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

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ABSTRACT

BACKGROUND: Oxytocin (OXT) modulates several aspects of social behavior. Intranasal OXT is a leading candidate for treating social deficits in patients with autism spectrum disorder, and common genetic variants in the human *OXTR* gene are associated with emotion recognition, relationship quality, and autism spectrum disorder. Animal models have revealed that individual differences in *Oxtr* expression in the brain drive social behavior variation. Our understanding of how genetic variation contributes to brain *OXTR* expression is very limited.

METHODS: We investigated *Oxtr* expression in monogamous prairie voles, which have a well-characterized OXT system. We quantified brain region–specific levels of *Oxtr* messenger RNA and oxytocin receptor protein with established neuroanatomic methods. We used pyrosequencing to investigate allelic imbalance of *Oxtr* mRNA, a molecular signature of polymorphic genetic regulatory elements. We performed next-generation sequencing to discover variants in and near the *Oxtr* gene. We investigated social attachment using the partner preference test.

RESULTS: Our allelic imbalance data demonstrate that genetic variants contribute to individual differences in *Oxtr* expression, but only in particular brain regions, including the nucleus accumbens, where oxytocin receptor signaling facilitates social attachment. Next-generation sequencing identified one polymorphism in the *Oxtr* intron, near a putative *cis*-regulatory element, explaining 74% of the variance in striatal *Oxtr* expression specifically. Males homozygous for the high expressing allele display enhanced social attachment.

CONCLUSIONS: Taken together, these findings provide convincing evidence for robust genetic influence on *Oxtr* expression and provide novel insights into how noncoding polymorphisms in *OXTR* might influence individual differences in human social cognition and behavior.

Keywords: Allelic imbalance, Autism, Individual differences, Prairie vole, Social behavior, Striatum

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Oxytocin (OXT) is a neuromodulator that influences reproductive and social behavior through signaling via a single G protein–coupled oxytocin receptor (OXTR) in the brain. The OXTR affects a range of social behaviors in animals, including maternal nurturing and bonding (1,2), social reward and gregariousness (3,4), social recognition (5), and pair bonding in monogamous species (6–8). It has been proposed that OXT influences these complex social behaviors by increasing the salience and reinforcing value of social stimuli (9).

In humans, intranasal OXT reportedly enhances many aspects of social cognition, including trust, emotion recognition, and eye gaze (1,10). Individual reports of the effects of intranasal OXT should be interpreted cautiously (11). Nevertheless, the OXT system is a leading candidate target for improving social function in patients with psychiatric disorders such as autism spectrum disorder (ASD) and schizophrenia (12–17), and one report suggested that *OXTR* expression may be reduced in ASD (18). Single nucleotide polymorphisms (SNPs) in noncoding regions of the human *OXTR* gene have been associated with pair bonding behaviors (19), parenting (1), face recognition skills (20), and autism (21). Some individuals have heterogeneous responses to OXT (22,23), which may involve interaction with *OXTR* genetic polymorphisms (24–27). Early life stress in humans can interact with *OXTR* variation to influence adult social behavior and emotional regulation (28–31). Despite these many associations with human social behavior and disorders, the neural mechanisms by which noncoding SNPs in *OXTR* could influence behaviors has yet to be explored.

One potential mechanism is that typical expression of *OXTR* is disrupted when such SNPs occur in regulatory elements (REs) that primarily lie within noncoding portions of the DNA (*cis*-REs). The OXTR is distributed throughout the brain of many vertebrates, and the pattern of OXTR distribution is diverse among species (32,33). Within regions of crucial behavioral circuits such as the mesolimbic reward (MLR) network and social decision making network, OXTR is often enriched and appears to modulate these networks to generate species-specific social strategies, such as monogamous social attachments (32) and social gregariousness (7). Thus, the manner in

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which the *Oxtr* gene is regulated among species appears to have profound consequences for the manner in which neural networks activate in response to the social environment.

