Mullerian Inhibiting Substance acts as a motor neuron survival factor *in vitro*

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The survival of motor neurons is controlled by multiple factors that regulate different aspects of their physiology. The identification of these factors is important because of their relationship to motor neuron disease. We investigate here whether Mullerian Inhibiting Substance (MIS) is a motor neuron survival factor. We find that motor neurons from adult mice synthesize MIS and express its receptors, suggesting that mature motor neurons use MIS in an autocrine fashion or as a way to communicate with each other. MIS was observed to support the survival and differentiation of embryonic motor neurons *in vitro*. During development, male-specific MIS may have a hormone effect because the blood–brain barrier has yet to form, raising the possibility that MIS participates in generating sex-specific differences in motor neurons.

Mullerian Inhibiting Substance type II receptor

Motor neurons are particularly prone to age-related deterioration (1–3), which, in the extreme, leads to motor neuron disease and to death by paralysis. The survival of motor neurons is controlled by multiple factors, each of which appears to have a different physiological role. Motor neurons are, for instance, regulated by skeletal muscle fibers and Schwann cells via cardiotrophin-1 (4), TGF-β2 (5, 6), and glial-cell-line-derived neurotrophic factor (GDNF) (7, 8). Motor neurons also receive protection against viral- and hypoxic-induced damage through IL-6 (9) and VEGF (10, 11), respectively. Variations in the VEGF gene cause adult-onset motor-neuron degeneration in some mice and have been linked to ALS in some human populations (10, 11). These findings have renewed interest in identifying nonclassical neuronal survival factors.

Mullerian Inhibiting Substance (MIS) is examined herein as a motor-neuron survival factor given that we found high expression of ligand and receptors in motor neurons. MIS is a member of the TGF-β superfamily, which includes motor-neuron survival factors, such as GDNF and TGF-β2. The known physiological actions of MIS are thought to be limited to sexual differentiation of males and to the function of mature reproductive tissues of both sexes (12). These studies introduce a possible function for this interesting molecule and its known signaling pathway.

TGF-β superfamily members signal through a complex of type I and type II receptors (13). MIS has a unique type II receptor (MISRII) but shares type I receptors with other members of the superfamily (12, 13). Genetic, organ culture, and cellular evidence implicates activin receptor-like kinase 3 (ALK3) (14) and ALK2 (Y. Zhan, D.T.M., and P.K.D., unpublished data) (15) as type I receptors for MIS in murine sexual differentiation, although ALK6 is likely to be involved in other cellular contexts (12, 16).

We find that adult motor neurons from male and female mice synthesize MIS and its receptors, with the MIS receptor mRNA in motor neurons being much more abundant than the mRNAs for the GDNF and TGF-β receptors. Our experiments show that MIS supports the survival of embryonic motor neurons *in vitro* at physiological concentrations, suggesting that mature motor neurons use MIS to communicate with each other and/or to maintain themselves. These findings identify MIS as an unanticipated and significant molecule of interest for motor neuron diseases.

Materials and Methods

Animals. All experiments were approved by the University of Otago's Animal Ethics Committee. The mice used in this study were 5- to 8-week-old adult C57Bl6 mice and were bred by the University of Otago. Murine fetuses were from time-mated dams.

Isolation of Motor Neurons. Lumbar spinal cords were embedded in OCT medium and snap frozen. Sections (10 μm) were cut in a cryostat, stained with cresyl violet, and dehydrated in ethanol, followed by xylene. The PixCell 2 laser capture microdissection (LCM) System and CapSure HS LCM Cap (Arcturus, Mountain View, CA) were used to dissect motor neurons. The motor neurons were identified based on their location and large nuclear diameter (>9–10 μm). Typically 4–500 motor neurons were collected from each mouse.

RNA Preparation, cDNA Synthesis, and Real-Time PCR. Total RNA fractions were isolated with TRIzol reagent (Invitrogen) for tissues or the PicoPure RNA isolation kit (Arcturus) for laser-captured motor neurons. The isolated RNA fractions were initially treated with DNase I (Promega) to remove genomic DNA contamination. The cDNA was synthesized with SuperScript II RNase H^- (Invitrogen) and oligo-d$(T)_{15}$ as the primer. The real-time PCRs were performed by using an Applied Biosystems Prism 7000, SYBR Green Master Mix (Applied Biosystems), and gene-specific primers (see Table 1, which is published as supporting information on the PNAS web site). A two-step PCR was carried out with denaturation at 95°C for 15 s and 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.

