

Title: Gene Expression Profiling of Hypothalamic Harmartomas...

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SUPPLEMENTARY INFORMATION

Supplementary Note 1

RNA Extraction

Total RNA from the HH samples was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA concentrations were determined spectrophotometrically, and RNA integrity was verified on denaturing agarose gels, and by electrophoretic trace using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA from human placenta and human fetal brain was purchased from BD Biosciences (San Jose, CA); total RNA from human hypothalamus was obtained from Ambion (Austin, TX).

The medial basal hypothalamus (MBH) and prefrontal cortex (FC) of female monkeys were dissected as described [1, 2] and frozen at -85°C until RNA extraction. The tissues were homogenized in TriReagent solution (MRC, Cincinnati, OH, USA) and the RNA was extracted following the manufacturer's protocol. In brief, the aqueous and organic phases were separated by the addition of 0.1 volume bromo-chloropropane (Sigma Chemicals, St Louis, MO, USA) per ml TriReagent, followed by centrifugation at 4°C. RNA was precipitated from the aqueous phase with 1 volume of isopropanol, overnight at -20°C followed by centrifugation at 13,000 rpm for 15 min at 4°C. After washing with 70% ethanol, the RNA was then resuspended in DEP-treated H₂O and treated with DNA-free DNase I (Ambion, Austin, TX, USA). RNA concentrations and integrity were determined as indicated for the HHs.

Supplementary Note 2

Sample Preparation and Microarray Hybridization

Microarray assays were performed in the Affymetrix Microarray Core of the OHSU Gene

Microarray Shared Resource. One hundred ng of each total RNA sample was amplified and labelled using the GeneChip Eukaryotic Small Sample Target Labelling Assay Version II (Affymetrix, Santa Clara, CA). This protocol uses two rounds of cDNA synthesis and in vitro RNA transcription to produce amplified, biotinylated cRNA targets. In the first round of RNA amplification, mRNA is converted to double-stranded cDNA using Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA) and an oligo-dT primer linked to a T7 RNA polymerase binding site sequence (Integrated DNA Technologies, Coralville, IA); cRNA is synthesized using the MEGAscript High Yield Transcription kit (Ambion, Austin, TX). The unlabeled cRNA is then reverse transcribed in the first strand cDNA synthesis step of the second cycle using random primers. A T7-oligo dT primer is used for the second strand cDNA synthesis to generate double-stranded cDNA containing T7 promoter sequences. Biotin-labeled cRNA is transcribed from the second round cDNA using the BioArray HighYield RNA Transcript Labeling Kit (ENZO Life Sciences, Farmingdale, NY).

The quality of each target was checked by analyzing 200 ng of each labelled cRNA on a RNA 6000 LabChip using the 2100 Bioanalyzer (Agilent Technologies) to measure the size range of amplified RNAs. Targets were then fragmented at 95° C in the presence of high magnesium concentration, and combined with biotinylated control oligomer (used for grid alignment during image processing) and hybridization control cRNAs for BioB, BioC, BioD and cre in hybridization solution (100 mM MES [2-(N-morpholino) ethanesulfonic acid], 1 M Na⁺, 20 mM EDTA, 0.01% Tween 20, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA). All solutions and hybridization assay procedures were as described in the Affymetrix GeneChip Expression Analysis Technical Manual.

Ten g of labeled cRNA were hybridized with the Human Genome_U133A GeneChip array (Affymetrix, Santa Clara, CA) containing 18,400 transcripts and variants, including 14,500 well-characterized human genes. After overnight hybridization at 45° C each array was washed, stained with streptavidin-phycoerythrin (SAPE, Molecular Probes, Eugene, OR), followed by signal amplification with biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA), and a final SAPE staining step. Array processing was performed on the Fluidics Station 400 (Affymetrix, Santa Clara,

CA). The distribution of fluorescent material on the array was measured using a confocal laser scanner (GeneArray laser scanner, Affymetrix).

