PNAS
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Supplementary Information for

Sex-specific neuroprotection by inhibition of the Y-chromosome gene, *SRY*, in experimental Parkinson's disease

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This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.1900406116

Supplementary Information Text

MATERIALS AND METHODS

Human cell culture model of PD Bone marrow derived human (*H. sapiens*) XY neuroblastoma BE(2)-M17 (M17) cells (CRL-2267, ATCC, Manassas, VA, USA), were cultured with Dulbecco's modified Eagle's medium with Ham's F12 nutrient in a 50:50 ratio supplemented with Glutamax (Life Technologies), 10% Fetal Bovine Serum (10100-139, Life Technologies) and 1% antibiotic/antimycotic at 37ºC with 5% CO_2 humidified atmosphere (Galaxy 170S incubator, New Brunswick, USA). Human M17 cells were plated onto 6 well plates and allowed to reach 80% confluency then treated with vehicle, 20µM 6-OHDA (Sigma-Aldrich, solubilized in sterile 0.1% ascorbic acid saline), or 100µM rotenone (Sigma-Aldrich, solubilized in 1% DMSO/saline). RNA was extracted from the cells at 0 to 72hrs post-treatment and processed for qRT-PCR or seeded at 24hrs and processed for immunocytochemistry.

Immunocytochemistry M17 cells were seeded on glass cover slips in 35 mm tissue culture wells, fixed with 4% paraformaldehyde and blocked for 1h at 37ºC (CAS block, Thermo Fisher). Cells were incubated with primary antibody against human SRY (1:100, rabbit polyclonal; AVIVA Systems Biology) overnight at 4ºC, washed and incubated with fluorescent secondary antibodies (1:1000 in block, Alexa Fluor 594 anti-rabbit; Thermo Fisher) for 1h at 37ºC. Coverslips containing the stained cells were mounted on slides with fluorescent nucleic acid stain, Hoechst 33342 (Thermo Fisher). Cells were imaged using a 60x objective on a confocal microscope (Olympus FV1200) and SRY-positive cells were assessed using the MetaMorph software version 7.1 (Molecular Devices). The intensity was calculated from the average values of the nuclear, cytoplasmic or whole cell fluorescence in six different fields from 4 independent experiments.

Animals All methods conformed to the Australian NHMRC published code of practice for the use of animals in research and were approved by the Monash University Animal Ethics Committee (MMCB13/02 and MMCB15/18). Adult Long-Evans male and female rats weighing between 280 and 350 g were used. Animals were housed in a 12h light:dark cycle room and had access to food and water *ad libitum.*

Stereotaxic implantation of cannula in the rat SNc Unilateral guide cannula (22 gauge, Plastics One) directed at the right SNc was implanted at 5.3 mm posterior, 2mm lateral from bregma, and 6.0 mm ventral to the surface of dura. The guide cannula was secured to the skull with stainless-steel screws and dental cement. Dummy cannulae that protruded <0.5 mm beyond the opening were placed in the guide cannulae.

Repeated Sry antisense oligonucleotide (ASO) infusions Nigral Sry expression in male rats was reduced by repeated intranigral infusions of Sry ASO. The Sry ASO used was a cocktail of three distinct ASOs directed against rat *Sry* mRNA added in equal proportions, as described previously (1). The ASO, which targets a highly conserved region of the rat Sry gene centered on the initiating methionine codon, is predicted to reduce expression of all known rat Sry gene transcripts. ASOs were HPLC-purified (Invitrogen, Australia) and dissolved in artificial cerebrospinal fluid (aCSF) vehicle to a final concentration of 2µg/µL. Infusions were made at a rate of 0.5µL/min followed by a 2 min equilibration period, during which the needle remained in place. All rats were infused unilaterally with ASO or SO daily (2µg in 1µL in aCSF) for 10 consecutive days.