Immunoprecipitation and Western Blotting. Murine spinal cords and testes were lysed in buffer containing 150 mM NaCl, 20 mM Tris·HCl (pH 7.4), 1% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, and 10% Complete solution (Roche Mo-

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Abbreviations: GDNF, glial-cell-line-derived neurotrophic factor; MIS, Mullerian Inhibiting Substance; MISRII, type II MIS receptor; TβRII, type II TGF-β receptor; rh-, recombinant human; LCM, laser capture microdissection; ALK, activin receptor-like kinase.

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lecular Biochemicals). Immunoprecipitation was carried out with an MISRII antibody (R & D Systems) overnight at 4°C followed by adsorption with protein G-Sepharose (Sigma) for 2 h at 4°C. Immunoprecipitates were washed three times in buffer (150 mM NaCl/20 mM Tris·HCl, pH 7.4) and eluted by boiling in NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen) and NuPAGE lithium dodecyl sulfate reducing agent (Invitrogen) for 5 min. Proteins were separated by electrophoresis of NuPAGE Bis-Tris gels (Invitrogen) and transferred onto 0.2-μm polyvinylidene difluoride membranes (Invitrogen). Blocking buffer containing 1% casein (Amersham Biosciences, which is now GE Healthcare) and 0.04% Tween 20, MISRII antibody (R & D Systems) and peroxidase-conjugated anti-goat IgG antibody (Jackson ImmunoResaerch) were used for Western blotting. The bands were visualized by enhanced chemiluminescence (ECL Plus kit, Amersham Biosciences).

Immunohistochemistry. Transverse sections of lumbar spinal cords, ovaries, and testes were cut in a cryostat at a thickness of 10 μm. The sections were stained by immunohistochemistry as described by ref. 17. Briefly, the sections were fixed in 1% or 4% neutral-buffered paraformaldehyde at 4°C, washed, and incubated in 5% donkey serum and then in either goat anti-MISRII (R & D Systems), rabbit anti-MISRII developed and affinity-purified at Massachusetts General Hospital (R. V. Pieretti, P.K.D., P. S. Szotek, T. Manganaro, M. Lorenzen, J. Lorenzen, E. F. Halpern, and D.T.M., unpublished data), or goat anti-MIS (R & D Systems) and goat anti-ALK3 (R & D Systems) overnight at 4°C. The slides were then sequentially incubated with a biotinylated donkey anti-IgG antibody (Sigma), methanol/H_2O_2, and a streptavidin-biotinylated horseradish peroxidase complex (Amersham Biosciences). The immunoreactivity was visualized with 3-amino-9-ethylcarbamide (Sigma) or NovaRed (Vector Laboratories) as the chromogen. In a few experiments, the immunoreactivity was visualized with a fluorescent 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid-conjugated secondary antibody (BODIPY-FL, Molecular Probes). Nonspecific binding was controlled for by replacing the primary antibody with nonimmune IgG (Sigma).

Motor Neuron Cultures. The spinal cords of 13- to 15-day-old (embryonic day 13 to embryonic day 15) fetuses were dissected and incubated for 15 min at 37°C in Dulbecco's PBS, pH 7.2 (Sigma) containing 10 μM 2-mercaptoethanol, 0.05% trypsin (Sigma), and 0.04% EDTA. Trypsin inhibitor at 0.033% (Sigma) was then added, and the cords were dissociated. The resulting cell suspension passed through a 100-μm mesh (Sigma) overlaid onto 10.4% OptiPrep (Sigma) in Dulbecco's phosphate-buffered saline and centrifuged for 20 min at 400 $\times$ g. The motor neurons (2,000 cells per cm^2) were cultured under serum-free conditions in neurobasal medium (Invitrogen) with B27 supplement and 500 μM glutamine at 37°C with 5% CO_2. Recombinant human MIS (rhMIS) and rhGDNF (Alomone Labs, Jerusalem, Israel) were added immediately after seeding. Half the volume of medium was changed after 2 days. Four days after plating, the cultures were stained with antibody to the motor neuron marker, anti-islet-1 (39.4D5, Developmental Studies Hybridoma Bank), and the immunoreactivity was developed as above by using biotinylated-anti-mouse IgG (Jackson ImmunoResearch). The numbers of surviving motor neurons were determined by counting islet-1^{+ve} neurons with a neurite in three randomly selected fields in each well. Three wells were used for each concentration of factor, and the experiment was replicated four times. Virtually all cells were stained by islet-1, indicating that the cultures were pure.

