Supplemental Data

Direct Regulation of Adult Brain Function by the Male-Specific Factor SRY

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Supplemental Experimental Procedures

cRNA Probe and Radioactive In Situ Hybridization

cRNA probes were transcribed after linearization of an *Sry* plasmid DNA encoding a 424 base pair (bp) fragment of mouse *Sry* (GenBank accession number X67204, bp 8304–8727). [^{33}P]-labeled riboprobes were synthesized in sense and antisense orientations, according to the manufacturer's protocol from the MAXIscript in vitro Transcription kit (Ambion, Austin, TX), with either T3 or T7 polymerases, respectively. Probes were subsequently purified to remove free nucleotides via NucAway spin columns (Ambion). Tissue sections were processed for in situ hybridization with the mRNAlocator in situ hybridization kit (Ambion) as recommended by the manufacturer's protocol. In brief, sections were pre-treated with Proteinase K (0.05 μg/μl) for 10 min, acetylated, and washed with 1× PBS. Slides were prehybridized for 2–4 hr at 60ºC to reduce nonspecific background and were incubated overnight at 60ºC with hybridization solution containing [^{33}P]-labeled sense or antisense riboprobes (1×10^7 cpm/ml). Posthybridization treatments included washes at 60ºC with decreasing stringencies of 4× SSC, 2× SSC for 30 min, RNase A treatment for 30 min, and 2× and 0.1× SSC for 30 min. Slides were then dehydrated with ethanol and air dried. Labeled sections were then apposed to Kodak BIOMAX MR film (Fisher Scientific, Pittsburgh, PA) for the appropriate lengths of time.

EGFP-Transgenic Mice

In brief, the *Sry* promoter region (from a plasmid subclone of L74) was subcloned into the pEGFP-1 promoter reporter vector (Clontech, Palo Alto, CA) and then microinjected into the pronucleus of C57BL/6 fertilized eggs according to standard protocols. Transgenic mice (line Tg92) expressing EGFP under the control of the *Sry* promoter were produced because mice were assessed to have both EGFP RNA expression and visible EGFP protein expression. EGFP-transgenic adult male and female mice were intracardially perfused with ice-cold saline followed by 4% paraformaldehyde (PFA) in 0.2 M Sorenson's phosphate buffer. Brains were removed, postfixed overnight, and transferred into 15% sucrose (in 0.1 M phosphate buffer). Upon arrival, brain coronal sections of 20 μm were cut with a cryostat (Zeiss Microm, Thornwood, NY) and direct mounted onto Superfrost Plus slides (Fisher). All samples were examined without enhancement via fluorescent microscopy.

Preparation of Mouse SRY Antibody

For generation of antibodies against mouse Sry in rabbits, a peptide that corresponds to amino acids 105–129 of the mouse Sry protein was used. For affinity purification of Sry antibodies from rabbit serum, the peptide covalently coupled to cyanogen bromide-activated Sepharose-4B (Amersham Pharmacia, Piscataway, NJ) was used. Antibody specificity was demonstrated with Sry-transfected COS7 cells, and protocols were further optimized with mouse gonadal ridges before use on brain sections with purified pre-immune sera as a negative control (data not shown).

Immunofluorescence on Mouse SN Sections

Mice were anesthetized deeply with sodium pentobarbital (100 mg/kg) before they were perfused transcardially with ice-cold saline, followed by 4% PFA in 0.2 M Sorenson's phosphate buffer. After perfusion, brains were removed from the skull and postfixed overnight, transferred into 15% sucrose (in 0.1 M phosphate buffer), and stored at 4ºC until they were ready to be cut on a cryostat. Coronal sections of 10 μm were cut serially through the SN and striatum. After sections were rinsed with PBS, nonspecific binding was blocked with 10% normal horse serum, 1% BSA, and 0.1% Triton X-100 in PBS for 1 hr at room temperature. Sections were incubated with rabbit anti-Sry antibody and anti-TH monoclonal antibody (1:1000; Sigma) for 24 hr at room temperature. After being washed, the sections were incubated with Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 594 donkey anti-mouse IgG (1: 800; Molecular Probes, Carlsbad, CA) for 1 hr at room temperature. After being rinsed tissue was mounted and cover-slipped with DAPI-containing fluorescence mounting medium (DAKO). In all cases, negative controls (omission of the primary antiserum and use of pre-immune serum) were run simultaneously to confirm the specificity of the immunostaining and lack of bleed through of the fluorescent markers as detected by confocal laser scanning microscopy.