Toxin-induced rat models of PD To assess the regulation of *Sry* expression in pre-clinical rat models of PD, a unilateral lesion of the right SNc was made by a single injection of DA toxins 6-hydroxydopamine hydrobromide (6-OHDA, 30µg dissolved in 1.5µL of 0.1% ascorbic acid saline)

or rotenone (30µg in dissolved 1.5µL of 1% DMSO/saline) into the right SNc in male rats. Motor function was assessed by the limb-use asymmetry test at various time points post-surgery (0 to 21 days post 6-OHDA or at 0 or 7 days post rotenone). At the end of the behavioural studies, rats were killed, and the brains were processed for measurement of nigral gene expression. To assess the function of *Sry* in pre-clinical rat PD models, male rats were repeatedly infused with Sry ASO (or SO) for 10 days prior to a single intranigral injection of 6-OHDA (30µg) or rotenone (30µg). Another group of male rats received a single intranigral injection of 6-OHDA (30µg) followed by repeated Sry ASO (or SO) infusion for 10 days. A group of female rats were infused with Sry ASO (or SO) for 10 days prior to a single intranigral injection of 6-OHDA (30µg) to account for any non-specific effects. Motor function was assessed by the limb-use asymmetry and amphetamine-induced rotation tests at various time points. At the end of the behavioural studies, the rat brains were processed for measurement of nigrostriatal gene and protein expression.

Limb-use asymmetry test The limb-use asymmetry test assessed spontaneous forelimb usage during vertical explorations in rats, where motor impairment is indicated by a reduction in limb-use contralateral (i.e. left) to the site of drug injection. The rat was lowered into a clear cylinder and forelimb contacts during vertical explorations were video recorded until a total of 30 touches were reached. The data was expressed as the percentage of left (impaired) forepaw contacts; where symmetric paw use (left ≈ right) was a measure of unimpaired limb use.

Rotarod test Rotarod test evaluated balance and motor coordination of rodents by assessing the ability of rodents to stay balanced on a rotating platform (Ugo Basile, Italy). Briefly, animals were trained for 3 consecutive days before the day of testing at a fixed speed (10 rpm) for 5 minutes. On the day of the testing, rats were placed on the moving rotarod which accelerated from 10 to 40 rpm over 3 minutes. The time after which the rat fell was determined and 4 experimental readings were averaged to obtain a single value for each animal.

Amphetamine-induced rotational test Rotational behaviour was measured by placing rats in a circular cage where they were tethered to an automated rotometer system and injected with amphetamine (2 mg/kg, intraperitoneally). The total number of rotations was measured for 90 mins at 10-minute intervals. The data are expressed as net rotations per minute, where rotation toward the side of the lesion was given a positive value.

Tissue processing Rat brains were either intracardially perfused and processed for immunohistochemistry or isolated fresh and processed for western blot or qRT-PCR. Coronal sections were cut serially through the SNc and striatum and stored at -80°C. Between each series, a 200µm slab was collected to isolate tissue for nigral mRNA and protein, or striatal DA and DOPAC measurements.

qRT-PCR Total RNA (100-300ng) isolated using TRI-Reagent (Sigma Aldrich) was reverse-transcribed into cDNA (Go Script, Promega). Equal amount of cDNA template was added to SensiMix**™**SYBR Hi-ROX Mastermix (Bioline) using primers listed in SI Appendix, Table S2 and real-time quantification of mRNA expression was performed by *QuantStudio 6 Flex* Real-Time PCR System (Applied Biosystems). Rat Sry primer is specific to the full-length rat Sry mRNA sequence and detects all copies of the rat Sry gene. The relative level of mRNA was interpolated from a standard curve prepared by serially diluting the cDNA reaction. All quantitative PCR reactions were conducted in triplicates. Final values represent fold change of gene expression relative to the housekeeping gene β2-Microglobulin (β2M, human) or TATA-box-binding protein (Tbp-1, rat).

Striatal DA and DOPAC measurements The striata was isolated and the levels of DA and DOPAC were measured, as described previously (2). In brief, the striata was homogenized and sonicated in perchloric acid (0.45N). The homogenate was centrifuged at (15,000*g*, 10 min, 4°C) and the supernatant was filtered through a syringe filter (0.22μm). DA and DOPAC (a metabolite of DA) were separated and quantified in the filtrate. Filtrate (20μL) was manually injected into reverse Phase C–18 high performance liquid chromatography (HPLC) column coupled with electrochemical detector (Waters, Milford, Massachusetts). The concentrations of DA and DOPAC were calculated as ng/mg tissue and expressed as a percentage of the intact control side.

TH protein expression Total cell protein was isolated from the rat SNc and amount of protein was quantified by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 10 μg of protein was run on a 10% acrylamide gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Primary sheep anti-TH primary antibody (1:1000, Pelfreez, P60101-150) was applied overnight at 4ºC. Secondary AlexaFluor488 antibody (1:200, Invitrogen, A11015) was incubated for 1h at room temperature. PVDF membranes were scanned using a Typhoon Trio variable mode imager (GE Healthcare).