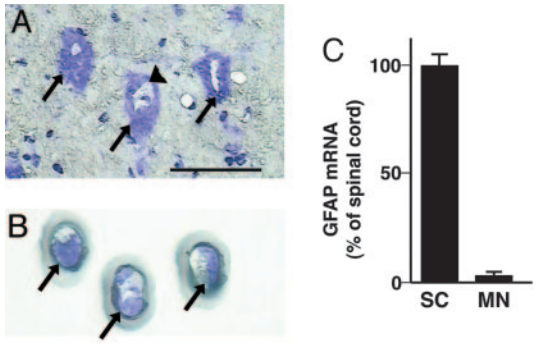

Fig. 1. LCM of motor neurons. (*A*) Cryostat section of lumbar spinal cord stained with cresyl violet. The arrows point to motor neurons; the arrowhead identifies the nucleus of one of the neurons. (Scale bar, 50 μm.) (*B*) The motor neurons removed by LCM. (*C*) The copy number of the glial marker, glial fibrillary acidic protein, in the isolated motor neurons (MN) and whole spinal cord (SC) was measured by real-time PCR and normalized relative to the abundance of GAPDH. The bars are the mean ± SEM of 11 motor neuron and four spinal cord samples.

Purification of rhMIS. Bioactive rhMIS was immunoaffinity-purified from Chinese hamster ovary cells as described in ref. 18, and its potency was validated in a MIS-specific Mullerian duct regression assay (19). The MIS produced by this method is 140 kDa (70-kDa disulfide-linked homodimer) that has been activated by proteolytic processing before secretion. The 25-kDa carboxyl domain, in which bioactivity resides, remains in noncovalent association with the amino terminus.

Results

MISRII Is Abundant in Motor Neurons. Motor neuron cell bodies have an unusually high abundance of mRNA. Consequently, we were able to obtain pure mRNA from the cell bodies of mature motor neurons by using LCM (Fig. 1). This method enabled us to determine the abundance (copy number) of MIS receptors and other mRNAs in the motor neurons from individual mice. MIS signals through MISRII, which works in concert with a type I receptor (12, 13). The isolated motor neurons were a comparatively rich source of MISRII mRNA (Fig. 2). The levels of MISRII transcripts in the isolated motor neurons were 30 times higher than in either the whole spinal cord or brain, indicating that most neurons and glia do not produce MISRII. This finding explains why MISRII has not previously been detected in the nervous system. The other parts of the neuromuscular system

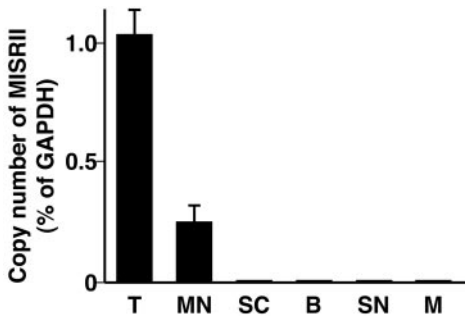

Fig. 2. The abundance of MISRII transcripts in laser-captured motor neurons and other tissues. The copy number of MISRII transcripts was measured by real-time PCR and normalized relative to that of GAPDH transcripts. The tissues examined were testes (T), laser-captured motor neurons (MN), spinal cord (SC), brain (B), sciatic nerve (SN), and skeletal muscle (M). The bars are the mean ± SEM of 11 motor neurons and four samples for each of the other tissues.

