Figure legends

Figure S1. The number of neurons in the hypoglossal nucleus is not sexually dimorphic and is not influenced by Mis genotype. Each bar is the mean number of hypoglossal neurons ± the standard error of the mean. The numbers of mice examined in each group were: 10 $Mis^{+/+}$ males; 7 $Mis^{-/-}$ males; 9 $Mis^{+/+}$ females and 9 $Mis^{-/-}$ females. The male $Mis^{+/+}$ mice had 3-4% more neurons than each of the other 3 groups, but there were no significant differences between any of the groups ($p > 0.25$), or between the $Mis^{+/+}$ males and the other 3 groups combined ($p = 0.20$).

Figure S2. Hypoglossal motor neurons contain MIS and its receptors. (**A**) The copy number of MIS and its receptors in the hypoglossal nucleus of male mice was assessed using qRT-PCR. (**B)** Photomicrograph of a section of the hypoglossal nucleus of a male MISRII-Cre^{+ve}, $LacZ^{+ve}$ mouse stained with X-gal. Similar staining was observed in female mice. Misrii-Cre^{+ve}, $LacZ^{+ve}$ mice express Cre recombinase under the control of the endogenous MISRII promoter (Jamin, et al., 2002), which activates lacZ expression in MISRII-expressing cells by cleaving a floxed-stop signal in the lacZ reporter (Soriano, 1999, Wang, et al., 2009). Hence, once the lacZ transgene is activated, a cell and its subsequent daughters will continuously express lacZ. The non-neuronal cells in the hypoglossal nucleus of the Misrii-Cre^{+ve}, $LacZ^{+ve}$ were lacZ negative, indicating that they do not express MISRII in the adult or during their development. The arrows point to lacZ positive motor neurons. The bar represents 100 μm.

Figure S3. MIS and its receptors are down regulated by avulsion (**A-C).** The levels of MIS (**A**), MISRII (**B**) and BMPR1A (**C**) protein were reduced in avulsed nuclei of male mice. Sections from control (D0), day 1 and 7 (D1, D7) are illustrated (see Fig. 1 for day 3). In each section, the left hypoglossal nucleus (arrowheads) was avulsed, with the right nucleus (arrows) acting as an internal control. (**D-F)** The copy number of MIS (**D**), MISRII (**E**), BMPR1A (**F**) mRNA in laser-dissected hypoglossal nuclei were measured by qPCR. The results are mean ± SEM of 4 mice, with the avulsed nuclei represented by the red bars, and the intact nucleus by black bars. The asterisks indicate significantly different to the intact nuclei (* $p<0.05$; ** $p<0.01$, *** $p<0.001$ one-way ANOVA.

Figure S4. MIS proteins are transported by motor neurons. Longitudinal section of a ligated sciatic nerve stained with anti-MIS. The MIS immunoreactivity was mainly observed at the proximal portion of the ligated nerves with trace level of MIS immunoreactivity at distal areas. The arrows point to the immunoreactivity of MIS in the axons. The “p” and “d” indicate proximal and distal ties. The scale bar = 300 μm.

Supplementary Methods

Motor neuron cell counts (Fig. S1). Isolated brainstems from adult male and female mice were dehydrated with ethanol and embedded in Technovit 7100. The brainstems were then serially sectioned transverse to the hypoglossal nucleus, with every third 40 µm section collected and stained with cresyl violet. The total numbers of motor neurons in the hypoglossal nuclei were estimated using the Fractionator (Gundersen, et al., 1988), with the counting particle being the nucleolus. All the cell counts were undertaken by C.L.T., who was blind to the identity of the mice. The ability of C.L.T. to correctly identify the boundaries of the hypoglossal nucleus was verified using sections where the hypoglossal neurons had been retrogradely labelled with fluoresbrite microspheres (Polyscience, Warrington, PA).

Hypoglossal nuclei mRNA (Fig. S2A). Adult mice were anesthetized and the brainstem rapidly removed, snap frozen in melting isopentane and stored at -80 ^{o}C. The brainstems were subsequently sectioned with a cryostat, and processed for laser microdissection as previously described (Wang, et al., 2005). The hypoglossal nuclei were dissected using a PixCell 2 LCM System and CapSure HS LCM Caps (Arcturus Engineering, Mountain View, CA).

Total RNA fractions were isolated using the CellsDirect kit from Invitrogen (Carlsbad, CA), as per the manufacturer's specifications. The isolated RNA fractions were initially treated with DNase I (Promega, Melbourne, Australia) to remove any genomic DNA contamination. The cDNA was synthesized using SuperScript III RNase H^{-} (Invitrogen) and oligo-d(T)$_{20}$ as the primer. The qPCRs used the SYBR Green Master Mix (Applied Biosystems, CA) method, with the gene-specific primers (Wang, et al., 2005). A two-step PCR was carried out with denaturation at 95°C for 15 seconds and annealing and extension combined at 60°C for 2 minutes in a total of 50 cycles. The uniqueness of the 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 calculated.

