Neuropeptide Y Receptor Gene Expression in the Primate Amygdala Predicts Anxious Temperament and Brain Metabolism

Supplemental Information

Supplemental Methods

All subjects (*N* = 238) were indexed for anxious temperament (AT) using a combination of behavioral and hormonal measures, and brain metabolic activity was subsequently assessed using [^{18}F]-fluorodeoxyglucose - positron emission tomography (FDG-PET) as described in detail below. A subset of animals (*n* = 24) were chosen for quantification of gene expression levels for the neuropeptide Y (NPY) family of genes. Robust regression analysis was used to test relations between AT and mRNA expression levels for the NPY family members that were detectable in the central nucleus of the amygdala (Ce). For those genes that had expression levels that predicted levels of AT (*NPY1R* and *NPY5R*), *in situ* hybridization analysis was used to define expression patterns in the amygdala and neighboring regions. Lastly, whole brain FDG-PET was used to determine if Ce gene expression levels for NPY1R or NPY5R predicted brain metabolic activity at distal sites.

Subjects

Behavior and brain metabolism were initially characterized in 238 young rhesus monkeys (*Macaca mulatta*) injected with FDG and exposed for 30-minutes to the No-Eye Contact (NEC) condition (described below) that elicits the AT phenotype. The details of the imaging of these monkeys have been previously described (1-3). At the time of behavioral testing/FDG-PET scans, the mean age was 2.41 years (SD = 0.92 years; 51.3% female), which is considered to be peripubertal. Animal housing and experimental procedures were in accordance with institutional guidelines. Monkeys were mother-reared and paired-housed at the

Wisconsin National Primate Research Center or Harlow Center for Biological Psychology. From this larger sample, 24 male monkeys were assessed two additional times using FDG-PET in the NEC condition for a total of three assessments over a period of 6 to 18 months (for additional details, see Ref. (3)). Between the second and third assessment, half of the animals were relocated every 5 days over a period of 3 weeks; the other half of the animals remained in their home cages. Relocation did not have any significant effects on behavior or physiology (3). The separation between the second and third assessments varied from 27 to 77 days with an average of 50 days and standard deviation of 10 days, and varied in duration from the initial assessment as previously described (3). Housing and experimental procedures complied with the animal care and use guidelines of the United States National Institutes of Health and were approved by the University of Wisconsin–Madison Institutional Animal Care and Use Committee.

Behavioral Assessment

During the NEC challenge, a human intruder enters the test room and presents his or her profile to the monkey while avoiding eye contact (4). Behavior was unobtrusively assessed via a closed-circuit television system by an experienced rater (4). Freezing was defined as a period of ≥ 3 sec of tense body posture, no vocalizations, and no locomotion except for slow movements of the head. Coo vocalizations were defined as audible calls made by rounding and pursing the lips with an initial increase and subsequent decrease in frequency and intensity. Locomotion was defined as one or more full movements at any speed in any direction, including such behavior as dropping from ceiling to floor.

Cortisol Assessment

Following 30 min exposure to the NEC challenge, animals were anesthetized and blood was collected. Blood sampling occurred between 08:45 and 14:45 hours, and approximately 6

min elapsed between the end of the NEC and blood collection. Plasma cortisol levels were quantified using the DPC Coat-a-count assay (Siemens, Los Angeles, CA). Samples were diluted 8-fold prior to being measured in duplicate, and the average ED_{80} and ED_{20} of the assay were 1.1 μg/dL and 36.9 μg/dL, respectively. The inter-assay and intra-assay coefficients of variation were 6.6% and 4.0%, respectively.

Computing Anxious Temperament Composite

AT is a composite of behavioral (freezing and cooing) and hormonal measures (cortisol) (1, 2, 5). Each appropriately-transformed measure [-1 x $cooing^{½}$, cortisol, log_e(freezing)] was residualized to remove variance linearly predicted by age and, in the case of cortisol, time-of-day. Cooing values were reflected to ensure consistent signs across the three component measures. The mean of the *Z*-transformed standardized measures obtained during the three assessments was used to compute average AT for each subject (1, 5).

[^{18}F]-FDG-PET Acquisition

The procedure for high-resolution FDG-PET assessment of brain metabolic activity has previously been described in detail (1-3). Scanning was performed using a microPET P4 scanner (Concorde Microsystems, Inc., Knoxville, TN; (6)) with an intrinsic resolution of approximately 2-mm full-width at half-maximum.

