Supporting Online Materials

Natural Antisense Inhibition Results in Transcriptional De-Repression and Gene Upregulation

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Many low abundance noncoding antisense RNAs, which are transcribed on the opposite strands of genomic loci, endogenously suppress corresponding sense gene expression; inhibition or removal of antisense transcript leads to locus-specific upregulation of sense mRNA, protein and function.

Supporting Online Material

List of content:

Supplementary Methods:

Mouse studies: We obtained approval for mouse studies from the Institutional Animal Care and Use Committee at The Scripps Research Institute, where the animal experiments were performed. We used 10 eight-week-old male C57BL/6 mice for *in vivo* experiments. We prepared mice with chronic indwelling cannulae in the dorsal third ventricle implanted subcutaneously with osmotic mini-pumps that delivered continuous infusions (0.11 microliter/h) of synthetic antisense oligonucleotide directed against *Bdnf*-AS (m*Bdnf*-AntagoNAT9) or control oligonucleotide (inert sequence that does not exist in human or mouse) at a dose of 1.5 mg/kg/d for 4 weeks. We connected tubing to the exit port of the osmotic minipump and tunneled it subcutaneously to the indwelling cannula, such that the treatments were delivered directly into the brain. At 5 d post-implantation all animals received daily intra-peritoneal (IP) injection of BrdU (80 mg/kg), for five consecutive days. At the 28th day post-surgery, we sacrificed the animals and excised three tissues (hippocampus, frontal cortex and cerebellum) from each mouse brain for quantitative RNA measurements. RNA extraction is described in the Supplementary Methods.

Design of modified AntagoNAT molecules: We designed and tested a number of DNA based antisense oligonucleotides, termed AntagoNATs, targeting noncoding *Bdnf*-AS and other antisense transcripts. We designed various AntagoNATs ranging from 12 to 20 nucleotides in length with or without full phosphorothioate modification plus/minus 2-O'-methyl RNA or LNA modified nucleotides. We observed highest efficacy on *Bdnf* mRNA level with 16-nucleotide phosphorothioate gapmer with three LNA-modified nucleotides (see, *e.g.*, ref. 15 of main manuscript) at each end (XXXnnnnnnnnnnXXX). For blocking interactions between human *BDNF* sense-antisense transcripts, we used 14-nucleotide mixmers containing both LNA and 2-

O'-methyl RNA molecules. Although these 2-O'-methyl RNA-modified oligonucleotides are suggested to only block the RNA, we observed marginal downregulation of targeted RNAs in this experiment (Supplementary Fig. 12). Sequences of various AntagoNATs, as well as all other siRNAs, primers and probes used for these studies are listed in supplementary table-1**.**

Rapid Amplification of cDNA Ends (RACE): Sequence information for the potential mouse *Bdnf*-AS was retrieved from the UCSC Genome Bioinformatics web site. Using RACE ready cDNA (Ambion catalog number 3200-3209) from the mouse brain we amplified the 5' and 3' ends of *Bdnf*-AS by nested PCR with gene specific and kit primers. Alternatively, using 250 ng poly "A" RNA from the testis as starting materials and utilizing the PRLM RACE kit (Applied Biosystems catalog number AM1700), we generated 5' and 3' end libraries and performed PCR followed by sequencing to identify the mouse *Bdnf*-AS transcript. We excised the 3' and 5' PCR products of both mouse and human from an agarose gel and cloned them into the T-Easy vector (Promega catalog number TM042). We sequenced positive colonies from each series.

Real-Time PCR (RT-PCR): We carried out RT-PCR with the GeneAmp 7900 machine (Applied Biosystems). cDNA synthesis reaction contained random hexamers, 200-400 ng of RNA, 2.5 mM mixture of dNTP, MgCl2, and appropriate buffer. The PCR reactions contained 20–40 ng cDNA, Universal Mastermix, 300 nM of forward and reverse primers, and 200 nM of probe in a final reaction volume of 15 µl. We designed the primer/probe set using FileBuilder software (Applied Biosystems). Primers were strand-specific for sense-antisense pairs and the probes covered exon boundaries to eliminate the chance of genomic DNA amplification. The PCR conditions for all genes were as follows: 50 ºC for 2 min then 95 ºC for 10 min then 40

cycles of 95 ºC for 15 s and 60 ºC for 1 min (50 cycles for mouse *Bdnf*-AS). The results are based on cycle threshold (Ct) values. We calculated the differences between the Ct values for experimental and reference genes (18S RNA) as ΔΔCt and graphed as a percent of each RNA to the calibrator sample. We assessed the relative expression of *BDNF* and *BDNF*-AS RNA transcripts in several human and mouse cell lines, by real time PCR (RT-PCR). We measured the expression of *Bdnf* and *Bdnf*-AS transcripts in several mouse brain regions and other mouse tissues, including: testis, ovary, liver, spleen, thymus, lung, kidney, heart, embryo and cerebellum in wild-type C57BL/6 mice (n = 3) by RT-PCR.

We measured the expression of both transcripts in a commercially available panel of human tissue RNA samples (Ambion), including: total brain, cervix, ovary, spleen, kidney, testis, esophagus, thyroid, adipose, skeletal muscle, bladder, colon, small intestine, liver, lung, prostate, heart, trachea, thymus and whole embryo. We measured expression of both transcripts in a panel of commercially available, 12-weeks embryonic RNA samples (Ambion), including: embryonic brain, liver, lung, heart, kidney, muscle and whole embryo.

We measured expression of *Bdnf* and *Bdnf*-AS in a panel of monkey brain RNA samples (n = 2) by RT-PCR using human primers and probe. The brain regions tested from the monkey samples were: 1) motor cortex 2) frontal cortex 3) hippocampus 4) amygdala 5) motor cortex 6) insula 7) temporal cortex 8) interior partial cortex 19) striatum 10) cerebellum 11) striatum 12) septum 13) occipital cortex 14) pituitary gland. Both *BDNF* and *BDNF*-AS transcripts were expressed in many tissues and cell lines tested, and the expression levels of *BDNF* mRNA was between 10 to 100-fold greater than the *BDNF*-AS transcript.

RNA extraction and RT-PCR of the mouse brain samples: We euthanized mice after 28 days and excised the brains. One hemibrain from each mouse was fixed in 4% formaldehyde overnight for histological studies. We excised another hemibrain for RNA quantitative measurement from the hippocampus, frontal cortex and cerebellum. We extracted RNA after homogenization in Trizol reagent (Invitrogen, 15596-026) according to the manufacturer's protocol. We separated the aqueous phase and added an equal volume of 70% ethanol before passing the samples through Qiagen RNeasy columns (QIAGEN, 74106) and we subjected those RNA samples to on-column DNAse treatment for removal of DNA contamination. We used 400 ng of each sample for the first strand cDNA synthesis and carried out RT-PCR measurements as described above. We plotted the percentile changes in RNA levels, for individual tissues as compared to control mice, in each graph.

Cell culture and transfection: We purchased human cortical neurons, HCN-1A, originating from the brain of an 18-month old female from ATCC (ATCC # CRL-10442). These cells were reported to be positive for a number of neuronal markers including neurofilament protein, neuron specific enolase (NSE) and gamma aminobutyric acid (GABA). We cultured cells in a DMEM medium supplemented with 10% FBS. We purchased human glial cells (M059K) that originated from the brain of a 33 year-old male with malignant glioblastoma, from ATCC (ATCC # CRL-2365). We cultured these cells in a mixture of DMEM and F12 plus 10% FBS, 1% NEAA, 2.5 mM L-glutamate, 15 mM HEPES, 0.5 mM sodium pyruvate, and 1% sodium bicarbonate. We cultured HEK293T, N2a, Human cortical neuron (HCN1) and human Glioblastoma MK059 cells in appropriate medium and transfected cells in logarithmic growth, with 5-20 nM of siRNA or antisense oligonucleotides using 0.2% Lipofectamine 2000 (Invitrogen), according to

manufacturer's instructions. Antisense oligonucleotides are single-stranded 14-nucleotide DNA strands with 2-O-Methyl and LNA modifications, complementary to the *BDNF*-AS sequence. We designed 14 functional and two inert control oligonucleotides (that do not have targets in the mammalian genome). Cells were incubated for various time points before RNA extraction, using Qiagen RNeasy columns.

Statistical analysis: We performed all experiments with 6–20 biological and 3–6 technical repeats. The data presented in the graphs is a comparison with control-treated groups after post-hoc analysis of the corresponding treatment factor using main effects in a two-way analysis of variance (ANOVA). We calculated the significance of each treatment as a p-value. As depicted in each graph, ($p < 0.05$) was considered significant.

Western blot: We transfected HEK293T cells with 10 nM of *BDNF*-AS, or control siRNA. We disrupted cells, 48 h post transfection, with 200 µl of Laemmli sample buffer (Biorad) containing 350 mM DTT. We separated 20 µl of the lysate on a 10% SDS PAGE and transferred it to a nitrocellulose membrane overnight. Then we incubated the membrane with primary antibody for MecP2 (Abcam), *BDNF* (Promega, catalog number G164B) and secondary antibody conjugated to HRP. After addition of HRP substrate, we detected the chemiluminescent signal with X-ray film. We stripped the same membrane and reused it for detection of β-Actin as a loading control.

ELISA: We transfected cells with 20 nM of *BDNF*-AS siRNA or control siRNA. The cell supernatant was collected for ELISA experiments. Alternatively, we extracted total protein from mouse brain tissues embedded in protein extraction buffer plus protease inhibitors (BCA kit,

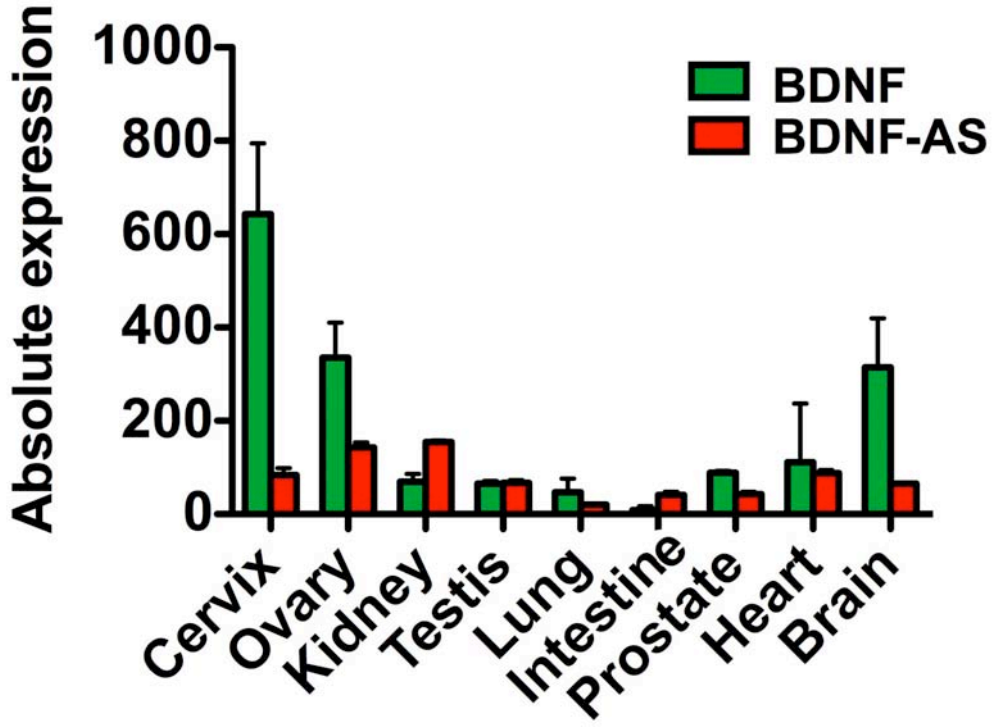
Fisher) and homogenized with the bioruptor and metal beads. Total protein was measured using BCA protein assay kit (Pierce catalog number 23227) and sample loads were normalized to total protein concentrations. We purchased the ELISA kits for human BDNF from Promega (catalog number G7611) or mouse Bdnf Millipore (catalog number CYT306) and we performed ELISA following the supplier's protocol. We subtracted average absorbance of three repeats at 450 nm from background and normalized it to the control sample.