MISRII lineage tracing (Fig. S2b). The Misrii-Cre mice had Cre recombinase knocked into the Misrii coding region, leading to a null Misrii allele and to the expression of Cre recombinase under the control of the endogenous Misrii promoter (Jamin, et al., 2002). These mice were a generous gift of Prof. Behringer, of the University of Texas. The ROS26-lacZ Cre reporter mice (Soriano, 1999) were obtained from a colony at the University of Otago.The Misrii-Cre mice were mated with the ROSA26-LacZ Cre mice to generate littermates that were Misrii-Cre^{+ve}, LacZ^{+ve} (for analysis) or Misrii-Cre^{-ve}, LacZ^{+ve} for controls. The brains of the mice were stained for LacZ, as previously described (Wang, et al., 2009).

MIS bioassay. The bioassay was based on the neuronal survival activity of MIS (Wang, et al., 2005). The spinal cords from dissected from embryonic day 14 mouse fetuses and incubated in Dulbecco's PBS, pH 7.2 (Sigma-Aldrich) containing 10 µM beta-mercaptoethanol, 0.05% trypsin (Sigma-Aldrich) and 0.4% EDTA for 15 minutes at 37ºC. Trypsin inhibitor (0.033%,

Sigma-Aldrich) was then added and the spinal cords dissociated by repeated passage through a 23-gauge needle. The resulting cell suspension was passed through a 100 μm mesh (Sigma-Aldrich) and overlaid onto 10.4% OptiPrep (Sigma-Aldrich) in Dulbecco's PBS and centrifuged for 20 minutes at 400 x *g*. Motor neuron suspensions were plated out (2,000 cell per cm^2) and cultured under serum-free conditions in Neuralbasal medium (Invitrogen) with B27 supplement (Invitrogen) and 500 μM glutamine at 37ºC and 5% CO_2.

Half the volume of medium was changed after 2-days, and at 4-days the cultures were stained using an antibody specific to motor neurons, anti-islet-1 (39.4D5 (Ericson, et al., 1992), Developmental Studies Hybridoma Bank, The University of Iowa). Immunoreactivity was developed as described in the accompanying paper. The number of surviving motor neurons was determined by counting islet-1^{+ve} neurons with a neurite in three randomly selected fields in each well.

The samples to be analyzed were serially diluted, with each dilution measured in triplicate. Each experiment included a sample that was known to cause maximal neuronal survival, enabling the concentration needed to produce 50% of maximal survival to be calculated for each sample. Each bioassay was replicated four times, with the inter-assay variability being less than 7%.