TH and DAT immunohistochemistry and stereology TH immunohistochemistry was performed by incubating 40μm-thick SNc sections in sheep anti-TH primary antibody (Pelfreez, P60101-150, 1:2000, overnight at 4ºC), followed by a biotinylated secondary antibody (rabbit, anti-sheep IgG, 1:1000, Vector Labs, BA-6000) and reacted with cobalt and nickel-intensified diaminobenzidine (DAB, Sigma-Aldrich). DAB-immunostained sections were counterstained with neutral red. DAT immunohistochemistry was performed by incubating 16μm-thick striatal sections in rat anti-DAT primary antibody (Chemicon, MAB369, 1:2000, 78 hrs at 4ºC) followed by a biotinylated secondary antibody (rabbit, anti-rat IgG, 1:500, Vector Labs, BA-4001) and reacted with DAB. DAB-immunostained sections were analysed by bright-field microscopy, using an Olympus microscope equipped with Olympus cellSens dimension v1.7.1 image analysis software. TH-immunoreactive and neutral-red positive neurons or DAT-immunoreactive terminals were quantified stereologically on regularly spaced sections covering the whole SNc or striatum. The fractionator design for estimating the number of TH-immunoreactive or neutral-red positive neurons and the number of striatal DAT-immunoreactive axonal varicosities were performed as previously (2).

TOM-20 and TH co-immunofluorescence TOM-20 (3) and TH co-immunofluorescence was performed by incubating 20μm-thick SNc sections in sheep anti-TH and rabbit anti-TOM-20 primary antibody overnight at 4°C (anti-TH, 1:1000, Pelfreez, P60101-150; anti-TOM-20, 1:200, Santa Cruz, SC-11415), followed by a 1hr incubation in donkey anti-rabbit 594 and donkey anti-sheep 488 (1:500, 1hr at RT, Invitrogen, A21207, A11015) and DAPI (1:1000, Invitrogen). Immunofluorescence staining was visualised by confocal microscopy (Nikon C1 macro laser confocal, Japan) equipped with NIS-Elements image software. For quantification of TH, TOM-20 and DAPI-positive labelling, confocal images at 20x magnification of SNc were analysed using IMARISx64 software (v7.6.5). Fluorescent intensity values of TH and TOM-20 above background threshold were given for all DAPI positive cells and were quantified for co-immunostaining.

TUNEL and iNOS staining TUNEL staining was performed by incubating 20μm-thick SNc sections in TdT enzyme and nucleotide mixture (1hr at 37°C, In Situ Cell Death Detection Kit, Roche, 11684795910), followed by incubation in anti-fluorescein antibody-peroxidase POD (30min at 37°C, Roche, 11772465001) reacted with cobalt and nickel-intensified DAB. Sections were counterstained with cresyl violet (Sigma-Aldrich Inc) iNOS immunohistochemistry was performed by incubating 20μm-thick SNc sections in mouse anti-iNOS primary antibody (Santa Cruz, SC-7271, 1:200, overnight at 4ºC), followed by a biotinylated secondary antibody (goat, anti-mouse

IgG, 1:1000, Vector Labs, BA-9200) and reacted with cobalt and nickel-intensified diaminobenzidine (DAB, Sigma-Aldrich). DAB-immunostained sections were counterstained with neutral red. TUNEL-positive or iNOS-positive SNc sections were analysed by bright-field microscopy (Olympus BX53F, Japan) equipped with Olympus cellSens dimension image analysis software (v1.7.1) and quantified stereologically on regularly spaced sections covering the whole SNc. Neurons with deep black nuclei were identified as TUNEL-positive or iNOS-positive neurons.

Statistical Analysis All values are expressed as the mean ± S.E.M. All data was analysed using tools within Graphpad Prism 5. Motor behaviour studies of the treatment groups across the days of testing was analysed by two-way analysis of variance (ANOVA) and Tukey's post hoc test. Histological and biochemical studies were analysed using two-tailed unpaired Student t-test or one-way ANOVA, where appropriate. The exact P-values of the ANOVAs are given in the Figure legends. Probability level of 5% ($P < 0.05$) was considered significant for all statistical tests.