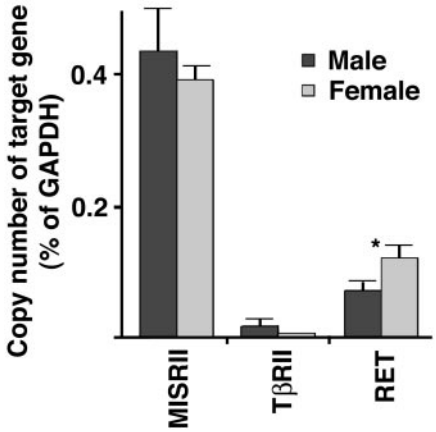

Fig. 3. Detection of MISRII, TβRII, and RET mRNA in spinal motor neurons. The motor neuron mRNA was collected from laser-captured motor neurons and quantified by using real-time PCR. The copy numbers of the target genes were calculated from a standard curve of each gene transcript using purified cDNA fragments. The values were normalized to the copy number of GDPDH and shown as the mean ± SEM. Six male and five female mice were examined. The asterisk indicates that the mean value of the male and female data were significantly different ($P < 0.05$, Student's *t* test).

(sciatic nerve and skeletal muscle) also had very low levels of MISRII (Fig. 2).

MISRII has a splice variant that lacks some of the exons that encode for part of the MIS binding domain (20, 21). The MISRII mRNA in motor neurons was therefore examined by RT-PCR analysis to determine whether exon 2 was present. The MISRII mRNA was predominantly full-length, with only trace levels of the spliced variant (data not shown).

We then assessed whether the level of MISRII expression was likely to be biologically significant by comparing the copy numbers of MISRII and other receptors in motor neurons. The abundance of MISRII was 40 times greater than that of TGF-β receptor type II (TβRII) and five times that of RET, the receptor for GDNF (22) (Fig. 3). Thus, at least at the level of mRNA, MISRII is possibly the most abundant growth factor receptor produced by motor neurons. Males have ≈50% higher incidence of motor neuron disease than females for reasons that are not apparent (23). Fig. 3 therefore illustrates data for male and female mice. Neither MISRII nor TβRII showed difference between the sexes. The abundance of RET, in contrast, was slightly higher in females than males. The motor neurons of females may therefore be more responsive to GDNF than those of males.

We then examined whether proteins are produced from the MIS receptor mRNAs. A 63-kDa protein was immunoprecipitated with an anti-MISRII antibody from both the spinal cord and testes (Fig. 4*A*). This molecular weight is consistent with previous studies of MISRII. Two anti-MISRII antibodies were then used to localize the MISRII protein. Strong MISRII immunoreactivity was detected in spinal lumbar motor neurons (Fig. 4*B*), with lesser levels being associated with some other spinal neurons and a minority of neurons in the brain (data not shown). The immunoreactivity in the motor neurons was mainly perinuclear (Fig. 4 *B* and *C*, double-headed arrows) and in scattered vesicle-like structures (arrows), which correlate with the endoplasmic reticulum/Golgi apparatus of the neuron; such staining is commonly seen with antibodies to growth factors and their receptors (24). Much lower levels of MISRII immunoreactivity were associated with the circumferences of the neurons (arrowheads), which is consistent with staining of the plasma membrane.