Dissecting mouse hippocampal neural stem cells in neurospheres: We separated neuronal stem cells from the hippocampus of mouse pups, P0-P1. The hippocampi were mechanically separated to single cells, collected by short spins and grown in a mixture of DMEM and F12, containing glutamine, antibiotics, B27 solution and 0.001 mM concentration of both EGF and FGF. After 3-4 days floating neurospheres formed (neurosphere cell processing and immunocytochemistry techniques have previously been published[50]). We plated 100,000 cells in 24-well plates coated with poly-L-Lysine (PLL). The plating of neurosphere cells onto PLL will start the differentiation process. On the third day post-plating, we removed growth factors from the medium and allowed the cells to grow for 4 more days (7 days post-plating). By this time, the cell culture had a mix of neural cell lineages consisting of astrocytes, neurons, oligodendrocytes and their progenitors making it more similar to mature brain tissue. We measured the expression of *Bdnf* and *Bdnf*-AS in floating neurospheres as well as in 3 and 7 days post-plating cultures. We performed knockdown experiments, using either 50 nM siRNAs or 20 nM antisense oligonucleotides targeting *Bdnf*-AS transcript, at 3 or 7 days post-plating.

Neural stem cells are also seeded in immunocytochemistry chambers, (18,000 cell per well) in a total volume of 80 µl. We next transfected neurospheres, using the same protocol, to assess the

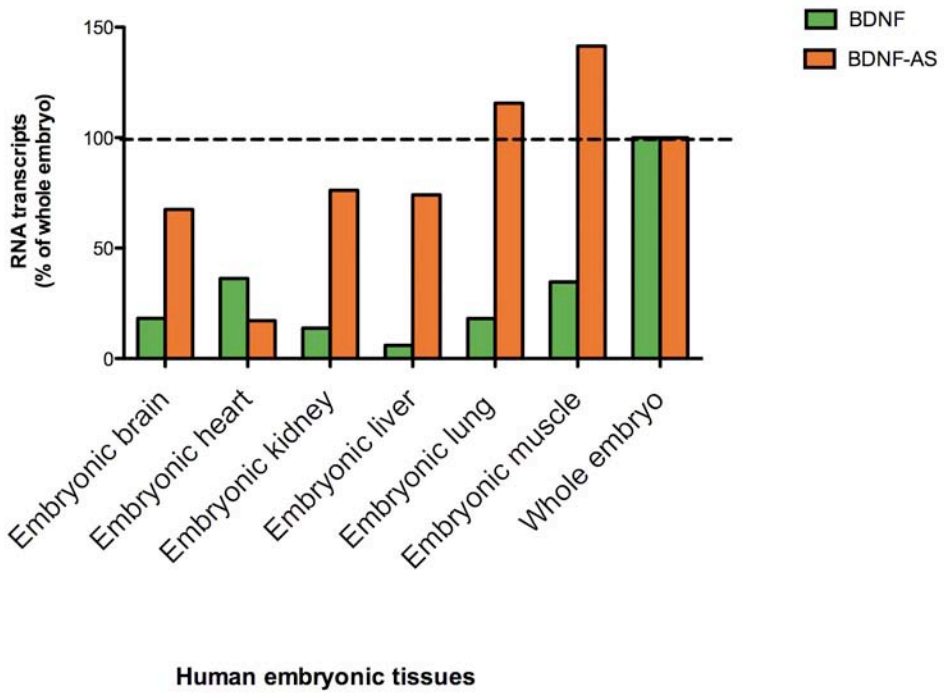
functional effects of *Bdnf*-AS knockdown on murine primary cells. After 48-72 h, cells were fixed with paraformaldehyde (4%) for 20 min and washed with 1X PBS several times. After blocking with FBS, neurospheres were incubated with primary antibody (Monoclonal Rabbit β tubulin III, TUJ1) at a 1:2000 concentration overnight. Fixed cells were incubated with secondary antibody, labeled with Alexafluor 568 (goat anti-rabbit IgG, 2mg/ml, at concentration of 1:5000). Nuclei were stained with Hoechst stain[50]. Images were obtained by immunofluorescence antigen detection microscopy.

Supplementary Figure 1: Expression of *BDNF* and *BDNF*-AS in human tissues:



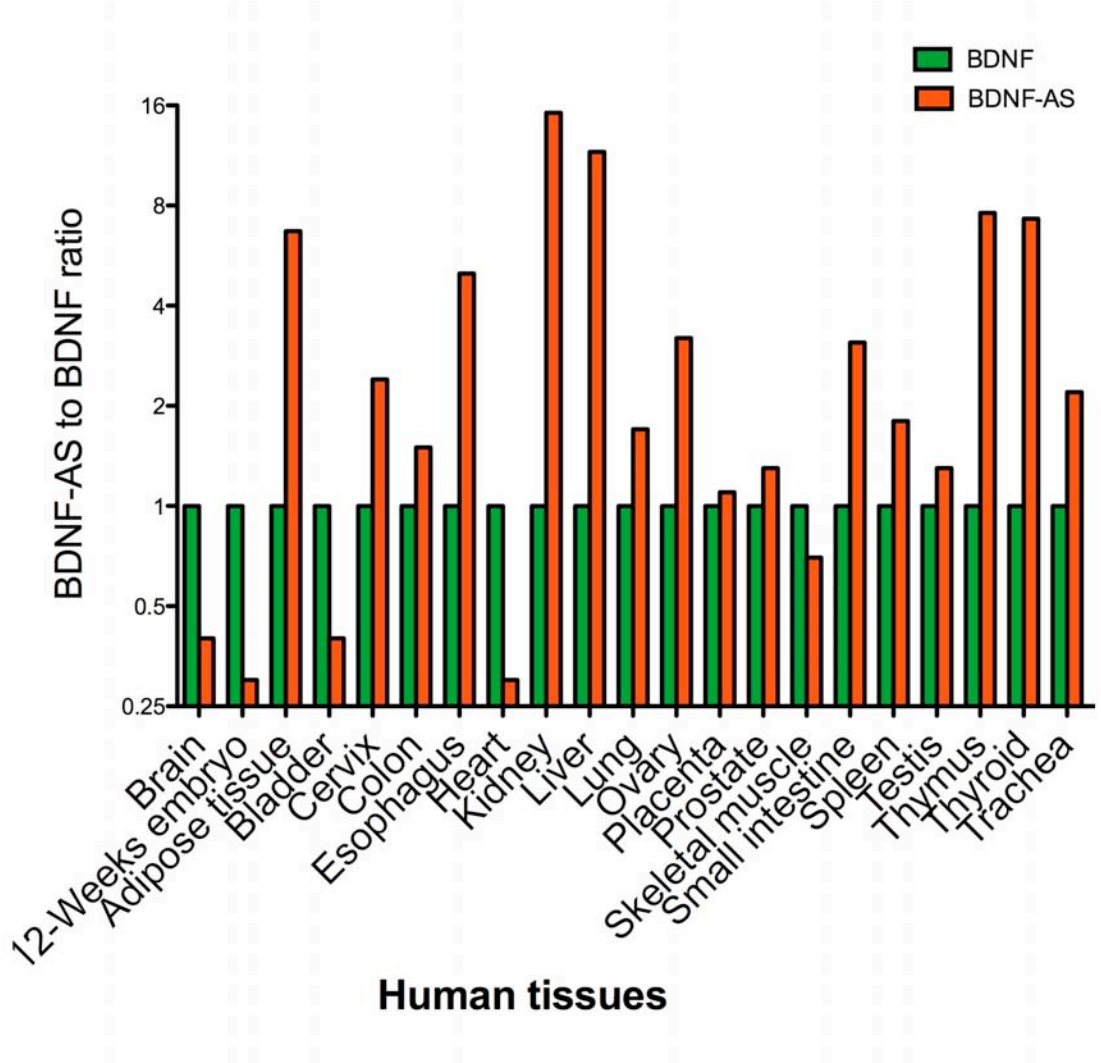
We assessed the absolute expression of *BDNF* and *BDNF*-AS transcripts in various human tissues by RT-PCR. Panel of commercially available RNA samples (Applied Biosystems) from various human tissues were analyzed for the presence of *BDNF* and *BDNF*-AS transcripts. Samples containing serial dilutions of cloned *BDNF* and *BDNF*-AS cDNA were used to draw individual standard curves for each transcript. Both sense and antisense transcripts are expressed in the brain and muscular tissues. *BDNF* mRNA levels are generally 10-100 folds higher than that of the *BDNF*-AS transcript, except for in testis, kidney and heart, which contain equal or higher levels of *BDNF*-AS. *BDNF* mRNA levels are low in post-natal tissues, except for in the brain, heart, cervix and ovary. *BDNF*-AS transcript levels are high in the kidney, testis, ovary and brain.

