Variation in the Oxytocin Receptor Gene Predicts Brain Region Specific Expression and Social Attachment

Supplemental Information

Supplemental Methods and Materials

Sanger Sequencing and Polymorphism Discovery for an Allelic Imbalance Marker

We collected brain and liver tissue from male prairies voles euthanized with CO_2. Tissue was frozen in crushed dry ice. PCR assays targeting *Oxtr* mRNA were designed using sequence from a BAC clone containing prairie vole *Oxtr* sequence, DP001215.2. The putative prairie vole transcript was identified by BLAST alignment of the mouse *Oxtr* transcript (NM_001081147.1) to the vole sequence.

The thermocycler program used for all five reactions was: initial denature: 94˚C – 5 m 30 s, 35x cycles: 1) denature: 94˚C – 30 s, 2) anneal: 51˚C – 30 s, 3) elongation: 72˚C – 30 s; final elongation: 72˚C – 10 min. The primers for the loci, in order and beginning at the 5' end of the gene were, locus 1, forward: 5'-ATAAGCAGCAGCAGGTAGG-3', reverse: 5'-ACACGCGTCCTAAGAGAAG-3'; locus 2, forward: 5'-TAGCAGAACTAGGGCCTACC-3', reverse: 5'-AGGTGCACATTTTCTCACTG-3'; locus 3, forward: 5'-CTGAAGATGGTTGAGAGCAG-3', reverse: 5'-AGTTCAGAGAAAGGGTGAGG-3', locus 4, forward: 5'-CAAGCATCAGCTTCTTCAAC-3', reverse: 5'-GTGCTTCTACTACCCCAACC-3'; locus 5, forward: 5'-GTGGAGTCTGAGGAAGGAAG-3', reverse: 5'-TGCTGTTCACTGAGTATTTGC-3'.

PCR amplicons were sequenced at Beckman Coulter Genomics (Danvers, MA).

Allelic Imbalance

Adult prairie voles used for this experiment were identified as heterozygous at NT204321. Subjects were euthanized with CO_2. Brains were frozen in crushed dry ice and stored at -80˚C. Brains were sectioned in a cryostat at 300 μm. A microdissecting tool was used to

collect specific brain tissues. Tissue was transferred to lysis buffer from the Qiagen mRNA/DNA Micro Kit. The mRNA and DNA was isolated then stored at -20˚C. We made cDNA from mRNA using poly-d(T) primers (Promega, Madison, WI), reverse transcription was performed with the Qiagen Omniscript Kit.

Sequences containing NT204321 PCR amplified from cDNA and genomic DNA (gDNA). PCR amplicons were pyrosequenced at the University of Alabama, Heflin Center for Genomic Sciences Genomics Core (Birmingham, AL). For each individual-brain region combination, two cDNA reactions were performed. For each individual brain region, we pyrosequenced 6 total cDNA replicates and 3 gDNA replicates. A sample was included in analysis if 66% of these reads passed a quality check.

Allelic ratios were calculated for cDNA (representing mRNA) and gDNA by dividing the T-allele allelic quotient by the C-allele quotient. A mean ratio for cDNA and gDNA was calculated for each individual-brain region combination. An average allelic ratio was calculated for all gDNA samples, and this mean gDNA ratio was used to normalize individual allelic ratios.

PCRs on brain-derived nucleic acid samples were performed using the FailSafe PCR kit master mix plus Buffer E (Epicentre, Madison, WI). All PCRs used the following touch-down PCR protocol: initial denature: 98˚C – 5 min; 30x cycles: 1) denature: 94˚C – 30 s, 2) anneal: *variable see below – 30 s, 3) elongation: 72˚C – 45 s; final elongation: 72˚C – 10 min. Annealing temperatures were variable, for each set of cycles, given temperatures were used: 1-2: 65˚C, 3-4: 63˚C, 5-6: 61˚C, 7-8: 59˚C, 9-10: 57˚C, 11-12: 55˚C, 13-14: 53˚C, 15-16: 51˚C, 17-30: 50˚C. The primers used were thus (note that reverse primers were biotin labelled at the 5' end), NT204321: forward, 5'-CTAGGCTTTGGTTGGGGAAATAAC-3',reverse: biotinylation-5'-TTGGGTCTTGTTATGGTCCTGAC-3', sequencing primer: 5'-GGAAATAACAAGAAATGG-3'.