Tissue analysis was carried out on a FV500 microscope (Olympus Corp., NY) equipped with a mercury light source and Texas Red and FITC band filter systems that allowed visualization of red and green immunofluorescence, respectively. Nuclear staining with DAPI was assessed with UV light. Images captured with confocal microscopes were combined into a plate with Adobe Photoshop 5.2 software (Adobe Systems, CA). In all cases, only brightness and contrast were adjusted. To obtain an estimation of the proportion of SRY-positive and TH-expressing cells in the SN, we visually counted cells (detected by DAPI staining) in two randomly selected areas in representative areas from four animals. The expression of SRY and staining for TH was also scored for each cell counted. All observations were made by a single investigator, and each cell counted had a clearly defined nucleus.

Oligodeoxynucleotides and Infusions

The antisense used for infusions was a cocktail of three distinct ODNs (Table 2 in the main text) added in equal proportions. The first and second ODN, a 21- and 23-mer phosphorothioate-endcapped oligo, were designed to correspond to the rat *Sry* mRNA (GenBank accession AF274872). The third ODN, a 20-mer, also corresponding to the rat *Sry* mRNA sequence, was not phosphorothioate-endcapped. Athough unmodified ODNs are less commonly used and rapidly degrade under physiologic conditions by single-stranded nucleases, there is less toxicity associated with them. The sense triple cocktail ODN corresponded to the complement sequences of the three antisense ODNs. ODNs were designed to be used in both rat in vivo and mouse in vitro studies because rat and mouse nucleotide sequences are 89% identical. ODNs were HPLC-purified (Invitrogen, Carlsbad, CA) and dissolved in artificial cerebrospinal fluid vehicle (0.1 M NaCl, 4 mM KCl, 1 mM $CaCl_2$, 870 μM NaH_2PO_4, and 430 μM MgSO4 in dH_2O) to a final concentration of 1 μg/μl. Infusions were performed with Hamilton syringes (Fisher) attached to a microinfusion pump (Harvard Instruments) at a rate of 1.0 μl/min (total volume of 1 μl/side). Male and female animals were injected unilaterally with either ODN for eight consecutive days with 1 μg per day, and behavioral testing was performed 4 hr after the last injection. This design produced a within-subjects control that allowed for direct comparisons of the effects of sense and antisense ODN treatments and eliminated confounding effects of a between-subjects design.

Surgery

Male and female rats were anesthetized with isoflurane and fixed in a stereotaxic frame. Bilateral guide cannulae (24 ga) directed at the SN were implanted via standard stereotaxic procedures. The coordinates, based on a rat stereotaxic atlas [S1], were as follows: −5.3 mm posterior, ±0.9 mm lateral from bregma, and −6.0 mm ventral from dura. The tooth bar was positioned at −.3 mm. The cannulae were secured to the skull with stainless-steel bone screws and

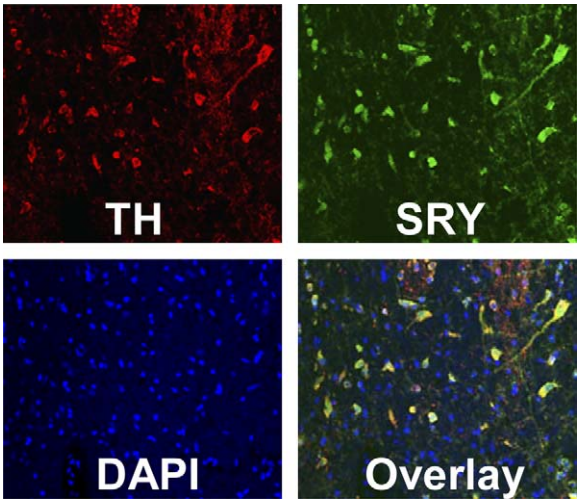

Figure S1. Sry Immunoreactivity in Male Rat SN

Sry staining (green) was observed in the adult male rat SN. As for mouse, all Sry-positive cells also stained for TH (red). The scale bar represents 20 μm.

dental cement. Stylets that protruded <0.5 mm beyond the opening were placed in the guide cannulae.