Supplementary Figure 2: Expression of *BDNF* and *BDNF*-AS in embryonic tissues:



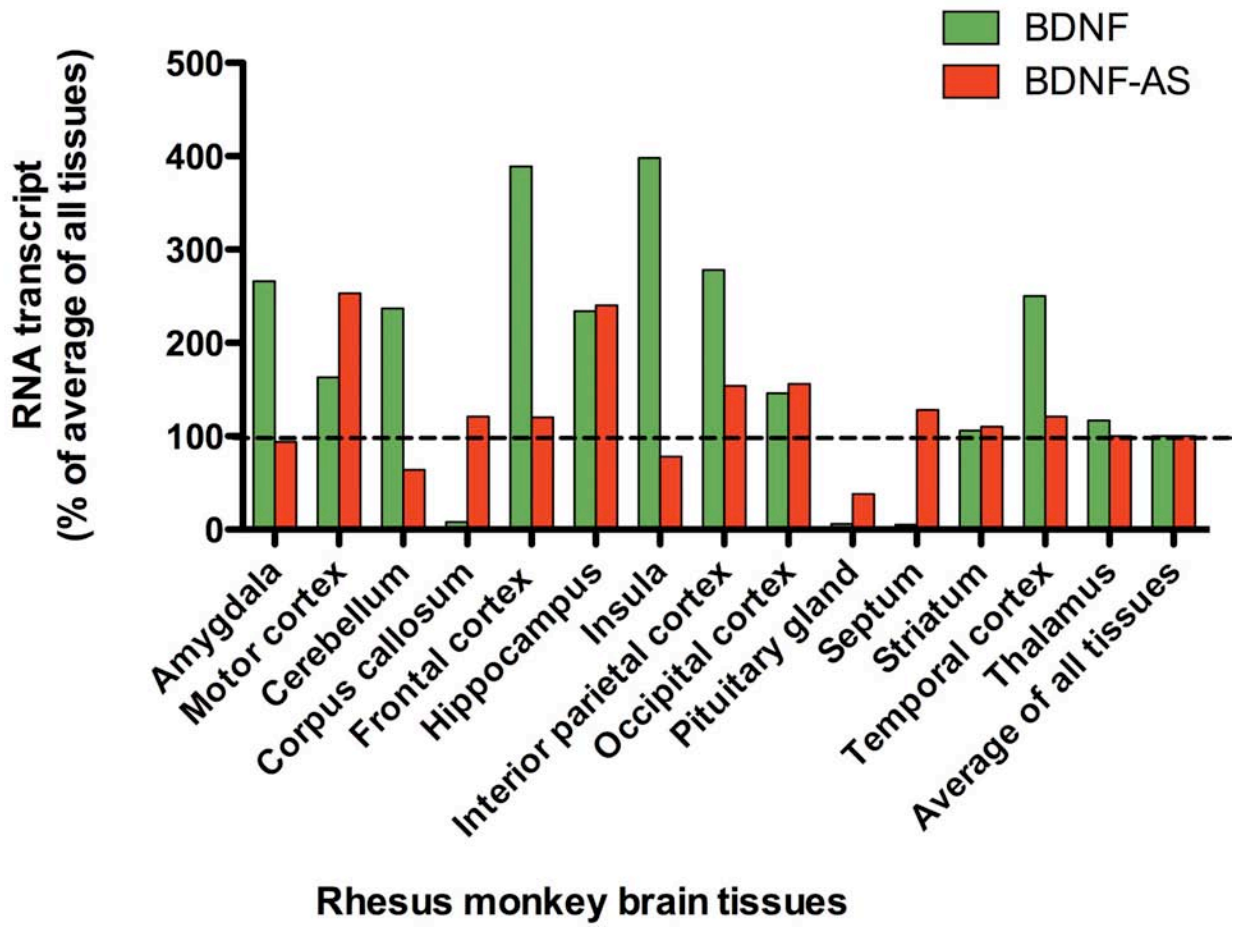
We observed high expression levels of *BDNF* and *BDNF*-AS transcripts in the 12-week embryo. Thus, we sought to determine the pattern of expression of both transcripts in human embryonic tissues. We bought a panel of RNA samples from various human embryonic tissues (Applied Biosystems). We assessed the expression of *BDNF* and *BDNF*-AS transcripts by RT-PCR, normalizing each transcript to the expression of the whole embryo. We found that *BDNF*-AS transcript levels are high in most of the embryonic tissues examined, with notably high levels in embryonic muscle and lung tissues.

Supplementary Figure 3: Ratio of *BDNF*-AS to *BDNF* in human tissues:



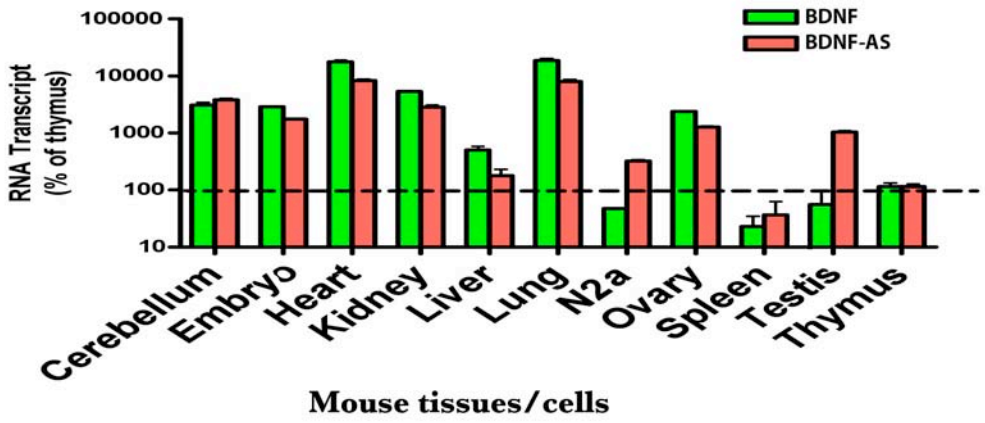
We performed RT-PCR and assessed the expression levels of *BDNF* and *BDNF*-AS transcripts in each tissue. Next, we normalized the expression of *BDNF*-AS to that of *BDNF* in the same tissue. We found that the *BDNF*-AS to *BDNF* ratio is vastly different amongst various tissues. The *BDNF*-AS to *BDNF* ratio is low in the brain, 12-week embryo, bladder and heart. This ratio is high (up between 2- to 16-fold) in the kidney, liver, thymus, thyroid, adipose tissue, esophagus, cervix, small intestine, ovary, and trachea. The *BDNF*-AS to *BDNF* ratio is equal to or less than 50% different in the colon, lungs, placenta, prostate, skeletal muscle, spleen and testis.

Supplementary Figure 4: Expression of *Bdnf* and *Bdnf*-AS in the monkey brain:



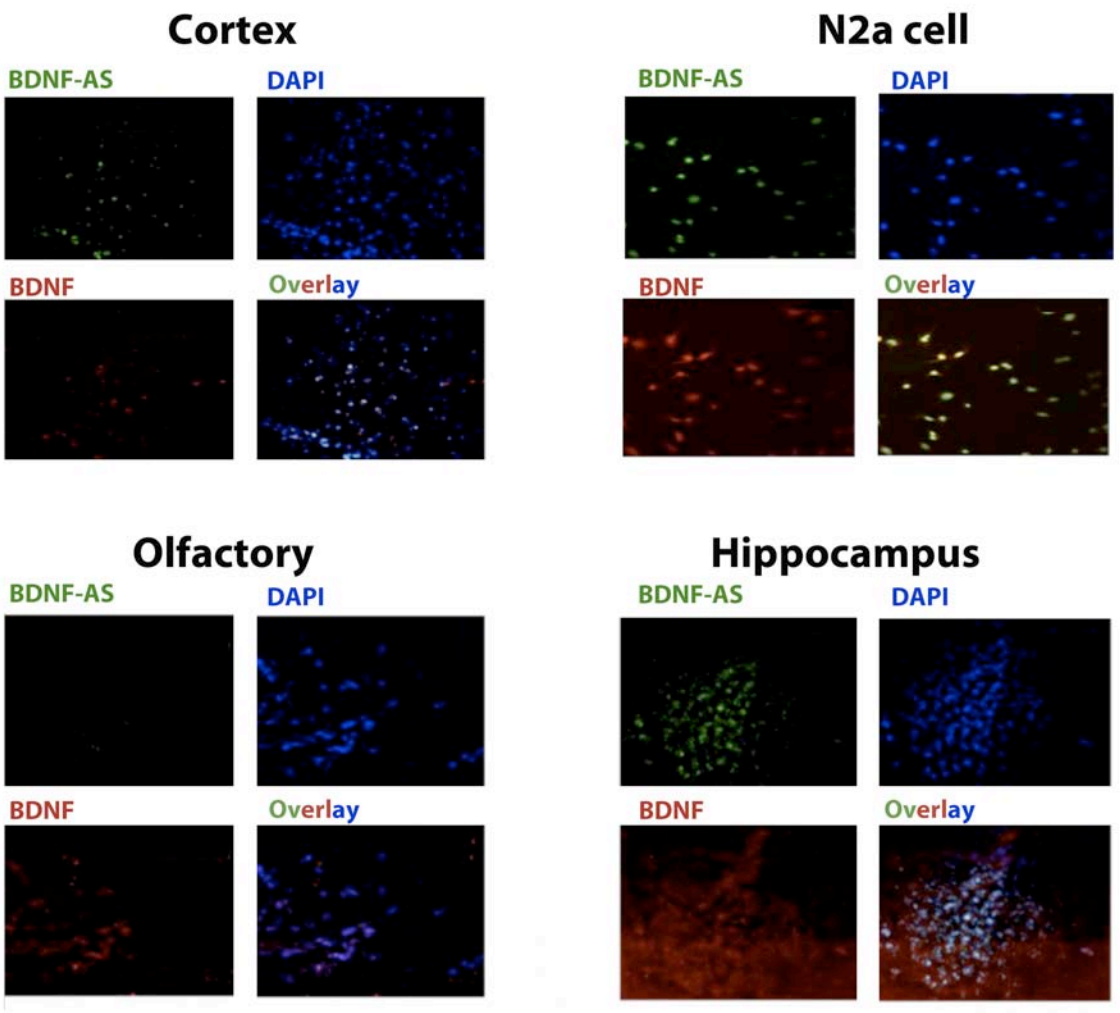
We determined the expression levels of *Bdnf* and *Bdnf*-AS transcripts in RNA samples from Rhesus monkey brain tissues (*n=2*), by RT-PCR. We used human primer/probe sets that contain one nucleotide mismatch to the Rhesus monkey genome. We found that both transcripts are expressed in various brain tissues; however, endogenous levels of *Bdnf*-AS are much lower than *Bdnf*. Expression in each tissue is normalized to the average of all tissues. Our data indicated the presence and differential expression of both *Bdnf* and *Bdnf*-AS transcripts in Rhesus monkey brain samples.