Supplementary Note 3

Microarray Data Analysis

Image processing and expression analysis were performed using Affymetrix Microarray Suite (MAS) 5.0 software. An absolute (single assay) expression analysis was performed for each GeneChip genome array hybridization. The GeneChip expression arrays contain control probe sets for both spiked and endogenous RNA transcripts (e.g., BioB, BioC, BioD, CreX and species-specific actin and GAPDH). Following image processing and absolute analysis of the array pattern with MAS 5.0, six parameters were examined to assess the overall assay performance: background, noise, average signal, % present, ratio of signal values for probe sets representing the 5' and 3' ends of actin and GAPDH transcripts, and total signal for BioC, BioD and CreX probe sets. Prior to further analysis, array data were globally scaled to a uniform, average target intensity for all assays prior to further analysis. Initial filters removed all absent genes and genes with no change across all data sets. The final group of genes was composed of genes presenting a 2-fold increase or more in HH1 compared to HH2-4.

Supplementary Note 4

Semi-Quantitative PCR Validation of Array Results

Total RNA (50 ng) from each HH were reverse transcribed using Omniscript RT Kit (Qiagen, Valencia, CA) for 1 h at 37°C followed by 5 min at 90°C. PCR was performed in a volume of 25 µl containing 1 µl of RT product, 2.5 µl of 10X buffer (HotStar Taq Polymerase Kit, Qiagen), 1 µl of 10 mM dNTPs (Promega-Fisher, Santa Clara, CA), 0.15 µl HotStar Taq Polymerase (Qiagen), 0.5 µl of each gene specific primer (50 µM) or 0.5 µl of a set of primers (50 µM) that amplify cyclophilin, a constitutive expressed gene used as to normalize the RNA values detected for each gene of interest. The primers were chosen using Primer Express software (PR Applied Biosystems, Foster City, CA). They were as follows:

Cyclophilin (NM_021130): sense 5'-GGCAAATGCTGGACCCAACACAAA-3' and antisense 5'-CTAGGCATGGGAGGGAACAAGGAA-3'; IA-1 (NM_002196.1): sense 5'-GTCTCGCCTCGCCTACCAATC-3' and antisense 5'-GGACCCTCGAAGCAGAATAAATGA-3' (annealing temperature: 51°C, 33 cycles); VILIP-1 (NM_003385.1): sense 5'-GGGGACGGCACCATTGA-3' and antisense 5'-CTCTTTGCAGCTTCTTTGA-3' (annealing temperature: 52°C, 29 cycles). TSG-6 (NM_007115): sense 5'-CGGCCATCTCGCAACTTACA-3' and antisense 5'-CAATAGGCATCCCATCTTTCACTC-3' (annealing temperature: 56°C, 33 cycles); mGluR1A (NM_000838.2): sense 5'-GAGAGCGGAAAATCAATGG-3' and antisense 5'-CAAGCCGAAGGAAAACACA-3' (annealing temperature: 52°C, 35 cycles); MEF2A (NM_005587.1): sense 5'-GCGGAAGAAAATACAAATCACACG-3' and antisense 5'-ATATCCGAGTTGGTTCTGCTTTCA-3' (annealing temperature: 52°C, 29 cycles).

After an initial incubation at 95°C for 15 min, the samples were amplified for the number of cycles specified above. Each cycle consisted of 30 sec at 94°C (denaturing), 30 sec at the annealing temperature indicated above, 1 min at 72°C (extension), and a final extension of 10 min at 72°C. VILIP-1 PCR products were resolved on a 2% MetaPhor agarose gel (Cambrex, Rockland, ME). All other PCR products were resolved on 2% regular agarose gels.

Other genes examined were GnRH, TGF α , KiSS1, and GPR54. The GnRH (NM_000825) primers used (sense 5'-CAAAAACCTCCTAGCTGGCCTT-3' and antisense 5'-CAGTTGACCAACCTCTTTGACT-3') were identified by searching the PrimerBank database [3]. The PCR program used (<http://pga.mgh.harvard.edu/primerbank/>) consisted of 2 min at 50°C, followed by 10min at 95°C and 38 cycles of 15 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C, with a final extension of 10 min at 72°C. The other primers were: KiSS1 (NM_002256): sense 5'-GCTACTGCTTTTCCTCTGTGCC-3' and antisense 5'-TAGCAGCTGGCTTCCTCTCG-3' (annealing temperature 50°C, 33 cycles); GPR54 (NM_032551): sense 5'-TGGGGAACCTCGCTGGTCATCTACG-3' and antisense 5'-CGCGCAACGGGAACACC-3' ((annealing temperature 60°C, 36 cycles); TGF α

(NM_003236): sense 5'-ATGACTGCCAGATTCCCACAC-3' and antisense 5'-TCGTGCCGGCAGATGAGG-3' (annealing temperature 60°C, 35 cycles).