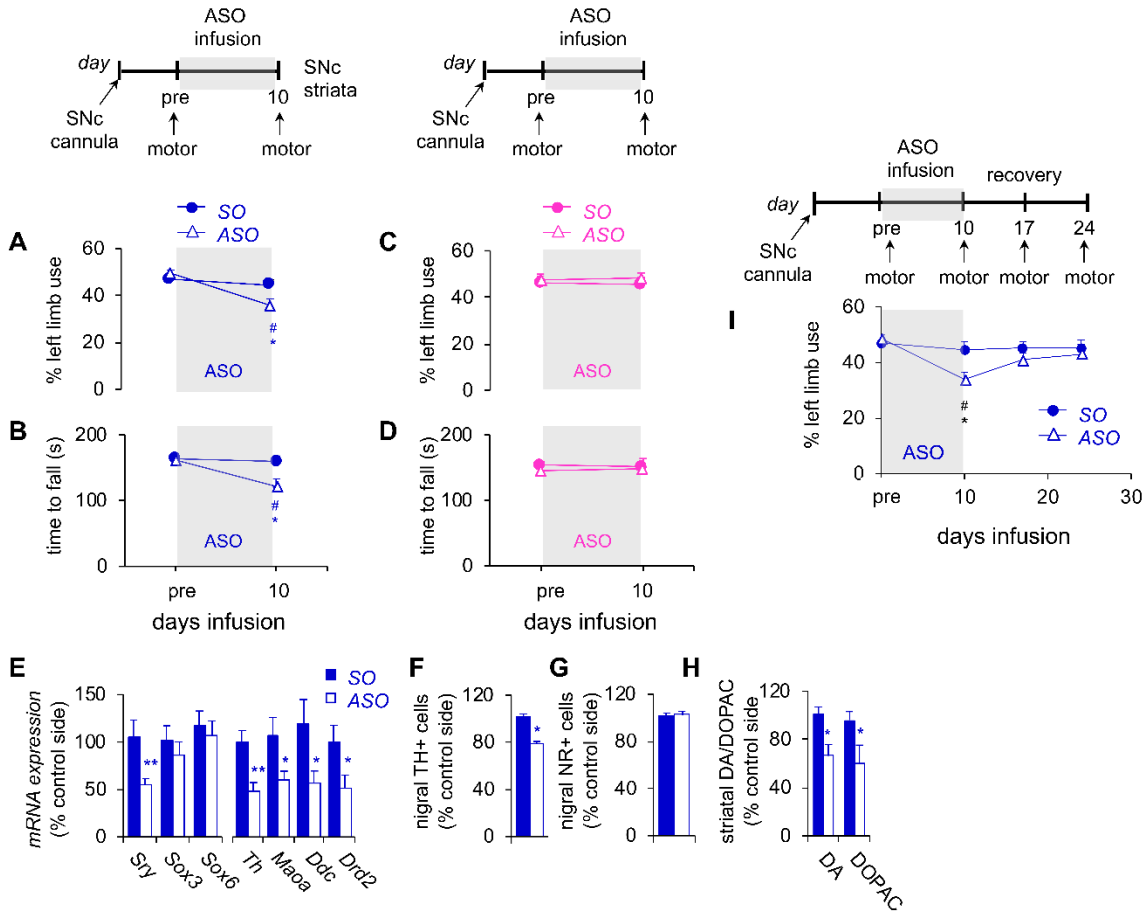

Fig. S1. Nigral Sry controls voluntary movement and nigrostriatal dopamine biosynthesis in male rats. Effect of ASO infusion in male (A, B) or (C, D) female rats on motor function in the limb-use asymmetry (A, C) and rotarod (B, D) tests (n=10/group; two-way ANOVA, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. SO; # $P < 0.05$ vs. pre). (E-G) SNc and striatal tissues were isolated from male rats and processed for measurement of (E) nigral *mRNA* expression, (F) total number of nigral TH+ cells or (G) neutral red (NR)+ neurons and (H) striatal DA and DOPAC levels as % of control side (n=10/group, two-tailed unpaired t-test, ** $P < 0.01$, * $P < 0.05$ vs. SO). (I) ASO-mediated reduction of limb-use in male rats is reversed at two weeks post last ASO-infusion. (n=5/group; two-way ANOVA, * $P < 0.05$ vs. SO; # $P < 0.05$ vs. day 0).