Supplementary Figure 5: Expression of *Bdnf* and *Bdnf*-AS in mouse tissues:



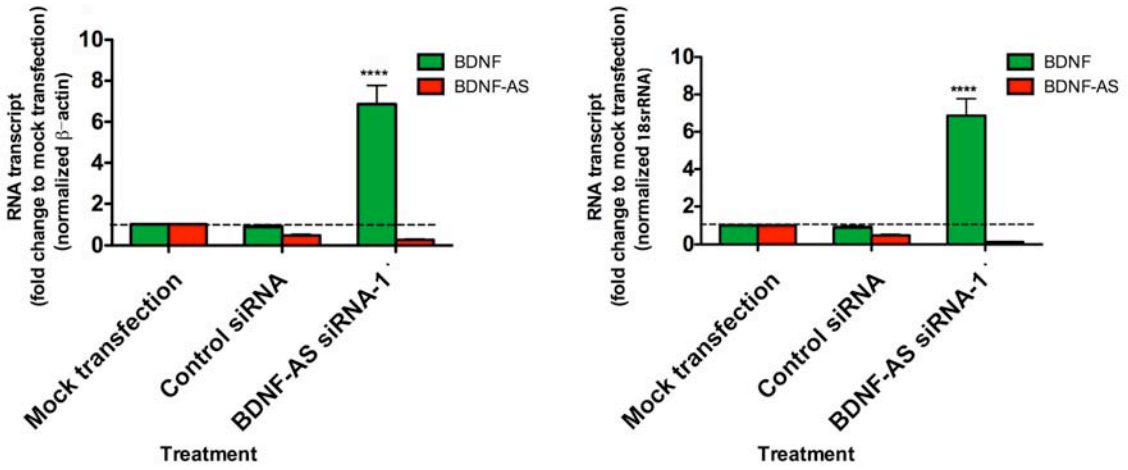
Utilizing 3' and 5' RACE approaches, we identified, for the first time, the *Bdnf*-AS transcript in the poly "A" RNA of mouse brain and testis. Next, we assessed expression of *Bdnf* and *Bdnf*-AS in mouse tissues, by RT-PCR. Both transcripts are expressed in many of the tissues examined; however, *Bdnf*-AS expression is much lower than *Bdnf* mRNA. The cerebellum and testis in addition to N2a cells show high levels of *Bdnf*-AS transcript expression.

Supplementary Figure 6: RNA FISH shows the expression of *Bdnf*-AS:



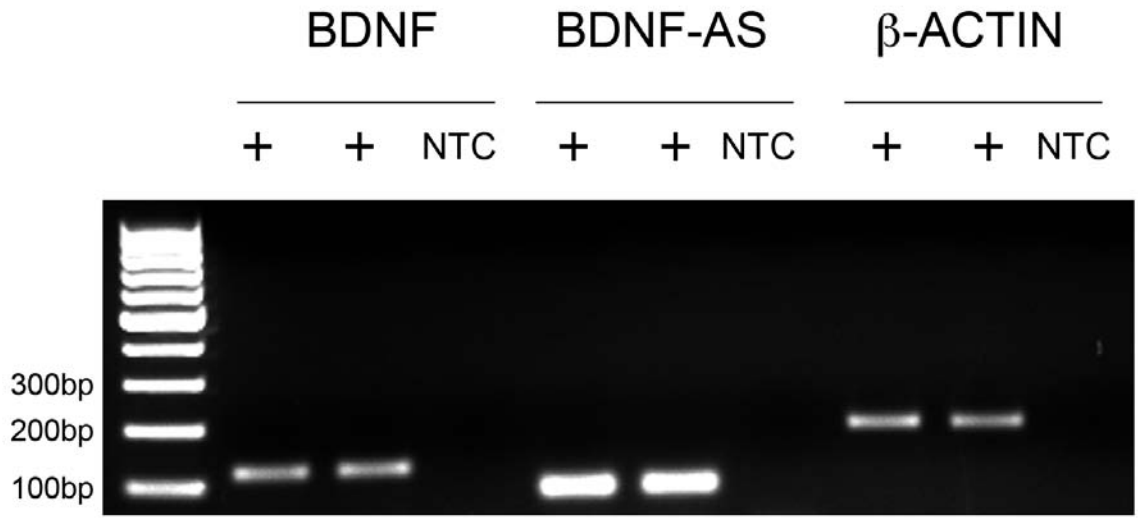
RNA FISH images with strand-specific probes in the mouse brain confirmed the presence of the *Bdnf*-AS transcript. We performed *in vitro* transcription of *Bdnf* and *Bdnf*-AS to generate strand-specific probes for the detection of *Bdnf* sense and antisense. We sliced, fixed and mounted mouse brain slices to the slides and incubated with specific RNA probes targeting *Bdnf* and *Bdnf*-AS. After several washes, we visualized the expression of *Bdnf*-AS (green) and *Bdnf* (red) transcripts. We documented the expression of *Bdnf*-AS in several brain regions, as well as in N2a cells. These data further confirm the presence of the *Bdnf*-AS transcript in mouse brain tissues.

Supplementary Figure 7: *BDNF*-AS knockdown leads to *BDNF* mRNA upregulation;



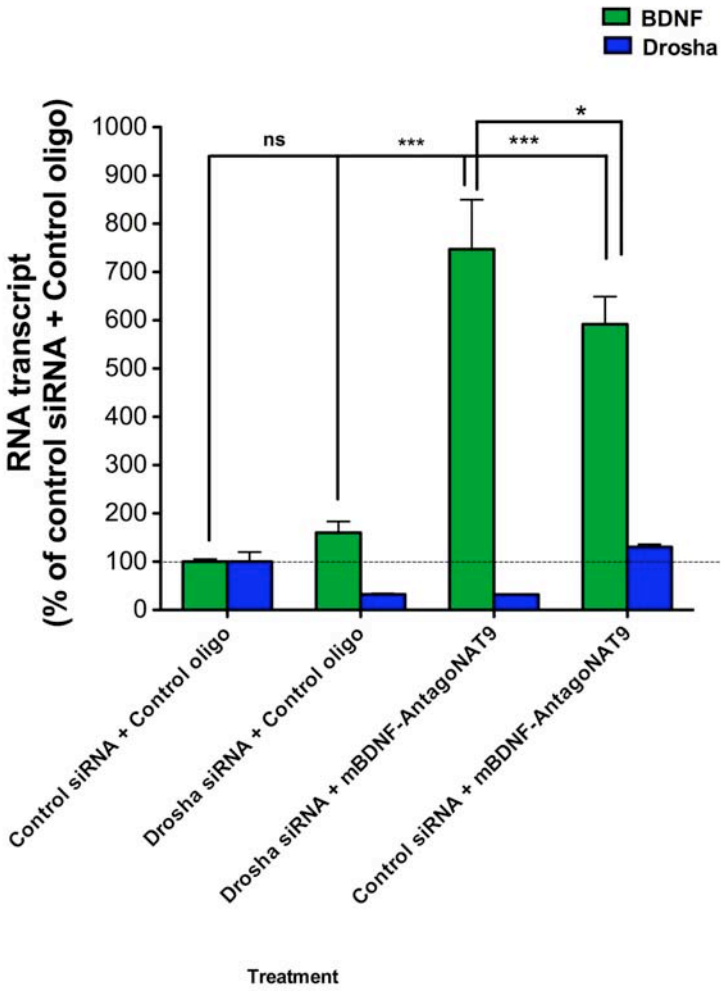
Knockdown of *BDNF*-AS, using siRNAs-1 (10 nM) targeting the non-overlapping region of the *BDNF*-AS transcript, caused a 6-fold upregulation of *BDNF* (sense) mRNA (****= $P < 0.0001$). Results depicted here were obtained from experiments in HEK293T cells, using beta actin (left panel) or 18S rRNA (right panel) as endogenous controls and the mock transfection as a reference sample. This experiment is intended to show that choice of endogenous controls or reference calibrator sample does not change the observed upregulation of *BDNF* mRNA.

Supplementary Figure 8: Controls for real-time PCR reactions:



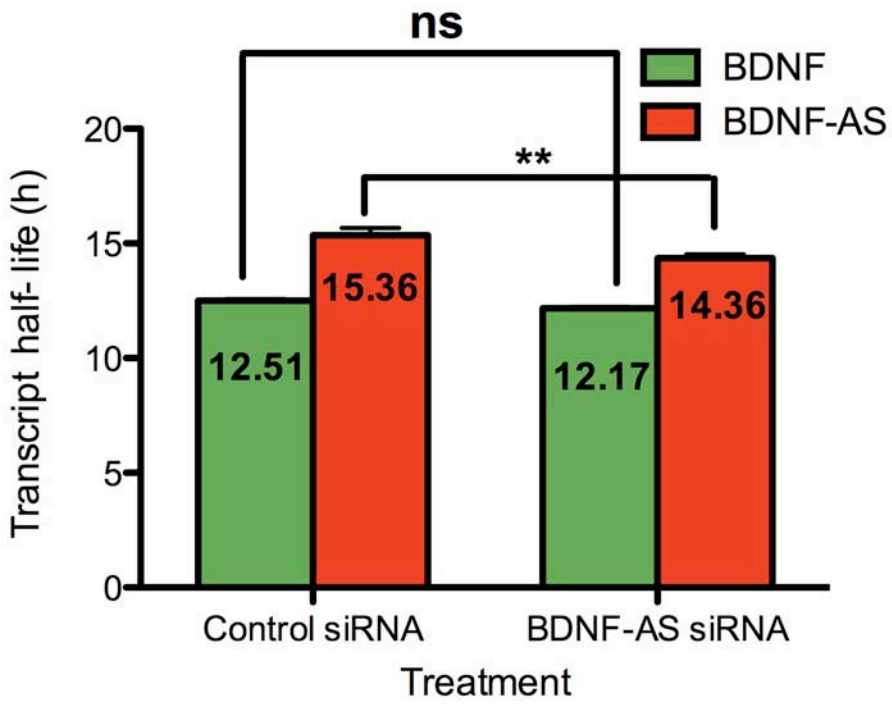
To ensure the validity of the RT-PCR, we included several controls. We included a no reverse transcriptase enzyme reaction (NRC) in all RT-PCR plates to make sure that there was no genomic DNA contamination. We also ran final RT-PCR products on a gel to ensure that only one product exists per reaction/probe. After completion of RT-PCR, final products from the *BDNF*, *BDNF*-AS and beta-actin probes, as well as corresponding no template controls (NTC) were separated on a 1.2% agarose gel and stained with ethidium bromide. There was only one band detectable in each lane, suggesting that RT-PCR probes were amplifying the correct product.

Supplementary Figure 9: Posttranscriptional regulation of *Bdnf* expression.