Supplementary Note 5

Semi-Quantitative PCR Analysis of IA-1, TSG-6 and mGluR1A mRNA Expression in Monkey Hypothalamus

Five hundred ng of total RNA from each monkey tissue sample were reverse transcribed as indicated in Supplementary Note 4. The PCR primers used were as follows: IA-1 (XM_001091915): sense 5'-ACGCCGCCAGGTGTTCC-3' and antisense 5'-AGATTGGTAGGCGAGGCGAGTAGA-3'; TSG-6 (XM_0011083111): sense 5'-TCTCCAGGCTTCCCAAATGAG-3' and antisense 5'-AGCCAAGCAACCTGGATCATC-3'; mGluR1A (XM_001085942): sense 5'-AGCCCCCTATGCCCATCTATCCT-3' and antisense 5'-TCCCTGTCCCTGCCTTCTTTCTTC-3'. The cyclophilin primers used were the same used to measure cyclophilin mRNA from the HHs. The PCR program consisted of an initial incubation at 95°C for 15 min, followed by followed by an amplification of 35 cycles for TSG-6, 33 cycles for mGluR1A and 31 cycles for IA-1, each cycle consisting of 30 sec at 94°C, 30 sec at 60⁰ C, 1 min at 72°C, and a final extension of 10 min at 72°C.

Supplementary Table 1. Genes Showing a 2-fold Decrease or More in an HH Associated with Precocious Puberty (HH1) in Comparison to HHs Not Accompanied by Sexual Precocity (HH2-4).

Accession Number	Gene identity	Function	1vs2 Fold decrease	1vs3 Fold decrease	1vs4 Fold decrease	Mean fold decrease
NM_004750.1	Cytokine receptor-like factor 1 (CRLF1)	Cell-cell communication	-7.46	-3.25	-10.56	-7.09
NM_005460.1	Synphilin	Cell-cell communication	-3.73	-2.64	-2.83	-3.07
AW157070	Epidermal growth factor receptor precursor	Cell-cell communication	-3.03	-2	-2.64	-2.56
AI017770	SEC14-like 1	Cell-cell communication	-2.46	-2	-2.83	-2.43
NM_003567.1	Breast cancer anti-estrogen resistance 3 (BCAR3)	Cell-cell communication	-3.48	-2.3	-3.48	-3.09
BE500977	Sulfatase 1	Metabolism	-2.64	-2.46	-2.3	-2.47
NM_000851.1	Glutathione S-transferase (GSTM5)	Metabolism	-9.19	-3.03	-14.93	-9.05
AF074331.1	PAPS synthetase-2 (PAPSS2)	Metabolism	-2.3	-6.06	-2.64	-3.67
NM_017512.1	Enolase superfamily member 1 (ENOSF1)	Metabolism	-3.73	-2.83	-2.64	-3.07
AB002384.1	KIAA0386	Cell differentiation	-3.03	-2.46	-2.64	-2.71
NM_021960.1	Myeloid cell leukaemia sequence 1	Cell differentiation	-2	-2.3	-3.73	-2.68
NM_017680.1	Asporin (ASPN)	Cell adhesiveness	-3.25	-3.03	-4.92	-3.73
NM_003319.1	Titin (TTN)	Structural protein	-4	-2.14	-3.48	-3.21
AI360875	Sex determining region Y-box 11	Transcriptional regulation	-2	-2.46	-2.3	-2.25
AL021977	Hypothetical protein	Unknown	-3.03	-2.3	-3.48	-2.94

References

- [1] Ma YJ, Costa ME, Ojeda SR: Developmental expression of the genes encoding transforming growth factor alpha (TGF) and its receptor in the hypothalamus of female rhesus macaques. *Neuroendocrinology* 1994;60:346-359.
- [2] Mastronardi C, Smiley GG, Raber J, Kusakabe T, Kawaguchi A, Matagne V, Dietzel A, Heger S, Mungenast AE, Cabrera R, Kimura S, Ojeda SR: Deletion of the *Tf1* gene in differentiated neurons disrupts female reproduction without impairing basal ganglia function. *J Neurosci* 2006;26:13167-13179.
- [3] Wang X, Seed B: A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res* 2003;31:e154.