have a persistent Müllerian duct, which prevents the descent of their testes (7). This malformation is readily detected, but *Mis*<sup>-/-</sup> and *Misrii*<sup>-/-</sup> men are very rare and have not been systematically studied. MIS has both positive and



Houston, TX). The *Mis*<sup>+/-</sup> mice were also generated from AB-1 stem cells (35) and backcrossed to C57BL/6 by The Jackson Laboratory, from whom they were purchased. Both the *Mis* and *Misrii* colonies have been maintained by brother-sister matings at the University of Otago. The ROS26-lacZ Cre reporter mice (36) were obtained from a colony at the University of Otago. The housing of the mice was as described in ref. 37, with the genotype determined by PCRs using the primers listed in Table S1.

**Neuronal Counts.** Wild-type, heterozygous, and null-mutant neonates from *Mis*<sup>+/-</sup> × *Mis*<sup>+/-</sup> and *Misrii*<sup>+/-</sup> × *Misrii*<sup>+/-</sup> matings were killed at birth by decapitation and fixed by immersion in 4% paraformaldehyde in 0.01 M sodium phosphate buffer, pH 7.4 (PB). The caudal spinal cords from the midthoracic region were removed for analysis of the lumbar LMC. Cell death in the murine LMC is complete by birth (14), and this age is the standard for estimation of LMC motor neurons (38). The spinal bulbocavernosus (SNB) motor neurons develop later than LMC motor neurons and may be dually influenced by prenatal and postnatal testosterone (18). The numbers of motor neurons in this nucleus were therefore determined by using spinal cords from 6-week-old mice that have been fixed by cardiac perfusion with 4% paraformaldehyde. The spinal cords were embedded in Technovit resin, serially sectioned at either 40 μm (LMC) or 30 μm (SNB) and stained with cresyl violet. The numbers of motor neurons were estimated by using an optical dissector (39), with the counting particle being the nucleolus. Every fifth section was examined through the entire LMC, whereas all sections containing the SNB were analyzed. Examples of LMC and bulbocavernosus motor neurons are illustrated in Fig. S3. The cell counts were done blind with respect to the sex and the genotype of the mice.

**MISRII-Cre-lacZ.** *Misrii*<sup>+/-</sup> dams were time mated with a ROSA26-lacZ Cre reporter stud, and the brains from the resulting MISRII-Cre-lacZ fetuses (Fig. S2) were collected at 13, 14, 16, or 20 days of gestation, or after the pups had matured to adulthood (6–8 weeks). The fetal brains were fixed on ice for 2 h in a solution containing 2% paraformaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P-40, and 0.01% sodium deoxycholate, and after washing they were stained overnight in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM MgCl<sub>2</sub>, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, and 0.1 M PB (40). The tails of the fetuses were used for genotyping, with the ROSA26-lacZ Cre, *Misrii*<sup>+/-</sup> pups serving as controls. The adult brains were snap-frozen in isopentane and serially sectioned in a cryostat, and the resulting sections were fixed for 10 min at 4 °C in 2% paraformaldehyde in 0.1 M PB containing 2 mM MgCl<sub>2</sub>, pH 7.3, after which they were washed in PBS containing 2 mM MgCl<sub>2</sub>, and stained overnight at room temperature with 2 mM MgCl<sub>2</sub>, 20 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 20 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, and 4 mg/ml X-Gal in PBS.

**Immunohistochemistry.** Transverse sections of lumbar spinal cords and testes from E12, E14, E16, and E18 embryos, neonates, and adult wild-type mice were cut in a cryostat at a thickness of 10 μm. The sections were stained by immunohistochemistry as described in ref. 41, using either goat anti-MISRII or goat anti-MIS (R&D Systems), with nonimmune IgG (Sigma) used as a control antibody. Sections of the brains of adult *Misrii*<sup>-/-</sup> mice were used as an additional negative control. The immunoreactivity was visualized by using 3-amino-9-ethylcarbamide (Sigma).

**Real-Time PCR.** Five 12-day-old C57BL/6 fetuses were dissected into the portions listed in Fig. S1, and total RNA fractions were prepared for each portion by using TRIzol reagent (Invitrogen). The isolated RNA fractions were treated with DNase I (Promega) to remove genomic DNA contamination and cDNA was synthesized by using SuperScript II RNase H<sup>-</sup> (Invitrogen) and oligo(dT)<sub>20</sub>. The real-time PCRs were performed by using an ABI Prism 7000 (Applied Biosystems), SYBR Green Master Mix (Applied Biosystems), and gene-specific primers (12). A 2-step PCR was carried out with denaturation at 95 °C for 15 s, annealing and extension combined at 60 °C for 1 min in a total of 40–50 cycles. The uniqueness of amplicons was analyzed by using dissociation curves and by sequencing. Standard curves were generated for each gene, and the copy number of the mRNA transcripts was calculated and normalized relative to the level of GAPDH.

**Behavioral Analysis.** The 9-week-old wild-type and null-mutant mice from 11 litters from *Misrii*<sup>+/-</sup> studs and dams were placed in an open-field apparatus (Opto-varimex; Columbus Instruments) under nocturnal lighting conditions (37) (40 mice; 10 male and 9 female *Misrii*<sup>+/+</sup>, plus 10 male and 11 female *Misrii*<sup>-/-</sup>). The mice from 10 of the 11 litters were subsequently tested on a Rotamex rotarod (Columbus Instruments) when 10–12 weeks of age (10 male and 7 female *Misrii*<sup>+/+</sup>, plus 8 male and 7 female *Misrii*<sup>-/-</sup>). The mice were tested by their regular caregiver in an anteroom attached to the room that housed the mice. The apparatus was cleaned after each mouse.

With the open-field test, each mouse was placed in the center of the 43 × 43 cm chamber and its movements were tracked for 20 min by an array of infrared beams. The movement of the mice across the floor of the apparatus was recorded by a computer, along with a record of the number of times the mice reared on their hindlimbs. As the test progressed, the mice spent less time traversing the floor of the apparatus and were more likely to rear in an attempt to move beyond the chamber. The data are presented as the distance traveled in the first 10 min and the number of rearings in the second 10 min of the test, because these correspond to the periods when the behavior was predominantly occurring. The observed effects of sex and genotype were similar when the data for the entire 20 min were analyzed.

Mice were trained to run on the rotarod for 3 consecutive days and tested on the fourth day. The initial speed of the rotarod was 5 rpm, with an acceleration of 0.5 rpm every 5 s, and with the mouse's presence on the rod being automatically detected by infrared beams. Each mouse was tested 3 times, and the average and maximum performances of the mice were analyzed. Both methods of analysis gave comparable results and only the maximum performance of the mice is presented here.

**Statistical Analysis.** The data were examined by 1-way ANOVA for sex differences (Fig. 1A) or by 2-way ANOVA for sex and genotype (Fig. 1B and C) (2, 4). Significant effects were confirmed by Student's *t* tests. The effect of gene dose on LMC motor neuron number was examined by linear regression (Fig. 1B).

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