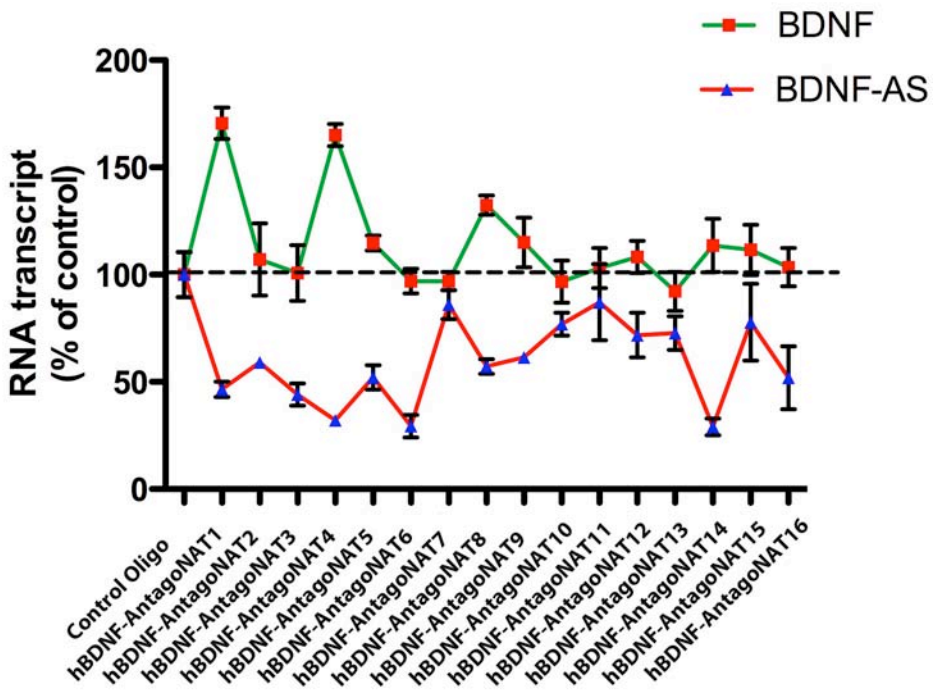
We transfected N2a cells with combination of mBdnf-AntagoNAT9 targeting mouse *Bdnf*-AS transcript and Drosha siRNA targeting Drosha protein, which is involved in microRNA (miRNA) processing. We observed *Bdnf* mRNA upregulation following treatment of cells with m*Bdnf*-ANtagoNAT9 (*** = p value <0.0001). Addition of Drosha siRNA marginally increased *Bdnf* transcript over m*Bdnf*-AntagoNAT9 treatment (*= p value <0.05). This experiment may suggest involvement of other post-transcriptional mechanisms, such as miRNAs in regulation of *Bdnf* transcript.

Supplementary Figure 10: *BDNF*-AS does not alter *BDNF* sense RNA stability:



We treated HEK293T cells with a siRNA targeting *BDNF*-AS or control non-targeting siRNA. To measure RNA stability, immediately following siRNA transfection, cells were treated with 50 μg/ml of α-amanitin, a drug that inhibits DNA polymerase II and blocks the synthesis of new RNA. RNA was then extracted and purified from harvested cells 24 h post treatment and RT-PCR was performed. The baseline half-life for *BDNF*-AS was 15 h 20 min, close to 3 h longer than the *BDNF* sense transcript. There was no significant change in *BDNF* sense RNA stability after reduction of the *BDNF*-AS transcript. This data indicates that the *BDNF*-AS transcript does not alter *BDNF* sense mRNA stability.

Supplementary Figure 11: Inhibition of the human *BDNF*-AS transcript by h*BDNF*-AntagoNAT:



The *BDNF*-AS transcript contains a 225-nucleotide overlapping region that has full complementarity to the *BDNF* mRNA. Our hypothesis is that RNA-RNA interactions may be responsible for the discordant regulation of *BDNF* by its antisense transcript. To determine the regulatory role of *BDNF*-AS on *BDNF* mRNA, we utilized gapmers (AntagoNATs) containing both LNA and 2'OMe RNA modification to block the interaction between sense and antisense transcripts. We covered overlapping region by tiling h*BDNF*-AatagoNATs. We found that the use of h*BDNF*-AntagoNATs upregulates the *BDNF* mRNA. We observed marginal downregulation of *BDNF*-AS transcript, which was not expected for 2'OMe-RNA containing blocking oligos. We tested 16 h*BDNF*-AntagoNATs (14-mers each with the sequences provided below) and found that blocking the first half of the *BDNF*-AS overlapping region has a greater effect on the upregulation of *BDNF* mRNA. Specifically, h*BDNF*-AntagoNAT1 and h*BDNF*-AntagoNAT4 caused significant upregulation of *BDNF* mRNA.

Unlike synthetic siRNAs, antisense oligonucleotides are single-stranded and can be shorter in

length; therefore, reducing non-specific (off-target) binding effects. Single-stranded locked nucleic acid (LNA)-modified oligonucleotides are generally more effective, *in vivo*, compared to unmodified siRNAs.

Sequence of h*BDNF*-AntagoNATs used in this study:

Bold letters indicate LNA bases and the remainder of the sequence consists of 2'-O-Me-RNA bases.

h*BDNF*-AntagoNAT1: CCA**G**GU**G**UGC**G**GA**C**

h*BDNF*-AntagoNAT2: CCA**U**GG**G**ACU**C**UG**G**

h*BDNF*-AntagoNAT3: AGA**G**CG**U**GAA**U**GG**G**

h*BDNF*-AntagoNAT4: CCCA**A**G**G**CAG**G**UU**C**

h*BDNF*-AntagoNAT5: AAG**A**UG**C**UUG**A**CA**U**

h*BDNF*-AntagoNAT6: CAU**U**GG**C**UGA**C**AC**U**

h*BDNF*-AntagoNAT7: UUC**G**AA**C**ACG**U**GA**U**

h*BDNF*-AntagoNAT8: AGAAGA**G**CUG**U**UG**G**

h*BDNF*-AntagoNAT9: AUG**A**GG**A**CCA**G**AA**A**

h*BDNF*-AntagoNAT10: GUU**C**GG**C**CCA**A**UG**A**

h*BDNF*-AntagoNAT11: AGA**A**AA**C**AAU**A**AG**G**

h*BDNF*-AntagoNAT12: ACG**C**AG**A**CUU**G**UA**C**

h*BDNF*-AntagoNAT13: ACG**U**CC**A**GGG**U**GA**U**

h*BDNF*-AntagoNAT14: GCU**C**AG**U**AGU**C**AA**G**

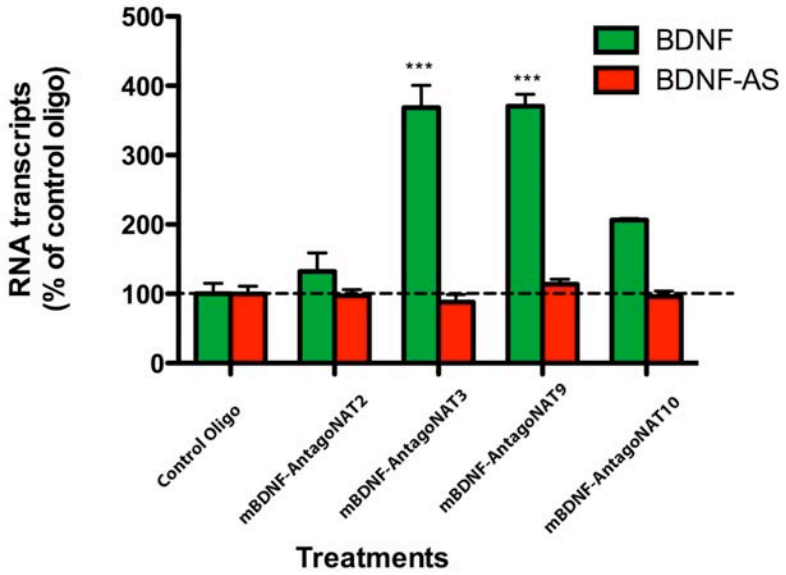
h*BDNF*-AntagoNAT15: UGC**C**UU**U**GGA**G**CC**U**

h*BDNF*-AntagoNAT16: CCU**C**UU**C**UCU**U**UC**U**

Human *BDNF* sense and antisense overlapping region:

CAGAAAGAGAAGAGGAGGCUCCAAAGGCACUUGACUACUGAGCAUCACCCUGGAC
GUGUACAAGUCUGCGUCCUUAUUGUUUUCUUCAUUGGGCCGAACUUUCUGGUCCU
CAUCCAACAGCUCUUCUAUCACGUGUUCGAAAGUGUCAGCCAAUGAUGUCAAGCA
UCUUGAACCUGCCUUGGGCCCAUUCACGCUCUCCAGAGUCCCAUGGGUCCGCACA
CCUGG

Supplementary Figure 12: Inhibition of the mouse *Bdnf*-AS transcript in N2a cells, by AntagoNATs:

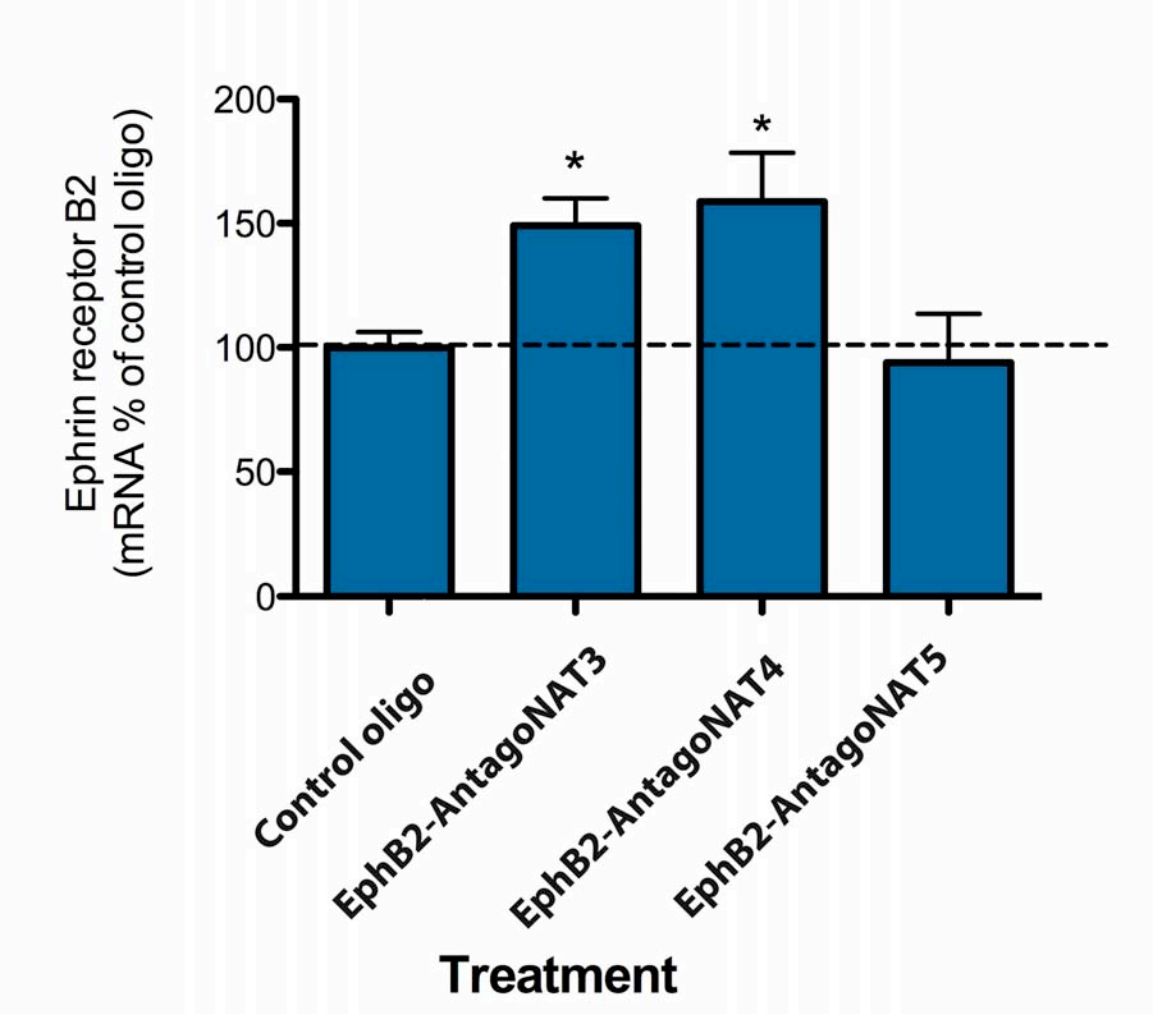


We showed that blocking of the overlap region between human *BDNF* sense and antisense transcripts upregulates *BDNF* mRNA levels. Next, we sought to determine if a similar regulatory mechanism exists in a mouse cell line and tested 11 m*Bdnf*-AntagoNATs that target the mouse *Bdnf*-AS transcript. m*Bdnf*-AntagoNATs contain a phosphorothioate backbone and three LNA-modified nucleotides at both 3' and 5' ends. Control oligonucleotides have a similar backbone and modifications, but do not target any sequence in the mammalian genomes. Two m*Bdnf*-AntagoNATs (m*Bdnf*-AntagoNA3 and m*Bdnf*-AntagoNAT-9) were able to increase *Bdnf* mRNA levels in N2a cells. In sum, blocking the mouse *Bdnf*-AS transcript with single-stranded AntagoNATs (16-mer) caused an upregulation of *Bdnf* mRNA levels in mouse N2a cells. These data suggest that the antisense transcript of *Bdnf* exerts a suppressive effect on *Bdnf* mRNA.

Sequence information: All PS backbone, 3 LNA each end

mBdnf-AntagoNAT2	GTATTAGCGAGTGGGT (overlap region)	AntagoNAT
mBdnf-AntagoNAT3	GTCTATGAGGGTTCGG (overlap region)	AntagoNAT
mBdnf-AntagoNAT9	CAACATATCAGGAGCC (second Exon)	AntagoNAT
mBdnf-AntagoNAT10	TGTATTCCCAGAACTT (second Exon)	AntagoNAT
Control AntagoNAT	CCACGCGCAGTACATG	AntagoNAT

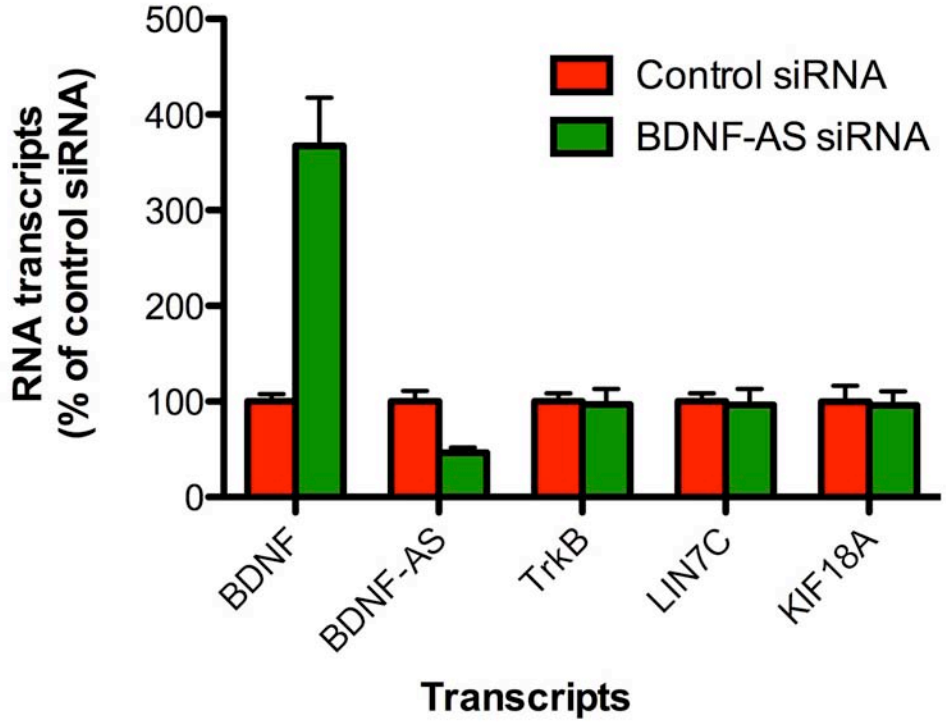
Supplementary Figure 13: Knockdown of antisense RNA to Ephrin receptor B2 (*EphB2*) causes upregulation of *EphB2*.



Selective knockdown of Ephrin receptor B2 antisense (*EphB2*-AS) increases *EphB2* mRNA: We treated cells with various AntagoNATs targeting a low abundance noncoding antisense RNA, *EphB2*-AS. We observed that *EphB2*-AntagoNAT3 and *EphB2*-AntagoNAT4 increase the *EphB2* mRNA (n=6 per treatment *= $P < 0.05$). Sequence information: Sequence information: All PS backbone, 3 LNA each end

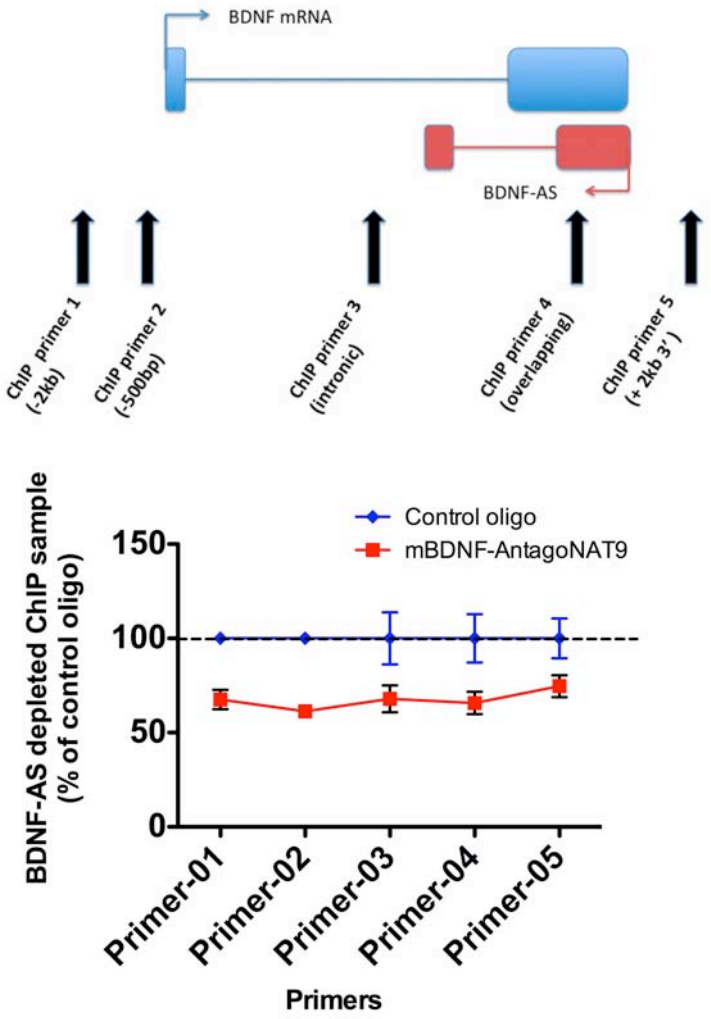
EphB2-AntagoNAT3	GATTTCAGAGCCGCAG	AntagoNAT
EphB2-AntagoNAT4	GACACATCCATCCCAG	AntagoNAT
EphB2-AntagoNAT5	CCTCGTCATGTCTGTG	AntagoNAT

Supplementary Figure 14: *BDNF*-AS knockdown neither changes the level of *TrkB* nor *BDNF* neighboring genes (*Let7C* and *KIF18A*) in both directions:



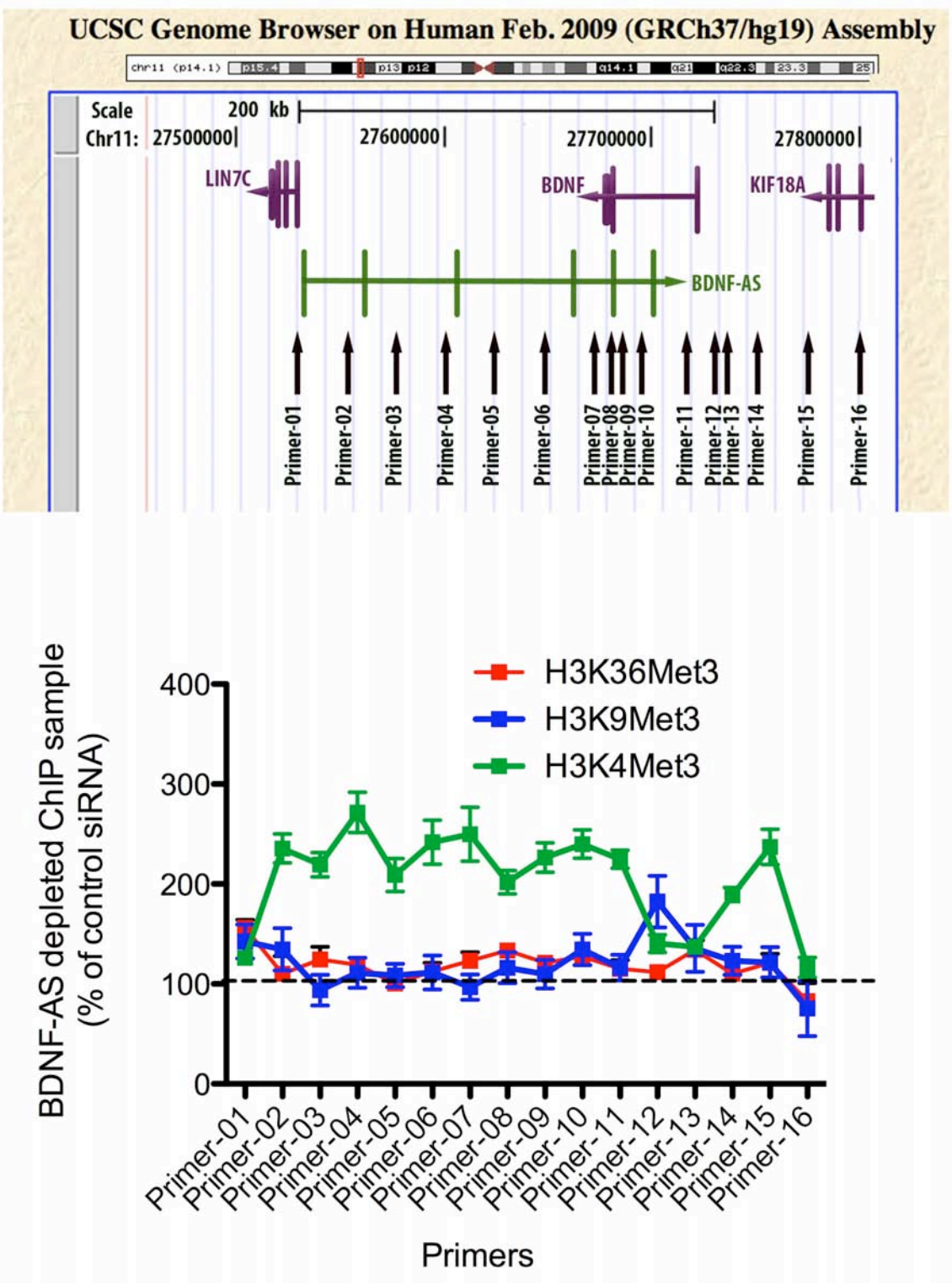
LIN7C and *KIF18A* are genes located 3' downstream and 5' upstream of *BDNF*, respectively. Neurotrophic tyrosine kinase, receptor, type 2 (*TrkB*) encodes a membrane-bound receptor for *BDNF* and is located on a different chromosome (Chr-9) as *BDNF*. We sought to determine whether these genes were altered upon depletion of the *BDNF*-AS transcript. We transfected HEK293T cells with control siRNA or *BDNF*-AS siRNA and measured several transcript levels. We observed that the *BDNF*-AS transcript was downregulated and that *BDNF* mRNA was upregulated as indicated elsewhere in this manuscript. We found that the knockdown of *BDNF*-AS has no effect on *TrkB* expression or on the neighboring genes Let7C and KIF18A. These data suggest that upon *BDNF*-AS depletion, there is a locus-specific alteration of *BDNF* expression.