Confirmation of Guide-Cannula Placement

After surgery, male and female animals were anesthetized with sodium pentobarbital (50 mg/kg) and intracardially perfused with saline followed by 4% PFA in 0.2 M Sorensons's phosphate buffer. Rats were decapitated, and brains were removed, postfixed overnight in 4% PFA, and then stored in 20% sucrose until they were sectioned with a cryostat. Coronal sections of 20 μm were melted onto Superfrost Plus slides, stained with thionin, dehydrated, and coverslipped with Cytoseal XYL (VWR, West Chester, PA). Injection sites were mapped and verified with bright-field illumination. Any rats with off-target cannulae placements were omitted from the study.

Animals and Behavioral Tests

Brains extracted from male and female C57BL/6J mice (approximately 120 days old) (The Jackson Laboratory) were used for in situ hybridizations. Male and female Long-Evans rats (approximately 90 days old) (Charles River) were used for infusions and motor-behavioral testing. All animals were housed individually in standard cages and provided with food and water ad libitum. All experimental procedures were approved by the Chancellor's Animal Research Committee at UCLA. Animals were screened for inclusion into the behavioral studies in advance. Animals that vocalized or showed any obvious signs of discomfort or stress in performing the motor-behavioral tests were omitted from the study. To assess preference for using the nonimpaired forelimb for weight-shifting movements in vertical exploration, limb-use asymmetry test was used as described previously [S2]. Forelimb use during explorative activity was analyzed by videotaping rats for at least 10 rearings. Animals were tested before surgery, before ODN infusion, 8 days after ODN infusion, and 7 days after the last ODN infusion. For the akinesia test, rats were held so that they bore weight solely with the strength of their opposing forelimb. The number of steps taken by the animal was manually counted and timed four times for each subject. Latency to initiate a step was recorded, and the number of steps was averaged separately for each forelimb. Forelimbs were tested in an alternating fashion to prevent any bias or adaptation. The results of the behavioral tests were compared by one-way ANOVA with Student-Newman-Keuls post hoc comparisons (Sigma Stat; Jandel Scientific, San Rafael, CA).

Immunohistochemistry

Male and female animals were anesthetized deeply with sodium pentobarbital (100 mg/kg) after the last behavioral tests were

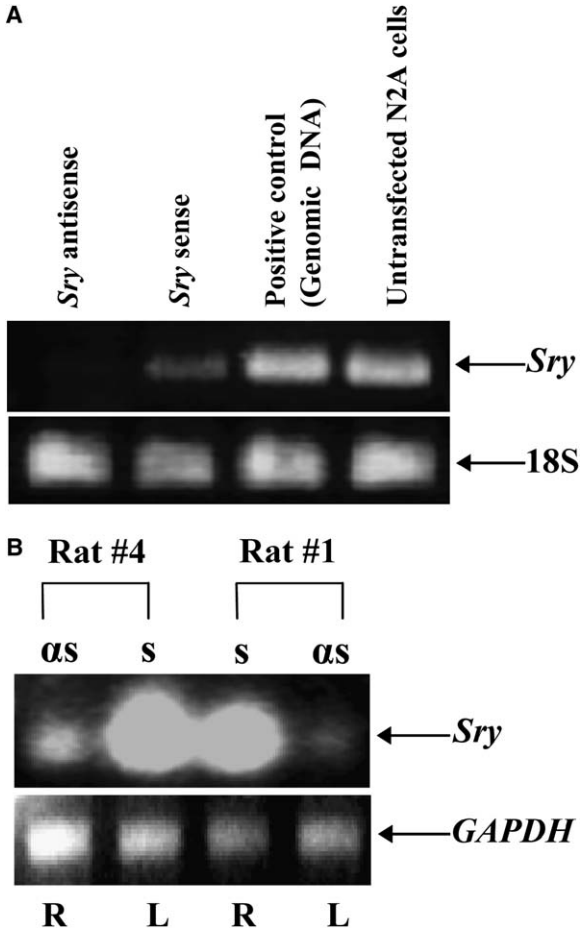

Figure S2. *Sry* Antisense ODN Downregulates *Sry* Expression In Vitro and In Vivo