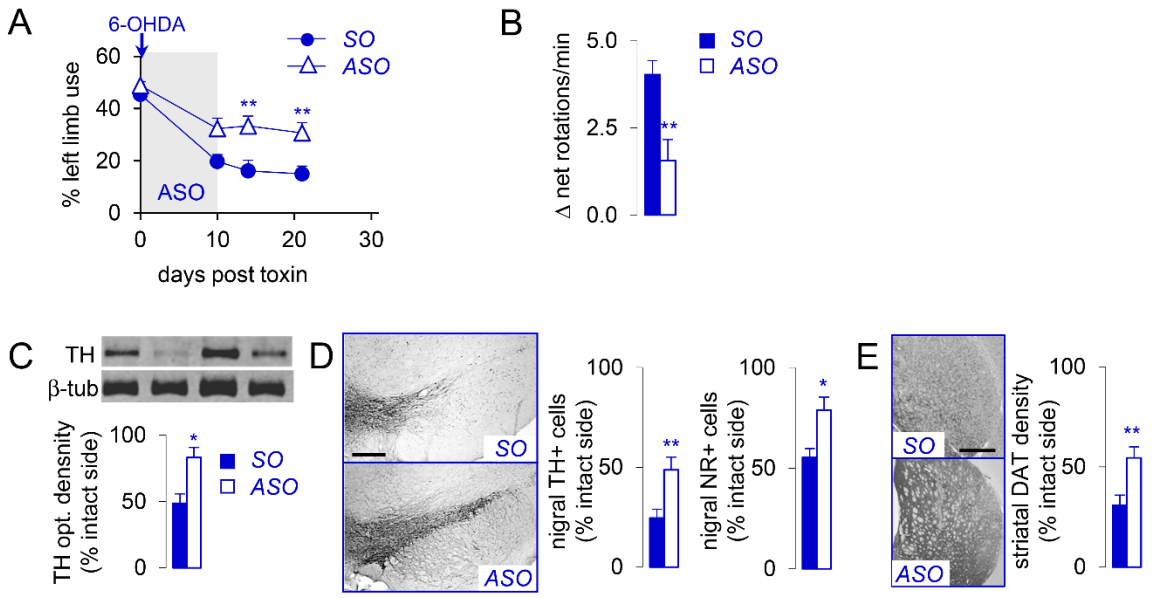

Fig. S2. Nigral *Sry* ASO infusion, following 6-OHDA injection, in male rats diminishes motor deficits and nigrostriatal degeneration. ASO (or SO) was infused at 8 hrs following a single 6-OHDA injection in male rats. (A, B) 6-OHDA-induced motor deficits were assessed by the (A) limb-use asymmetry or (B) amphetamine-induced rotation test (n=12/group; limb-use: two-way ANOVA, * $P < 0.05$, ** $P < 0.01$ vs. SO; rotation test: unpaired t-test, ** $P < 0.01$ vs. SO). (C-E) 6-OHDA-induced nigrostriatal degeneration was assessed by (C) nigral TH protein expression, (D) total number of nigral TH+ or neutral red (NR)+ cells and (E) striatal DAT density as % of intact side (n=12/group; unpaired t-test, * $P < 0.05$, ** $P < 0.01$ vs. SO; scale bars = 400µm)

Table S1. Comparisons of motor behaviour, nigrostriatal DA, or PD pathogenesis marker expression between male and female rats infused with ASO (or SO) prior to 6-OHDA injection (one-way ANOVA; n=8 to18 / group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs. Male SO 6-OHDA).

Parameter	Fig	Male SO 6-OHDA	Female SO 6-OHDA	Male ASO 6-OHDA	Female ASO 6-OHDA
limb use (% left limb use)	2	17.5 ± 3.1	40.0 ± 3.9 ***	31.8 ± 3.1 **	39.9 ± 1.8 ***
rotations (Δ net rotations)	2	3.1 ± 0.4	2.0 ± 0.3	0.3 ± 0.7 **	1.5 ± 0.4
TH protein (% intact side)	3	26.1 ± 4.7	64.5 ± 11.6 ***	53.2 ± 5.7 **	69.2 ± 1.9 ***
TH cell counts (% intact side)	3	51.7 ± 5.8	81.6 ± 6.8 *	80.4 ± 7.2 **	81.9 ± 7.1*
striatal DAT density (% intact side)	3	29.2 ± 4.0	65.4 ± 7.8 *	67.8 ± 14.3 *	69.5 ± 5.4 *
TH/TOM-20 + cells (% intact side)	5	64.8 ± 7.9	73.1 ± 7.7	89.5 ± 5.4 *	67.4 ± 3.1
TUNEL + cells	5	15.2 ± 2.1	3.0 ± 0.4 ***	7.4 ± 2.3 *	2.3 ± 0.2 ***
iNOS + cells	5	4.4 ± 1.1	0.7 ± 0.2 **	0.6 ± 0.2 **	0.7 ± 0.1 **

Table S2. List of primers use in the study.