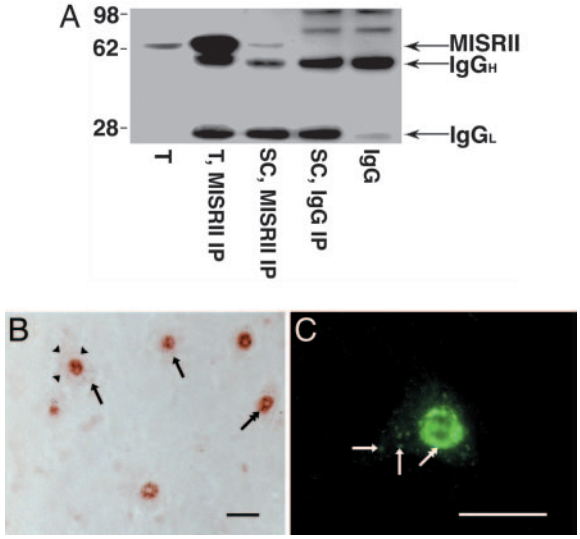

Fig. 4. MISRII proteins are produced by spinal motor neurons. (*A*) Western blot analysis of MISRII proteins in the testis (T) and the whole spinal cord (SC) after immunoprecipitation with goat anti-MISRII (MISRII IP) or nonimmune IgG (IgG IP). The positions of MISRII, IgG heavy chain (IgG_H), and IgG light chain (IgG_L) are indicated. The molecular masses are in kDa. (*B* and *C*) Motor neurons stained with goat anti-MISRII. The sections were visualized by using a color reaction product (horseradish peroxidase/3-amino-9-ethylcarbazole) (*B*) or fluorescence (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid) (*C*). The double-headed arrows point to perinuclear immunoreactivity, the single-headed arrows indicate stain in vesicle-like structures, whereas the arrowheads identify faint staining of the circumference of motor neurons. Similar staining was observed with a rabbit anti-MISRII antibody. (Scale bars, 50 μm.)

ALK3 Is the Most Abundant Type I Receptor in Motor Neurons. MISRII share type I receptors (ALK) with other type II receptors of the TGF-β superfamily. The most abundant of the ALK receptor mRNAs in motor neurons was ALK3 (Fig. 5*A*), which is a known type I receptor for MIS (12, 14). ALK3 mRNA was slightly more abundant in motor neurons isolated from females (Fig. 5, $P = 0.05$). ALK2 and ALK6 have also been implicated in MIS signaling (25–27) (Y. Zhan, D.T.M., and P.K.D., unpublished data). The mRNAs for both of these receptors were present in motor neurons but with fewer copies than for ALK3. TGF-βs signal through ALK5 (28) and/or ALK1 (13). The abundances of these receptors were less than the MIS-associated receptors (Fig. 5*A*), which mirrors the greater abundance of MISRII compared with TβRII (Fig. 3).

Motor neurons were also stained by an antibody to ALK3. In apparent reflection of the mRNA abundance, the intensity of the ALK3 immunoreactivity was noticeably less than that of the MISRII immunoreactivity. The ALK3 immunoreactivity was associated with the plasma membrane of the motor neurons (Fig. 5*B*).

MIS Promotes Motor Neuron Survival *in Vitro*. We then sought proof that MIS is a neural regulator by testing its potency in a classical *in vitro* survival assay. In the cultures with no added growth factor, only a few motor neurons survived and extended neurites (Fig. 6*A*). Addition of MIS to the medium caused a dose-dependent increase in neuronal survival, with the maximum extent of survival being similar to that achieved by the classical motor neuron survival factor, GDNF (Fig. 6*C*). The MIS- and GDNF-treated motor neurons had more elaborate neurites than the control motor neurons, which tended to have a single unbranched neurite (Fig. 6*B*). The effect of MIS was biphasic, with the maximum effect being at a concentration of 50–100