Supplementary Figure 15: Reduction of mouse *Bdnf*-AS by m*Bdnf*-AntagoNAT9 is associated with modification of chromatin marks;



Mouse N2a cells were treated with control oligonucleotide or m*Bdnf*-AntagoNAT9. 48 h post-transfection cells were harvested, fixed with formaldehyde, sonicated and incubated with antibodies against H3K27met3. Immunoprecipitation was performed followed by DNA extraction. DNA samples were analyzed using 5 primer sets covering the entire mouse *Bdnf* gene locus, and the *Bdnf* promoter region, as indicated in inset data. There was a decrease in association of the repressive chromatin marker, H3K27met3, upon treatment of the cells with m*Bdnf*-AntagoNAT9 in all regions examined (n=6 for each data point). These results suggest that there is a local antisense-mediated chromatin repression of *Bdnf* locus and further show reduction of H3K27Met3 association following treatment of cells with m*Bdnf*-AntagoNAT9.

Supplementary Figure 16: *BDNF*-AS knockdown alters chromatin modifications associated to the *BDNF* locus:



BDNF-AS is a potent regulatory RNA transcript, despite its low abundance in the cell. Although the *BDNF*-AS cycle threshold (Ct) value is typically above 30, the knockdown of this transcript leads to a 2-6-fold increase in *BDNF* mRNA. We postulated that the *BDNF*-AS transcript is a local modulator of chromatin structure. This proposed mechanism might explain the potent functionality of *BDNF*-AS, despite the low abundance of the antisense RNA molecules. Unlike RNAs that have multiple copies present in a single cell, there are only two copies of DNA for any given gene in a cell. Therefore, only two molecules of antisense RNA per cell are sufficient to bind to the corresponding DNA strand and exert a regulatory function. To test this hypothesis, we performed chromatin immunoprecipitation (ChIP), using antibodies for H3K9Met3, H3K4Met3, H3K27Met3 and H3K36Met3 modifications. We found that the removal of the *BDNF*-AS transcript does not change the association of H3K9met3 at the *BDNF* genomic locus, except for one peak that indicates an increase at the *BDNF* promoter region. *BDNF*-AS depletion resulted in a significant increase in H3K4met3 association to the entire *BDNF* locus but not at the *BDNF* promoter regions. Upon *BDNF*-AS depletion, there was no significant alteration in the association of H3K36met3 to the entire *BDNF* locus. On the other hand, we found that the removal of the *BDNF*-AS transcript causes a reduction in the H3K27met3 repressive chromatin mark at the *BDNF* genomic locus (main text figure 6). These data suggest that the *BDNF*-AS transcript might directly impact H3K27 trimethylation.

Method: HEK293T cells were treated with control or *BDNF*-AS siRNA followed by ChIP analysis using antibodies against H3K4Met3, H3K36Met3, H3K27Met3 or H3K9Met3 chromatin marks. Immunoprecipitation was followed by RT-PCR of the extracted DNA, using 16 primer sets that covered the entire *BDNF* locus as indicated in the schematic above. The observed chromatin modification did not extend toward neighboring genes. These results suggest antisense-mediated chromatin modification, which is unlike H3K27met3 not extended to the promoter region.

Our findings show that the repressive or active chromatin marks can be induced or modified by ncRNA expression. We assessed the expression of immediate neighboring genes and found that removal of *BDNF*-AS does not affect their expression, suggesting that the antisense RNA-

mediated regulation of gene expression is a highly specific event. Considering the low abundance and high potency of *BDNF*-AS, we believe that antisense RNA-mediated regulation of *BDNF* occurs at the nuclear and transcriptional levels. Local accumulation of antisense RNA or formation of sense-antisense RNA duplexes, which can be processed to small RNA intermediates, trigger the cascade of events that result in modification of chromatin structure and the activation or deactivation of *BDNF* transcription. We find that the removal of *BDNF*-AS causes a reduction in suppressive H3K27met3 chromatin marks over the promoter and body of the *BDNF* gene locus. This modification is local and is expanded only to the promoter of the *BDNF* transcript. Identification of the components and the cascade of events that result in antisense RNA-mediated modification of chromatin structure are currently being investigated.

Supplementary Table S1:
List and sequence of AntagoNATs, siRNAs, primers and probes utilized for experiments. (F: Forward, R: Reverse, P: Probe)

Primer name	Sequence	Application
Human BDNF-AS siRNA-1	GGCTCACCAGTTGTTTGTT	siRNA
Scramble siRNA-1	AGCTCGCCAGTCGTTTATT	Scrambled control
Human BDNF-AS siRNA-2	GCAATGTATCTTAGGCTCA	siRNA
Human BDNF-AS siRNA-3	GCTAATCTTACAACAGCAC	siRNA
Scramble siRNA-3	ACTAAGCTTACAGCAGCGC	Scrambled control
Human BDNF-AS siRNA-4	TCCCTACAAACATGTCATT	siRNA
Scramble siRNA-4	GCCCGACAAACAAGTCAAT	Scrambled control
Control siRNA	CCUCUCCACGCGCAGUACATT	Non-targeting control
Human BDNF siRNA-1	GCCAACTGAAGCAATACTT	siRNA
Human BDNF siRNA-2	GCTGGCGATTCATAAGGAT	siRNA
Human BDNF prob	AGTAGTGACTGAAAAGTTCCACCAG	Real time PCR
Human BDNF-AS primer-F	AGTGGCTAATCTTACAACAGCACAA	Real time PCR
Human BDNF-AS primer-R	CTCAGTAGTCAAGTGCCTTTGGA	Real time PCR
Human BDNF-AS probe	CCTCCTCTTCTCTTTCTGGTTAG	Real time PCR
hBDNF-AntagoNAT1	CCAGGUGUGCGGAC	Blocking
hBDNF-AntagoNAT2	CCAUGGGACUCUGG	Blocking
hBDNF-AntagoNAT3	AGAGCGUGAAUGGG	Blocking
hBDNF-AntagoNAT4	CCCAAGGCAGGUUC	Blocking
hBDNF-AntagoNAT5	AAGAUGCUUGACAU	Blocking
hBDNF-AntagoNAT6	CAUUGGCUGACACU	Blocking
hBDNF-AntagoNAT7	UUCGAACACGUGAU	Blocking
hBDNF-AntagoNAT8	AGAAGAGCUGUUGG	Blocking
hBDNF-AntagoNAT9	AUGAGGACCAGAAA	Blocking
hBDNF-AntagoNAT10	GUUCGGCCCAAUGA	Blocking
hBDNF-AntagoNAT11	AGAAAACAAUAAGG	Blocking
hBDNF-AntagoNAT12	ACGCAGACUUGUAC	Blocking
hBDNF-AntagoNAT13	ACGUCCAGGGUGAU	Blocking
hBDNF-AntagoNAT14	GCUCAGUAGUCAAG	Blocking
hBDNF-AntagoNAT15	UGCCUUUGGAGCCU	Blocking
hBDNF-AntagoNAT16	CCUCUUCUCUUUCU	Blocking
Control AntagoNAT1	CCACGCGCAGUACA	Blocking
Control AntagoNAT2	UGCGCGUGGAGAGG	Blocking
Mouse Bdnf-AS 3' outer primer1	TCAGCAGTCAAGTGCCTTTG	RACE Reaction
Mouse Bdnf-AS 3' outer primer2	AACTGTGGGAAGGAAGCAGA	RACE Reaction
Mouse Bdnf-AS 3' inner primer1	CCATTCACGCTCTCCAGAGT	RACE Reaction
Mouse Bdnf-AS 3' inner primer2	TTAGAACTTTCTTTCTCGGG	RACE Reaction
Mouse Bdnf-AS 5' outer primer1	GGTTCGAGAGGTCTGACGAC	RACE Reaction
Mouse Bdnf-AS 5' inner primer1	TCAGCAGTCAAGTGCCTTTG	RACE Reaction
Mouse Bdnf-AS 5' inner primer2	ATTGGCTGGCGATTCATAAG	RACE Reaction
Mouse Bdnf-AS 5' outer primer2	AACTCGCA ATGCCGAACTAC	RACE Reaction
Mouse Bdnf-AS primer-F	TGGGAAGGAAGCAGAGACAGA	Real time PCR
Mouse Bdnf-AS primer-R	AAGCTGGGACAGAAGGAATGAG	Real time PCR
Mouse Bdnf-AS probe	CCAACCATCCTGTTCTGTG	Real time PCR
mBdnf-AntagoNAT1	CCCGGTATCCAAAGGC (overlap region),	AntagoNAT
mBdnf-AntagoNAT2	GTATTAGCGAGTGGGT (overlap region)	AntagoNAT
mBdnf-AntagoNAT3	GTCTATGAGGGTTCGG (overlap region)	AntagoNAT
mBdnf-AntagoNAT4	CCTCCTCTACTCTTTC (overlap region)	AntagoNAT
mBdnf-AntagoNAT5	GGCAGGTTCGAGA GGT (overlap region)	AntagoNAT
mBdnf-AntagoNAT6	TTCCTTCCCACAGTTC (overlap region)	AntagoNAT
mBdnf-AntagoNAT7	CGGTTGCATGAAGGCG (overlap region)	AntagoNAT
mBdnf-AntagoNAT8	TGGCTGGCGATTCATA (overlap region)	AntagoNAT
mBdnf-AntagoNAT9	CAACATATCAGGAGCC (second Exon)	AntagoNAT
mBdnf-AntagoNAT10	TGTATTCCCAGAACTT (second Exon)	AntagoNAT
Control AntagoNAT	CCACGCGCAGTACATG	AntagoNAT
EphB2-AntagoNAT3	GATTTCAGAGCCGCAG	AntagoNAT
EphB2-AntagoNAT4	GACACATCCATCCCAG	AntagoNAT
EphB2-AntagoNAT5	CCTCGTCATGTCTGTG	AntagoNAT
Mouse Bdnf-AS siRNA-1 (#49)	GCAACCGAAGTATGAAATAACCATA (On second Exon)	Stealth siRNA
Mouse Bdnf-AS siRNA-2 (#4)	GCCTTGTCCGTGGACGTTTACTTCT (On second Exon)	Stealth siRNA