References

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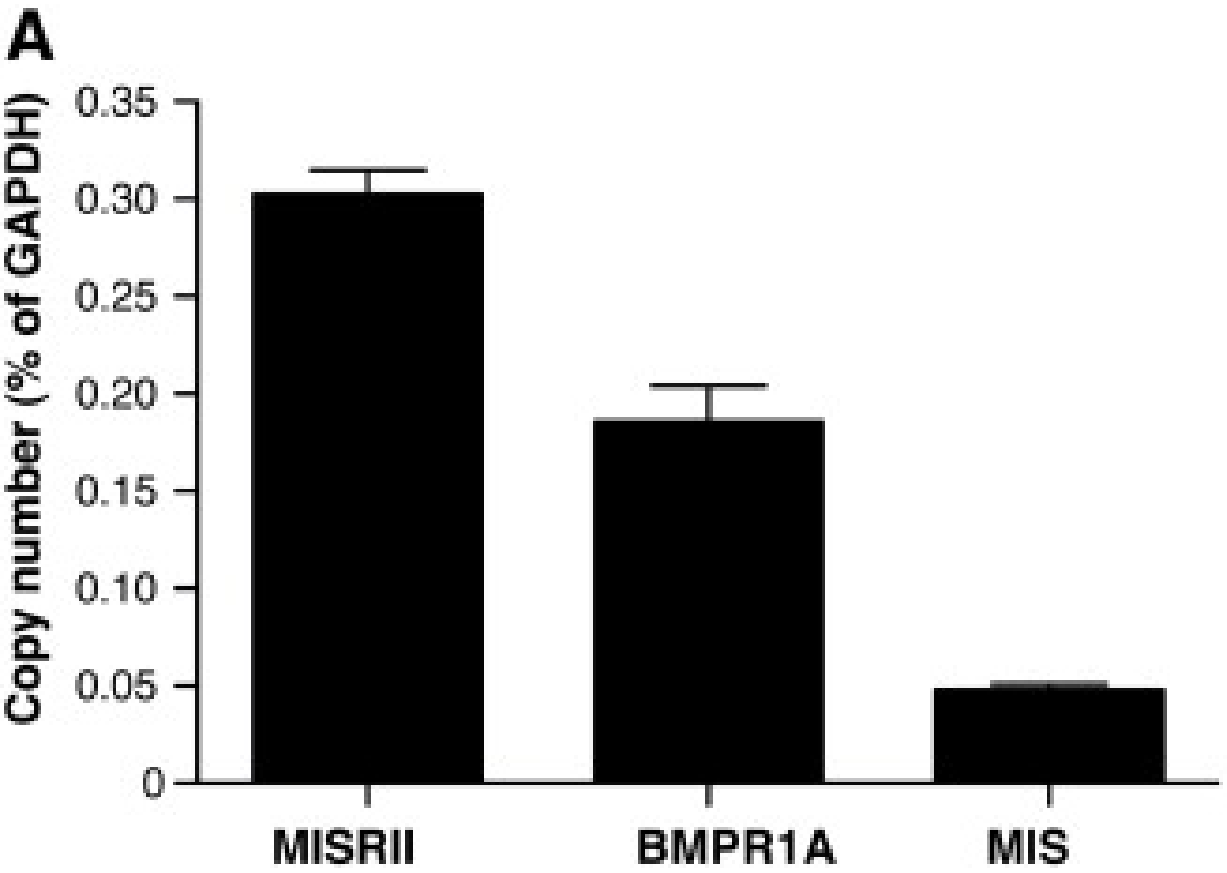
Number of neurons
500
1,000
1,500
Male
Female
Mis+/+
Mis-/-
Mis+/+
Mis-/-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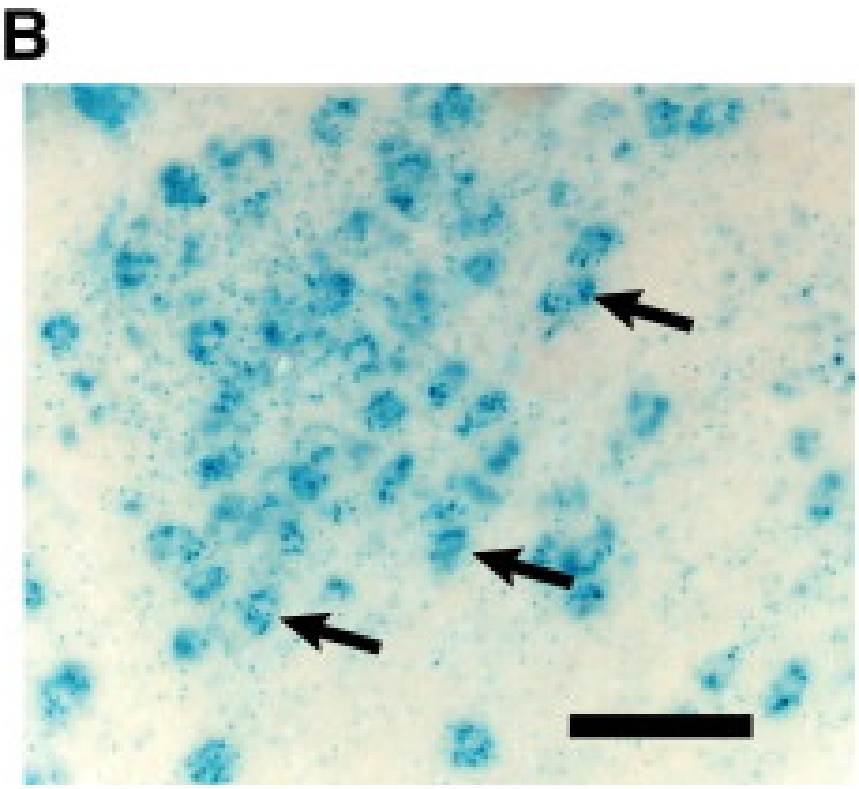