Gene	Full Name	Species	Forward sequence (5' – 3')	Reverse sequence (5' – 3')	GenBank Accession No.
Sry	Sex determining Region Y	Rat	ttccaggaggcgcagagactga	tgttgaggcaacttcacgctgca	NM_012772.1
Th	Tyrosine hydroxylase	Rat	actgtggaattcgggctatg	cattgaagctctcggacaca	NM_012740
Ddc	Dopa decarboxylase	Rat	tgactatctggacggcattgagg	ggaagtaagcgaagaagtagggg	NM_001270852.1
Maoa	Monoamine oxidase A	Rat	acgctcaggaatgggacaagatg	cccacactgcctcacataccaca	NM_033653.1
Dbh	Dopamine beta hydroxylase	Rat	agccccttcccttaccaca	tgcgttctccatctcacctc	NM_013158.2
Drd2	Dopamine D2 receptor	Rat	gtcctctacagcgccttcac	atgaggtctggcctgcatag	NM_012547.1
Sox3	SRY-related HMG-box 3	Rat	aacgctttcatggtgtggtcc	gtccgggtactccttcatgtg	XM_008773577.2
Sox6	SRY-related HMG-box 6	Rat	ctgcctctgcaccccataatg	ttgctgagatgacagaacgct	NM_001024751.1
Bax	Bcl-2-associated X protein	Rat	ctcaaggccctgtgcactaaa	cccggaggaagtccagtgt	NM_017059.2
Bcl2	B-cell lymphoma 2	Rat	ggaggctgggatgcctttg	ctgagcagcgtcttcagagaca	NM_016993.1
Sod1	Superoxide dismutase 1	Rat	cactgcaggacctcattttaatcc	gtctccaacatgcctctcttcat	NM_017050.1
Sod2	Superoxide dismutase 2	Rat	cgctggccaagggagat	ccccgccattgaacttca	NM_017051.2
Gpx1	Glutathione peroxidase 1	Rat	ctcggtttcccgtgcaat	tcggacatacttgagggaattca	NM_030826.4
iNos	Inducible nitric oxide synthase	Rat	cggaagagacgcacaggcagaggtt	aaggcagcaggcacacgcaatgatg	NM_012611.3
Pgc1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	Rat	cagtcaagctgtttttgacgac	cggagagttaaaggaagagcaa	NM_031347.1
Cyt c	Cytochrome c oxidase	Rat	actacccctgcctgatgtg	actcattggtgcccttgttc	NM_017202.1
Nd1	NADH-ubiquinone oxidoreductase chain 1	Rat	ttaattgccatggccttcctcacc	tggttagagggcgtatgggttctt	X56833.1
Txnrd1	Thioredoxin reductase 1	Rat	tcaaggtgaccgctaagtcc	tcttcccggtcttttcattg	NM_031614.2
Gadd45g	Growth arrest and DNA-damage-inducible protein 45 gamma	Rat	gctgcgagaacgacattgaca	cggctctcctcgcagaacaa	NM_001077640.1

Puma	p53 upregulated modulator of apoptosis	Rat	agtgcgccttcactttgg	caggaggctagtggtcaggt	NM_173837.2
Tbp1	TATA-box-binding protein 1	Rat	gggagctgtgatgtgaagt	gtggtcttcctgaatccccttta	NM_001004198.1
Tnfa	Tumour necrosis factor alpha	Rat	gccctaaggacacccctgagggagc	tccaaagtagacctgcccggactcc	NM_012675.3
Il1b	Interleukin 1 beta	Rat	aaaaatgcctcgtgctgtct	tcgttgcttgtctctccttg	NM_031512.2
Il10	Interleukin 10	Rat	taagggttacttgggttgcc	tatccagagggtcttcagc	NM_012854.2
Tgfb1	Transforming growth factor beta 1	Rat	tggaaatcaatgggatcagtc	ggagctgtgcaggtgttgag	NM_021578.2
SRY	Sex determining Region Y	Human	ttcccgcagatcccgcttcggtactctg	ttttttttttttttgaaatgaataag	NM_003140.2
GADD45G	Growth arrest and DNA-damage-inducible protein gamma	Human	actagctgctggttgatcgc	caactcatgcagcgctttc	NM_006705.3
B2M	Beta-2-Microglobulin	Human	tgaattgctatgtgtctgggt	cctccatgatgctgcttacat	NM_004048.2

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