Tissue and RNA Isolation

Twenty-four monkeys were sacrificed and brain tissue was obtained and cut into slabs prior to freezing for storage at -80°C. From each monkey, one hemisphere was dissected into 14.5 mm slabs in preparation for cryostat sectioning for *in situ* hybridization. The remaining hemisphere was dissected into 4.5 mm slabs for tissue punches. The hemisphere used for each procedure was counterbalanced across monkeys. At the start of the molecular analyses, tissue

slabs were subsequently thawed to obtain punches of the Ce region as previously described (3). In addition to the Ce, a control brain region corresponding to the primary motor cortex was obtained. The Ce region that was punched corresponds to the amygdalar FDG-PET signal that was most predictive of AT (Figures 1A and 1B in the main report), whereas the primary motor cortex is not a core component of the neural circuit that underlies AT (1). RNA was extracted from the Ce and motor cortex samples using Qiagen RNeasy plus mini kit (Valencia, CA).

Gene Expression Analysis

For quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis, the cDNA was reverse transcribed from the RNA using SuperScript Vilo (Life Technologies, Carlsbad, CA). The cDNA served as template for qRT-PCR using TaqMan probes and the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). The TaqMan probe and primer sets were custom designed by Applied Biosystems to target the same regions targeted by the Affymetrix probe sets on the Affymetrix GeneChip rhesus macaque genome arrays. These regions corresponded to the following: NPY (GenBank #NM_001032814; bases 57-384; AffyID MmugDNA.43201.1.S1_at), peptide YY (GenBank #NM_001113958; bases 113-543; Affy ID MmugDNA.31444.1.S1_s_at), NPY receptor 1 (NPY1R) (GenBank # NM_001032833; bases 984-1152; Affy ID MmuSTS.3013.1.S1_at), NPY receptor 2 (NPY2R) (GenBank # NM_001032832; bases 595-1141; Affy ID MmuSTS.3014.1.S1_at), and NPY receptor 5 (NPY5R) (GenBank # NM_001032833; bases 949-1338; Affy ID MmuSTS.1973.1.S1_at). Because NPY receptor 4 (NPY4R) is not included on the Affymetrix GeneChip, the probe set was custom designed by Affymetrix to target the published sequences (GenBank # AY149475).

To minimize qRT-PCR assay variability, we used geNorm (7) to identify housekeeping genes for normalization that showed the least variability between samples for each brain region. In each case, we confirmed that the expression levels for these genes did not correlate with AT. The Ce expression levels of the NPY system genes were normalized to the expression level of

a housekeeping gene, succinate dehydrogenase complex, subunit A, (SDHA) flavoprotein variant using a custom-designed TaqMan probe set targeting bases 757-1091 (GenBank # XM_001094170). For each sample, the results for each member of the NPY system were divided by the levels of SDHA expression. For qRT-PCR performed on motor cortex tissue, NPY system gene expression was normalized to the geometric mean of the expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin (ActB) using TaqMan probes sets targeting bases 302-388 (GenBank # NM_001195426) and 830-952 (GenBank # NM_001033084), respectively.

In Situ **NPY1R and NPY5R Assessment**

Because NPY1R and NPY5R mRNA levels were associated with AT levels we assessed the expression pattern of these two mRNAs in the amygdala and surrounding tissue by radiolabeled *in situ* hybridization. The 530 bp rhesus NPY1R probe was amplified from rhesus amygdala cDNA using forward (5'GAGAGACTTGCAGTTCTTCTTTAACTTT 3') and reverse (5'TAATCTAATGGCAGTATTGGATGGCAAGT 3') PCR primers. The 373 bp rhesus NPY5R probe was amplified from rhesus amygdala cDNA using forward (5'CTGTAAGAAGTCAGCTCTCTTCATC 3') and reverse (5'CAGTGTATAAGGGACATTAAATCAGC3') PCR primers. The sequences were based on the Affymetrix probe sets MMUSTS.3013.1.S1_AT and MMUSTS.1973.1.S1_AT and were 96% and 99% identical to the human NPY1R and NPY5R, respectively (GenBank #s NM_000909 and NM_006174). The riboprobes were prepared and *in situ* hybridization was performed using previously published procedures (8). Phosphor screens were scanned using Typhoon 9410 Imaging System and the signal was quantified using ImageQuant 5.2 software (GE Healthcare, Piscataway, NJ).

Acetylcholinesterase (AChE) Staining

AChE staining was used to identify the structural details of the amygdala nuclei. The method was based on a previously published procedure (9). The slides were then mounted using Distrene Plasticiser Xylene (DPX) mountant (Sigma-Aldrich, St. Louis, MO).