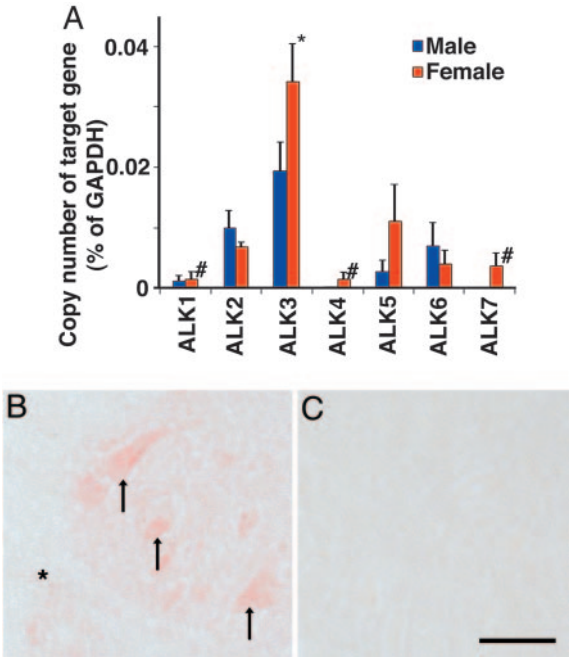

Fig. 5. ALK3 mRNA is abundant in motor neurons. (*A*) The abundances of ALK1–ALK7 mRNA in isolated motor neurons were measured as described in Fig. 2. *, the mean value of the male and female data were significantly different ($P = 0.05$). #, not significantly different to zero (Student's *t* test). (*B*) ALK3 immunoreactivity in the adult lumbar spinal cord. The arrows point to immunoreactive motor neurons in the lateral motor column. The asterisk indicates the white matter lateral to the motor column. (*C*) IgG control for nonspecific stain. (Scale bar, 20 μm.)

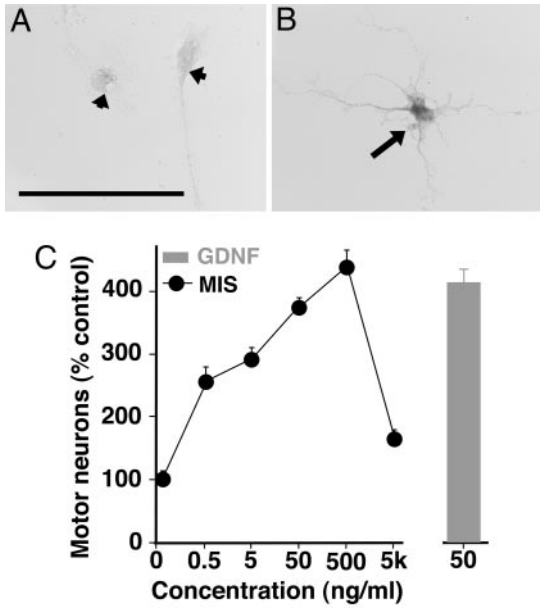

Fig. 6. MIS promotes motor neuron survival *in vitro*. (*A* and *B*) The motor neurons were cultured in either serum-free medium (*A*) or medium plus 50 ng/ml rhMIS (*B*). The neurons were stained with anti-MISRII. The control neurons either died or had a thin neurite (arrowheads), whereas those with MIS had elaborate neurites, which exhibited punctate staining with the anti-MISRII antibody (arrow). (Scale bar, 50 μm.) (*C*) A dose–response curve for the effect of MIS, with GDNF included as a positive control. The surviving motor neurons had neurites and were stained with the motor-neuron-specific marker islet-1. The values are the mean ± SEM of triplicate wells. Similar data were obtained in three other replicate experiments.

ng/ml. The MIS-induced survival persisted for up to 2 weeks, the longest time tested (data not shown).

MIS Transcripts and Proteins Are Present in Motor Neurons. If the ALK3/MISRII receptors have a physiological function in motor neurons, then a source of MIS should be present in the mature neuromuscular system. We therefore examined the abundance of MIS mRNA in the neuromuscular system. Only trace levels of MIS mRNA were detected in skeletal muscle, the sciatic nerve, the spinal cord and the brain, using real-time PCR (Fig. 7*A*). The levels of MIS transcripts in the isolated motor neurons were, in contrast, comparable with the abundance of MIS in the testes (Fig. 7*A*). No sex difference was observed in the level of MIS in motor neurons. The presence of MIS in motor neurons was then further verified by using immunohistochemistry. The anti-MIS antibody stained motor neurons (Fig. 7*C*) and a minority of other neurons, as well as the classical sources of the MIS: the Sertoli cells of the testes (data not shown) and the granulosa cells of the adult ovary (Fig. 7*B*) (29, 30).

Discussion

MIS May Regulate Mature Motor Neurons. Our study strongly suggests that MIS is a regulator of mature motor neurons. Motor neurons produce MISRII, which is the unique receptor for MIS, as well as the three type I receptors that associate with MISRII. Furthermore, MIS supported the survival and differentiation of embryonic motor neurons *in vitro*, indicating that activation of MIS receptors leads to downstream functional consequences in motor neurons. In the TGF-β superfamily, the type II receptor determines ligand specificity, whereas the type I receptors controls downstream pathway activation (13). The presence of multiple type I receptors in motor neurons thus raises the possibility that MIS is able to activate diverse biochemical pathways in motor neurons.