(A) RT-PCR verifies the downregulation actions of *Sry* antisense in Neuro-2A cultures. Lanes 1 and 2: N2A cells transfected with 5 μg of *Sry* antisense and sense ODN, respectively. Lane 3: genomic rat DNA was used for a positive control for rat *Sry* primers. Lane 4: untransfected N2A cells have endogenous *Sry* expression. 18S was amplified as a test for amplification efficiency and template control. The PCR product size for *Sry* and 18S should be 110 bp and 125 bp, respectively. Lower levels of 18S in the "Sry sense" lane accounts for a Sry/18S signal ratio similar to the ratio in the "untransfected" control lane.

(B) RT-PCR confirms the decrease in *Sry* mRNA expression in vivo in two separate rats infused with *Sry* ODN. R and L represent right and left sides of the rat brain in which either antisense (αs) or sense (s) was infused (shown as indicated). Sides of brain in which antisense was infused exhibited less *Sry* expression than sides infused with sense in both cases. GAPDH was amplified as a test for amplification efficiency and template control.

completed. Animals were perfused transcardially with ice-cold saline, followed by 4% PFA in 0.2 M Sorenson's phosphate buffer. Brains were removed and postfixed overnight, transferred into 15% sucrose (in 0.1 M phosphate buffer), and stored at 4ºC until they were ready to be cut on a crystostat. Free-floating coronal sections of 40 μm were cut through the SN and striatum. One in four sections was incubated in blocking solution for 1 hr at room temperature before incubating with monoclonal TH primary antibody (1:1000 for SN and 1:10,000 for striatum; Sigma) or Nurr1 (N-20, 1:250; Santa Cruz Biotechnology, Santa Cruz, CA) for 24 hr at 4ºC. Sections were then incubated with biotinylated secondary antibody (goat anti-mouse IgG F(ab′)2 from MP Biomedicals, Irvine, CA, or goat anti-rabbit from ICN, respectively) diluted 1:500 for 1 hr at room temperature and avidin/biotin complexed (Vector Laboratories,

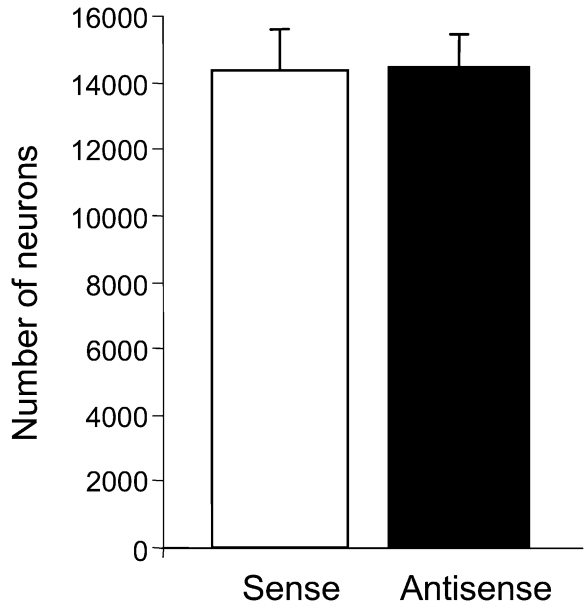

Figure S3. Effects of Sry ODN on the Overall Number of Neurons in the SNc

Nissl staining of slides where Sry antisense and sense ODNs were unilaterally infused into the rat brain showed no difference between the two sides of the brain in the overall number of neurons (n = 6). The total number of neurons in the SN was more than the number of TH+ neurons because of the presence of non-TH neurons, which are mostly GABAergic interneurons, in the SNc.