Mouse Bdnf-AS siRNA-3 (#217)	GGATTACACTTGGTCTCGTAGAAAT (Overlap)	Stealth siRNA
Mouse Bdnf-AS siRNA-4 (#504)	GGAAGTGTACAAGTCCGCGTCCTTA (Overlap)	Stealth siRNA
Mouse Bdnf-AS siRNA-5 (#833)	CAGAACAGGTTAGAACTTTCTTTCT (Overlap)	Stealth siRNA
BDNF IP Primer01-F	TGGAGTCTTCTACTGAAGAACAGAAA	Chromatin IP
BDNF IP Primer01-R	TGTGGAACTTCTGTGGCATT	Chromatin IP
BDNF IP Primer02-F	TTGACTTTGGGTTGCCCTAC	Chromatin IP
BDNF IP Primer02-R	TAGGGAGTGCTGGCTAGGAA	Chromatin IP
BDNF IP Primer03-F	GAAGTGGCAGAGGAAAATGC	Chromatin IP
BDNF IP Primer03-R	AGGCAGACCTGGTCACCTTA	Chromatin IP
BDNF IP Primer04-F	TTAGTTTGTTCCTTTATAACCAAGC	Chromatin IP
BDNF IP Primer04-R	AGAGCCTTTGCCATCAACTG	Chromatin IP
BDNF IP Primer05-F	AAGGCGAATGCATCTTTTTG	Chromatin IP
BDNF IP Primer05-R	CTGGCCCTGAATCCTCATTA	Chromatin IP
BDNF IP Primer06-F	TTTGCTTTAAGATTGACCCTGA	Chromatin IP
BDNF IP Primer06-R	GGAGCAGTGTTGGCAATGTA	Chromatin IP
BDNF IP Primer07-F	TGACTTGTGAGGCTGAGGTG	Chromatin IP
BDNF IP Primer07-R	CCTTCTCTCCCCCATTCTTC	Chromatin IP
BDNF IP Primer08-F	AAAGTTCGGCCCAATGAAG	Chromatin IP
BDNF IP Primer08-R	CCTCATGGACATGTTTGCAG	Chromatin IP
BDNF IP Primer09-F	AGAAGCAAACATCCGAGGAC	Chromatin IP
BDNF IP Primer09-R	TCCAACAGCTCTTCTATCACG	Chromatin IP
BDNF IP Primer10-F	TGCTTTCAGCCAGATGTCTC	Chromatin IP
BDNF IP Primer10-R	GAGTTGGTTCCCTCTGTTGC	Chromatin IP
BDNF IP Primer11-F	CACAGGGAGATGCAAGTTGA	Chromatin IP
BDNF IP Primer11-R	GAAAGGCACTCCCATTTCAG	Chromatin IP
BDNF IP Primer12-F	GCGCTGAATTTTGATTCTGG	Chromatin IP
BDNF IP Primer12-R	GAAAGTGGGTGGGAGTCCA	Chromatin IP
BDNF IP Primer13-F	AACGCACACACACAGAAAGC	Chromatin IP
BDNF IP Primer13-R	CCCTCCTCCTGAAATTGTGA	Chromatin IP
BDNF IP Primer14-F	GCTTTTTAAGGGCGACACAG	Chromatin IP
BDNF IP Primer14-R	ACAGAGCCAACGGATTTGTC	Chromatin IP
BDNF IP Primer15-F	CGGGAAACTGAAAATTCACC	Chromatin IP
BDNF IP Primer15-R	TCAATTTTGACAGAGGACCAGA	Chromatin IP
BDNF IP Primer16-F	GGCCCAAGATGTATTTGGTT	Chromatin IP
BDNF IP Primer16-R	TTAAAGTATTTTTGAAAAGGAAAGTCA	Chromatin IP
Mouse BDNF IP Primer01-F	ctgtaccccaagacctctgaag	Chromatin IP
Mouse BDNF IP Primer01-R	catccatgaattagccagcag	Chromatin IP
Mouse BDNF IP Primer02-F	atcttcccctcctagcctacac	Chromatin IP
Mouse BDNF IP Primer02-R	tgagagttaacagcctctgtgc	Chromatin IP
Mouse BDNF IP Primer03-F	taagcttggtttggtccatagg	Chromatin IP
Mouse BDNF IP Primer03-R	ctataagcatactccgcaaacc	Chromatin IP
Mouse BDNF IP Primer04-F	CAAACATGTCTATGAGGGTTCG	Chromatin IP
Mouse BDNF IP Primer04-R	TGTGACCCACTCGCTAATACTG	Chromatin IP
Mouse BDNF IP Primer05-F	caccttgaactgcaacaagag	Chromatin IP
Mouse BDNF IP Primer05-R	gtaataacgttgccaggaatgc	Chromatin IP

Additional Data File 1: Human *BDNF*-AS sequence

One splice variant of human *BDNF*-AS

Exon 5 or 6 has the overlap with *BDNF* (Red letters 225 bp)
Blue letters: Exon-exon boundaries

Gtcatcgctgtctggaacagcgatgactcgatcgcgagatcaggaaggtggccgagtgtgtcgccgcggccatcaggcacttctccttcct
gcccttgtatgaagaaggatgtgtttgcttccccttgtgccatgattgtaaatttcctgaggcctcctcagccctgcagaactggggttatagcc
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tggggcaagacttttcagtgtaaacttcttttaaatttttattttgatttttgaataatgtaaattaactgtcaaataattaaattaaaaataaccaatttat
taacaaaaaaaaaaaaaaaa

Additional Data File 2: Mouse *BDNF*-AS sequence

Mouse *Bdnf*-AS sequence (Varient-1):
Red letters shows overlapping region with mouse *Bdnf* transcript
Green highlighted part indicates common overlapping region between human and mouse

CGCTGTCTCAATATAATCTATACAACATAAATCCACTATCTTCCCCTTTTAATGGTCA
GTGTACATACACAGGAAGTGTCTATCCTTATGAATCGCCAGCCAATTCTCTTTTTGCT
ATCCATAGTAAGGGCCCGAACATACGATTGGGTAGTTCGGCATTGCGAGTTCCAGTG
CCTTTTGTCTATGCCCCTGCAGCCTTCCTTGGTGTAACCCATGGGATTACACTTGGTC
TCGTAGAAATACTGCTTCAGTTGGCCTTTGGATACCGGGACTTTCTCTAGGACTGTG
ACCGTCCCGCCAGACATGTCCACTGCAGTCTTTTTATCTGCCGCTGTGACCCACTCGC
TAATACTGTCACACACGCTCAGCTCCCCACGGCGGGCAGGGTCGGAGTGGCGCCGA
ACCCTCATAGACATGTTTGCGGCATCCAGGTAATTTTTGTATTCCTCCAGCAGAAAG
AGTAGAGGAGGCTCCAAAGGCACTTGACTGCTGAGCATCACCCGGGAAGTGTACAA
GTCCGCGTCCTTATGGTTTTCTTCGTTGGGCCGAACCTTCTGGTCCTCATCCAGCAGC
TCTTCGATGACGTGCTCAAAAGTGTCAGCCAGTGATGTCGTCGTCAGACCTCTCGAA
CCTGCCCTGGGCCCATTCACGCTCTCCAGAGTCCCATGGGTCCGCACACCTGGGTAG
GCCAAGTTGCCTTGTCCGTGGACGTTTACTTCTTTCATGGGCGCCGCCTTCATGCAAC
CGAAGTATGAAATAACCATAGTAAGGAAAAGGATGGTCATCACTCTTCTCACCTGGT
GGAACTGTGGGAAGGAAGCAGAGACAGACACAGAACAGGTTAGAACTTTCTTTCTC
GGGGACAGCATGTGGCCCATCTGCTTCAATAATTTAATTTAAAAAAAAA

Mouse *Bdnf*-AS sequence (Two exons splice variant, varient-2):
Red letters shows overlapping region with mouse *Bdnf* transcript
Green highlighted part indicates common overlapping region between human and mouse
Blue **GG** **indicates the exon boundary**

CGCTGTCTCAATATAATCTATACAACATAAATCCACTATCTTCCCCTTTTAATGGTCA
GTGTACATACACAGGAAGTGTCTATCCTTATGAATCGCCAGCCAATTCTCTTTTTGCT
ATCCATAGTAAGGGCCCGAACATACGATTGGGTAGTTCGGCATTGCGAGTTCCAGTG
CCTTTTGTCTATGCCCCTGCAGCCTTCCTTGGTGTAACCCATGGGATTACACTTGGTC
TCGTAGAAATACTGCTTCAGTTGGCCTTTGGATACCGGGACTTTCTCTAGGACTGTG
ACCGTCCCGCCAGACATGTCCACTGCAGTCTTTTTATCTGCCGCTGTGACCCACTCGC
TAATACTGTCACACACGCTCAGCTCCCCACGGCGGGCAGGGTCGGAGTGGCGCCGA
ACCCTCATAGACATGTTTGCGGCATCCAGGTAATTTTTGTATTCCTCCAGCAGAAAG
AGTAGAGGAGGCTCCAAAGGCACTTGACTGCTGAGCATCACCCGGGAAGTGTACAA
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TCTTCGATGACGTGCTCAAAAGTGTCAGCCAGTGATGTCGTCGTCAGACCTCTCGAA
CCTGCCCTGGGCCCATTCACGCTCTCCAGAGTCCCATGGGTCCGCACACCTGGGTAG
GCCAAGTTGCCTTGTCCGTGGACGTTTACTTCTTTCATGGGCGCCGCCTTCATGCAAC
CGAAGTATGAAATAACCATAGTAAGGAAAAGGATGGTCATCACTCTTCTCACCTGGT
GGAACTGTGGGAAGGAAGCAGAGACAGACACAGAACA**GG**ATGGTTGGCTCCAGCC
TCATTCCTTCTGTCCCAGCTTCTGAAGTTCTGGGAATACAACATGTACCATCATGCCA
AGCTCTACTTTTTGAAATCATGGCTCCTGATATGTTGGGCAGCCCTGTTGTGCTTTGA
AGATAAAATGCTCCACAAA