Genotyping

DNA was isolated using a Qiagen DNeasy Kit. A 140 bp amplicon including NT204321 was amplified by PCR, using a Qiagen Taq PCR Master Mix Kit. The thermocycler program used was, initial denature: 94˚C – 5 min 30s; 35x cycles: 1) denature: 94˚C – 30 s, 2) anneal: 53˚C – 30 s, 3) elongation: 72˚C – 30 s; final elongation: 72˚C – 10 min. Amplicons were digested for 1.5 hours at 37˚C with the SSP1 restriction enzyme (New England Biolabs, Ipswich, MA). SSP1 cuts the T-allele of NT204321 but not the C-allele. Thus, resultant banding patterns were used to identify genotypes. The primers used for this reaction were, forward: 5'-CTAGGCTTTGGTTGGGGAAATAAC-3', reverse: 5'-TTGGGTCTTGTTATGGTCCTGAC-3'.

For the intronic SNP at NT213739, a 117 bp amplicon including was amplified by PCR, using a Qiagen Taq PCR Master Mix Kit. The thermocycler program used was, initial denature: 94˚C – 5 min 30s; 35x cycles: 1) denature: 94˚C – 30 s, 2) anneal: 55˚C – 30 s, 3) elongation: 72˚C – 30 s; final elongation: 72˚C – 10 min. Amplicons were digested for 1.5 hours at 65˚C with the BsiHKAI restriction enzyme (New England Biolabs). BsiHKAI cuts the C-allele of NT213739 but not the T-allele. The primers used for this reaction were, forward: 5'- GGGACGTTCACGTTACATGG -3', reverse: 5'- AGACGGGACAGAGTCTCCAG -3'.

Long-range PCRs for Target Enrichment of 70kb Surrounding *Oxtr*

Loci 2 through 10 were run with the same thermocycler program, initial denature: 93˚C – 3 min; 35x cycles: 1) denature: 93˚C – 15 s, 2) anneal: 62˚C – 30 s, 3) elongation: 68˚C – 10 min 20 s. Loci 2 and 4 through 10 were amplified with a single reaction. Locus 1 and 3 required an initial amplification followed by a second amplification using internally nested primers. Locus 3 was run with the same above program for initial and nested reactions. Locus 1 required six replicates run on the central wells of the thermocycler with a 5˚C gradient. All initial amplicons were then run with nested primers on the same program without a gradient.

Primers for each loci are listed below: Locus 1 – initial, forward: 5'-TTTGGACACTGTGACTTGGCATTG-3', reverse: 5'-ACGTCCACCTTGGGTATCGTTTTG-3', Locus 1 – nested, forward: 5'-GGTGGGTCATCTGTCTATCTGTTGC-3', reverse: 5'-GGCTCCTGATTTTCCCAGGTACAAG-3', Locus 2, forward: 5'-CGAAGGTCAGGGGAGAAAAGTGAC-3', reverse: 5'-ACTCCAGTCCTTGTGGAACTAATGTGG-3', Locus 3 – initial, forward: 5'-CAATAAGCAGCTAGACAGGGCCCA-3', reverse: 5'-CCCTGGATCTACATGCTGTTCACG-3', Locus 3 – nested, forward: 5'-CGCTGCAGTAGTGGGAAGACATTG-3', reverse: 5'-ACGAACTTGTGCAGCGCTTTCTC-3', Locus 4, forward: 5'-GACCCTCTGATGGCTGAGTGACTG-3', reverse: 5'-CCCAGAGGGAACTGCATCTGAGTC-3', Locus 5, forward: 5'-TCAGCCCTCAGAAACTTTTTCAAACAC-3', reverse: 5'-GAAGGGTGCCTGTCTTCTTTGGTC-3', Locus 6, forward: 5'-AAGGGGAGTGACTTTCAGGGGAAG-3', reverse: 5'-AGTGTGTGACAGCATTGGGACTTTG-3', Locus 7, forward: 5'-CCAAGGGATGACACAGCTTTGAGAG-3', reverse: 5'-CCAGCTTTGCTACAGAGGATCAGC-3', Locus 8, forward: 5'-CCAGGGCAGCTTTATTCATGTGTG-3', reverse: 5'-TGCTGCTACCAGTCATGTCTCTGC-3', Locus 9, forward: 5'-AAATCCTGGATGGTGATATTGTCTGC-3', reverse: 5'-AGTAACATGCCTGCTCCTGTGTGTG-3', Locus 10, forward: 5'-GGCGAAACTACTTTCCACGTTTGC-3', reverse: 5'-TGTGCTAGCCAGTTCACCATCAGC-3'.