Human and murine MIS$^{-/-}$ or MISRII$^{-/-}$ mutants do not

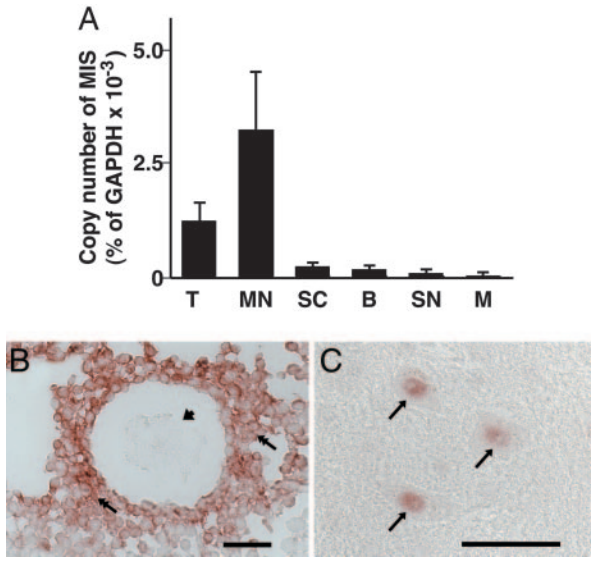

Fig. 7. MIS is produced by motor neurons. (*A*) The abundance of MIS transcripts in laser-captured motor neurons and other tissues was measured by real-time PCR. The abbreviations are as for Fig. 2. The bars are the mean ± SEM of 11 samples for the motor neurons and four samples of each of the other tissues. (*B* and *C*) MIS proteins were localized in the granulosa cells of an ovary (*B*) and motor neurons (*C*) by immunohistochemistry. (*C*) The arrows point to a motor neuron in the lumbar spinal cord. (*B*) The double-headed arrows and arrowhead indicate the granulosa cells and oocyte, respectively. (Scale bar, 50 μm.)

exhibit overt motor symptoms for two possible reasons. First, the differentiation and maintenance of motor neurons appears to be controlled by multiple redundant factors (31). The absence of any one of these factors only produces a partial loss of motor neurons, which is subclinical (e.g., GDNF$^{-/-}$; see ref. 7). This phenomenon contrasts with dorsal root ganglion or sympathetic neurons where the null mutation of their neuronal survival factors produces massive cell death (32). Second, some neuronal survival factors appear to have no role in the day-to-day function of the neuron but are vital when the neuron is subject to an insult. For instance, IL-6 is a potent neuronal survival factor, but the brains of IL-6$^{-/-}$ mice are normal (33) until a virus penetrates the blood–brain barrier (9). Similarly, the importance of VEGF to motor neurons appears to be limited to transient episodes of hypoxia. Consequently, VEGF deficiency only leads to motor neuron loss and motor disease once the nervous system has aged (10).

The ability of motor neurons to produce MIS and its receptors may indicate that MIS is an autocrine regulator of neurons. However, this pattern of expression would also enable motor neurons to use MIS to communicate with neighboring motor neurons. The extent to which motor neurons interact with each other during normal physiology is poorly understood. Because such interactions are known to be more prevalent after damage to motor axons/nerves (34), it is possible that MIS may be important for protection of motor neurons after injury. Therefore, MIS should be considered as a potential therapy for motor neuron disorders.

Does MIS Induce Sex-Specific Differences in Neurons? MIS is involved in male sexual differentiation and is produced by the testes in the urogenital ridge, where it acts on the Mullerian ducts via an endocrine mechanism. During development, the brain and nervous system may be exposed to MIS because the formation of the blood–brain barrier is a late event. Verification of this postulate may be possible in primate or human embryos for whom appropriate MIS species specific ELISAs are available, but such experiments will require extensive collaborative efforts given the rarity of available specimens. The effect of MIS on cultured motor neurons (Fig. 5) was maximal at the concentration of MIS that is present in the blood of young murine (35) and human (36) males. The generation of sexual differences in motor neurons may therefore be influenced by MIS as well as steroid hormones (37).

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