Statistical Analyses

Gene Expression Correlational Analysis. Gene expression analysis was performed as previously described (3). The primary analysis of interest was the relationship between variation in the mean level of AT across the three assessments and individual differences in gene expression levels, indexed using qRT-PCR. This was tested using robust regression techniques that attenuate the influence of high-leverage outliers, minimizing the likelihood that a small number of observations exerted disproportionate effects on the regression estimate (2, 10).

FDG-PET Statistical Analyses. Because NPY1R and NPY5R mRNA levels predicted AT, we assessed the relationship of these two signals to brain metabolism. Voxelwise robust regressions were performed between qRT-PCR-measured Ce and motor cortex NPY1R and NPY5R mRNA levels and mean FDG-PET across three assessments. These analyses were performed using an adaptation (11) of Fmristat (http://www.math.mcgill.ca/keith/fmristat/) (12, 13). Regressions were performed across the whole brain controlling for nuisance variation in mean-centered age, change in age across assessments, relocation, and voxelwise gray-matter probability. Separate regression analyses were performed between qRT-PCR-measured Ce and motor cortex NPY1R and NPY5R mRNA levels and the 95% confidence interval in the Ce region. This is the region within the Ce that, with 95% certainty contains the peak voxelwise correlation between FDG metabolism and AT. The 95% confidence intervals were calculated based on the entire set of 238 monkeys as previously described (1, 14). The mean of the three FDG-PET signals extracted from the 95% confidence intervals most predictive of AT were used

to perform regressions with NPY1R and NPY5R gene expression levels co-varying for mean-centered nuisance variance in age, change in age across assessments, and relocation.

Supplemental References

1. Oler JA, Fox AS, Shelton SE, Rogers J, Dyer TD, Davidson RJ, *et al.* (2010): Amygdalar and hippocampal substrates of anxious temperament differ in their heritability. *Nature* 466:864-868.
2. Shackman AJ, Fox AS, Oler JA, Shelton SE, Davidson RJ, Kalin NH (2013): Neural mechanisms underlying heterogeneity in the presentation of anxious temperament. *Proc Natl Acad Sci U S A* 110:6145-6150.
3. Fox AS, Oler JA, Shelton SE, Nanda SA, Davidson RJ, Roseboom PH, *et al.* (2012): Central amygdala nucleus (Ce) gene expression linked to increased trait-like Ce metabolism and anxious temperament in young primates. *Proc Natl Acad Sci U S A* 109:18108-18113.
4. Kalin NH, Shelton SE (1989): Defensive behaviors in infant rhesus monkeys: environmental cues and neurochemical regulation. *Science* 243:1718-1721.
5. Fox AS, Shelton SE, Oakes TR, Davidson RJ, Kalin NH (2008): Trait-like brain activity during adolescence predicts anxious temperament in primates. *PLoS One* 3:e2570.
6. Tai C, Chatziioannou A, Siegel S, Young J, Newport D, Goble RN, *et al.* (2001): Performance evaluation of the microPET P4: a PET system dedicated to animal imaging. *Phys Med Biol* 46:1845-1862.
7. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, *et al.* (2002): Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:0034.0001 - 0034.0011.
8. Hsu DT, Lombardo KA, Herringa RJ, Bakshi VP, Roseboom PH, Kalin NH (2001): Corticotropin-releasing hormone messenger RNA distribution and stress-induced activation in the thalamus. *Neuroscience* 105:911-921.
9. Lim MM, Hammock EA, Young LJ (2004): A method for acetylcholinesterase staining of brain sections previously processed for receptor autoradiography. *Biotech Histochem* 79:11-16.
10. Huber PJ (2004): *Robust Statistics*. Hoboken, NJ: Wiley Interscience.
11. Oakes TR, Fox AS, Johnstone T, Chung MK, Kalin N, Davidson RJ (2007): Integrating VBM into the General Linear Model with voxelwise anatomical covariates. *Neuroimage* 34:500-508.
12. Friston KJ, Holmes AP, Poline JB, Grasby PJ, Williams SC, Frackowiak RS, *et al.* (1995): Analysis of fMRI time-series revisited. *Neuroimage* 2:45-53.
13. Worsley KJ, Poline JB, Friston KJ, Evans AC (1997): Characterizing the response of PET and fMRI data using multivariate linear models. *Neuroimage* 6:305-319.
14. Ma L, Worsley KJ, Evans AC (1999): Variability of spatial location of activation in fMRI and PET CBF images. *Neuroimage* 9:S178.