Burlingame, CA). TH immunoreactivity was visualized with 3,3′-diaminobenzidine (DAB; Sigma). For Nissl staining, adjacent sections were stained with thionin. Sections were mounted on Superfrost Plus slides, dehydrated through a series of graded alcohol and xylene, and cover-slipped with Permount (Fisher).

Optical-Fractionator Sampling Scheme

The number of neurons in the SN was estimated using an optical-fractionator sampling design [S3, S4]. The boundaries of the SN were delineated with a rat brain atlas [S1] and intrinsic landmarks. The ventral margin of the SN, the substantia nigra pars reticulata, was marked by sparsely packed neurons with a soma size larger than those found in the substantia nigra compacta (SNc). The rostromedial border, the ventral tegmental area (VTA), was identified by neurons that were smaller than those found in the SNc. An internal grid of counting frames was superimposed onto the delineated region to sample the SNc in a random and non-biased manner. The thickness of each section was measured with a *z*-axis micrometer, and the volume of the SNc (*V*) was calculated according to the formula: $V = \Sigma P \cdot a(P) \cdot t$, where a(*P*) is the area (corrected for magnification) between grid points, *P* is the number of grid points within the boundaries of the SNc, and *t* the total thickness of the SNc. Cells were observed and counted with a 40× objective lens. The optical-dissector method was used to eliminate the possibility of counting the same cell twice regardless of the morphology and orientation of each cell. Cells were counted by an investigator blind to the samples and confirmed by another investigator.

Cell Cultures and Cellular Uptake of Antisense Oligodeoxynucleotides

Mouse Neuro-2a (N2A) cells were grown in minimum essential medium (Eagle), supplemented with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/liter sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. N2A cells were split into 12-well plates and grown to 90% confluency at 37ºC in a humidified atmosphere of 5% CO_2 in air. Cells were then transfected via LipofectAMINE 2000 Reagent (Invitrogen) with either 5 μg of *Sry* antisense or sense and incubated for 48 hr, according to the manufacturer's recommended protocol. Untransfected cells served as controls. Subsequently, all cells were harvested with TRIzol (Invitrogen), and RNA was extracted. Total RNA was isolated from cells as per the manufacturer's recommended protocol, and quantity and quality were determined by spectrophotometry with 260/280 absorption ratios.

RT-PCR Analysis

Total RNA (1 μg) from *Sry*-antisense and sense-treated and untreated N2A cell cultures was used to synthesize cDNA in a 20 μl reaction using SuperScript II RNase H^- reverse transcriptase and a combination of random priming and oligo dT (Life Technologies, Rockville, MD). The reverse transcription was performed at 42ºC for 1 hr. cDNAs were subjected to PCR with primers specific to rat *Sry* (Genbank accession #NM012772; Fwd: 5′-GCAGCGTGAAG TTGCCTCAAC-3′ and Rev: 5′-TGCAGCTCTAGCCCAGTCCTG-3′). Primers were designed with Primer3 software (The Whitehead Institute, Boston, MA). PCR conditions used for amplification were as follows: initial denaturation at 94ºC for 4 min, 35 cycles of denaturation at 94ºC for 45 s, annealing at 60ºC for 45 s, and elongation at 72ºC for 1 min, with a final extension at 72ºC for 7 min. Amplified products were separated by electrophoresis on a 1.5% agarose gel with ethidium bromide and visualized under UV light to confirm product size. Gapdh or 18S was also amplified at 25 cycles to serve as a template control (Figure S3).

Supplemental References

S1. Paxinos, G., and Watson, C. (1986). The Rat Brain in Stereotaxic Coordinates, Second Edition (Orlando, FL: Academic Press).

S2. Schallert, T., Fleming, S.M., Leasure, J.L., Tillerson, J.L., and Bland, S.T. (2000). CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology *39*, 777–787.

S3. Gundersen, H.J., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., et al. (1988). The new stereological tools: Disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. APMIS *96*, 857–881.

S4. West, M.J., Slomianka, L., and Gundersen, H.J. (1991). Unbiased stereological estimation of the total number of neurons in thesubdivisions of the rat hippocampus using the optical fractionator. Anat. Rec. *231*, 482–497.