Oxytocin Receptor Autoradiography

All animals analyzed for genotype-phenotype associations were confirmed for *Oxtr* genotypes through additional genotyping of DNA isolated from caudal brain tissue. The first set included 31 adult prairie voles aged 60 – 120 days, 17 females and 14 males. Since we did not detect a sex difference in NAcc OXTR density, and we recently demonstrated that OXTR signaling is critical for partner preference formation in males (1), all subsequent

studies focused only on males. The second set included 86 adult male prairie voles aged 60 – 190 days, Brain tissue was unavailable to confirm the genotype of 8 subjects from this second set, thus only 78 of these animals were used for genotype-phenotype associations. Freshly frozen brains were stored at -80˚C. Coronal sections were cut in a cryostat at temperatures between -16˚C and 18˚C. Twenty µm sections were collected and then stored at -80˚C until use in autoradiography or *in situ* hybridization. Tissue was removed from -80˚C storage and air dried, then dipped into 0.1% paraformaldehyde in 7.4 pH PBS. Next, tissue was rinsed in 50 mM Tris buffer before a 1 h incubation in 50 pM ^{125}I-OVTA (NEX 254050UC, PerkinElmer). Finally, sections were dipped through four washes 50 mM Tris with 2% MgCl and underwent a rinse in ddH_20 and then air dried. Dry sections were exposed to BioMax MR film (Kodak) for 72 h. Processed film was imaged on a light box. Images were taken and analyzed with MCID software. Digital images were obtained with a light box and a 12-bit QICAM camera (QImaging, Surrey, BC, Canada).

Evaluators were blind to genotype during scoring. A semi-quantitative measure of OXTR binding density was calculated: disintegrations per minute per milligram of tissue (dpm/mg) was estimated by comparing raw optical density (ROD) values to a ^{125}I standard. Background binding was captured from regions of the brain with consistent lack of signal such as the corpus callosum. Specific OXTR binding density was calculated by subtracting mean background binding from values of regions of interest. Brightness and contrast of representative images were equally adjusted for all autoradiography images within a panel using Adobe Photoshop CS3.

In situ Hybridization

The antisense RNA probe was complementary to the prairie vole sequence corresponding to base pairs 101–1272 of predicted *Oxtr* mRNA (RefSeq accession number XM005364985). Twenty µm cryosections adjacent to the slices used for OVTA autoradiography were hybridized with the probes, and were exposed to BAS-TR2025 phosphoimaging plate (Fujifilm, Tokyo, Japan) for a month and then, to Kodak BioMax MR films for 7 months. For

quantitative analysis, phoshoimaging plates were scanned with BAS-5000 and analyzed using Multi Gauge V3.1 software. ROD was captured for NAcc and a region of each section with background, such as the corpus callosum Evaluators were blind to genotype during scoring. Specific NAcc ROD was calculated by subtracting mean background from mean NAcc for each section. Representative images were taken from film autoradiograms, digital images were obtained using a light box and a QICAM camera connected to a computer. Brightness and contrast of representative images were equally adjusted for all *in situ* images within a panel using Adobe Photoshop CS3.

Partner Preference Test

Subjects were toe clipped at postnatal day PND 7 and housed with 1-2 aged-matched males until testing at adulthood (PND 60-190). Stimulus animals for the partner preference tests were sexually naïve females. Female stimulus voles were cohoused in cages of 2-3 until and were sexually naïve at the time of pairing with each subject. Subjects were cohabitated with a female vole (partner) for 6 h. After cohabitation, females were removed from the cage and tethered to serve as stimuli for the partner preference test.

Partners and female strangers unfamiliar to the subject were tethered to opposing ends of a three-chamber Plexiglass arena, total dimensions: 75 cm x 20 cm x 30 cm. The subject was placed in the center of the arena and allowed to freely roam and interact with stimulus animals for 3 h. Behavior was recorded from a digital camera above the arenas and side-by-side huddling duration between the subject and each stimulus is scored automatically with SocialScan 2.0 (Clever Sys Inc, Reston, VA, USA).

Statistical Analysis

Analyses for partner preference data were performed using 2-way ANOVAs, with time spent huddling as the outcome measure and the interaction between genotype and stimulus animal as the main predictor. Allelic imbalance data were analyzed as previously described: cDNA allelic ratio was considered to have significant allelic imbalance if it was greater than

the mean gDNA allelic ratio plus three gDNA standard deviations for that region (2). For linear regression, genotypes were modelled as 0, 1 and 2, with the number representing the number of high OXTR-associated alleles: the T-allele in the case of NT204321 and C-allele in the case of NT213739. For all reported associations using linear regression we investigated the residuals and found they were normally distributed. We used the following VCF tools functions: --maf, --min-meanDP, --minQ and --max-missing, for filtering and quality control of the sequence data. All figures were generated using the data visualization package ggplot2 in R.

Supplemental References

1. Johnson ZV, Walum H, Jamal YA, Xiao Y, Keebaugh AC, Inoue K, *et al.* (2015): Central oxytocin receptors mediate mating-induced partner preferences and enhance correlated activation across forebrain nuclei in male prairie voles. *Hormones and behavior.*
2. Barrie ES, Weinshenker D, Verma A, Pendergrass SA, Lange LA, Ritchie MD, *et al.* (2014): Regulatory polymorphisms in human DBH affect peripheral gene expression and sympathetic activity. *Circulation research.* 115(12):1017-25.