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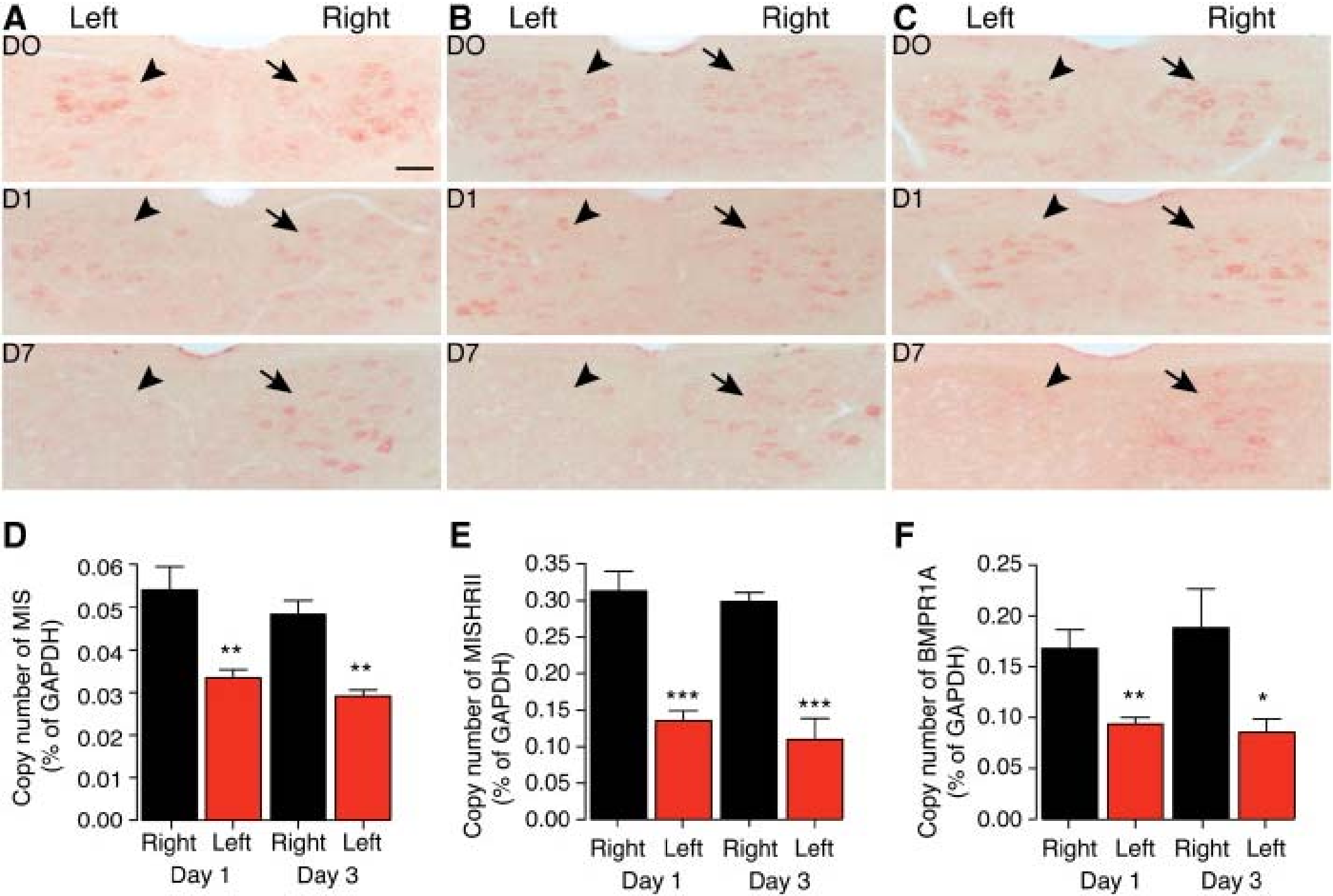
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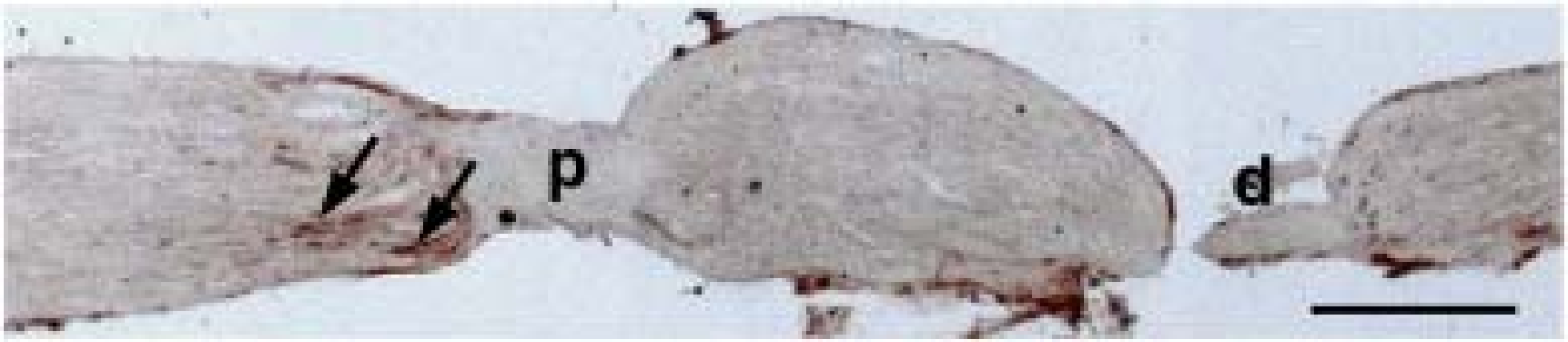
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