In a socially monogamous rodent, the prairie vole (*Microtus ochrogaster*), OXTR is enriched in important MLR regions such as the nucleus accumbens (NAc) and prefrontal cortex that constitute part of a neural network for pair bonding (2,6,8). The OXTR density is much higher in the NAc of prairie voles than promiscuous vole species, and OXTR signaling in the NAc is required for mating-induced partner preference formation, a laboratory proxy of pair bonding (6). Infusion of an OXTR antagonist into the NAc or the prefrontal cortex, but not the caudate putamen (CP), blocks mating-induced partner preferences in females (34) and males (A.C. Keebaugh, Ph.D., and L.J.Y., unpublished data, 2015). The OXTR density also varies among individual prairie voles, especially in the NAc and the CP (35). Increasing OXTR density in the NAc using viral vector–mediated gene transfer facilitates partner preference formation, whereas decreasing OXTR density in the same region using RNA interference inhibits such bonding (36–38). Variation in NAc OXTR density is correlated with individual differences in monogamy-related behavior in males in naturalistic settings (39). Furthermore, variation in prairie vole NAc OXTR confers susceptibility or resilience to the effects of daily neonatal isolations, a model of neglect, in relation to the ability to form social attachments as adults (40). Mechanisms responsible for OXTR diversity in the NAc in the prairie vole may be important determinants of individual differences in social behavior as well.

One likely causal explanation for OXTR diversity is that genetic polymorphisms in *cis*-REs regulating *Oxtr* generate variation in expression in a brain region–specific manner. Variation in gene expression mediated by *cis*-REs plays an important role in evolutionary phenotypic change (32,41–45). In prairie voles, a microsatellite in the 5′ flanking region of the vasopressin receptor gene (*Avpr1a*) containing *cis*-REs has been shown to have functional influence over species differences and individual variation in *Avpr1a* expression and is associated with variation in social behavior (46,47). The influence of *cis*-REs can be detected by assaying for allelic imbalance, which is observed when two alleles of a gene in a heterozygous individual are expressed at different rates, creating an imbalance in the respective messenger RNAs (mRNAs) (48–50). Any differences in mRNA levels between alleles occur in the same nuclear environment, where both alleles should be affected equally by environmental, hormonal, or epigenetic factors, unless *cis*-REs proximal to the alleles are variable. Allelic imbalance is commonly observed in a tissue-specific manner (50,51).

To determine whether prairie vole *Oxtr* gene expression is influenced by polymorphic *cis*-REs, we analyzed brain region–derived mRNA for allelic imbalance in animals heterozygous for a SNP in the *Oxtr* transcribed region. We found that robust allelic imbalance of *Oxtr* occurs within the striatum, but not in several other brain regions. Voles with alternative homozygous genotypes for this SNP had significant differences in OXTR density in NAc. Finally, to gain a more thorough understanding of the relationship between genetic polymorphisms in the prairie vole *Oxtr* and neural OXTR density, we sequenced 70 kb of DNA around the gene in 45 voles. We observed strong associations between several genetic markers and OXTR density that were particularly robust in the NAc. A bioinformatics analysis using ENCODE (ENCyclopedia Of DNA Elements) data suggests that an intronic SNP is the most likely functional candidate for further investigation. This intronic SNP is strongly associated with OXTR density in the NAc and was found to be associated with the propensity to form social attachments. Our results demonstrate for the first time that noncoding SNPs in the *Oxtr* can profoundly predict OXTR density and *Oxtr* expression in a brain region–specific manner. These findings implicate that *cis*-regulation drives the remarkable variation in *Oxtr* transcription and has a more modest, but significant, influence on social behaviors. This is the first study to demonstrate that SNPs in the noncoding region of the *OXTR* robustly affect receptor density in the brain.

METHODS AND MATERIALS

Animals

Prairie voles (*Microtus ochrogaster*) were housed in same-sex groups with two or three voles per cage from postnatal day 21. Housing consisted of a ventilated 36 cm × 18 cm × 19 cm plexiglass cage filled with Bed-o'Cobs laboratory animal bedding (The Andersons Inc., Maumee, Ohio) under a 14/10 hour light/dark cycle (lights on 7:00 AM–9:00 PM) at 22°C with access to food (rabbit diet; LabDiet, St. Louis, Missouri) and water ad libitum. Our laboratory breeding colony was originally derived from field captured voles in Illinois. All procedures were approved by the Emory University Institutional Animal Care and Use Committee.

Sanger Sequencing and Polymorphism Discovery for an Allelic Imbalance Marker

DNA was isolated using a QIAGEN DNeasy kit (Germantown, Maryland). We designed primers to amplify five loci spanning the two coding exons plus the 5′ untranslated region (UTR) and parts of the 3′ UTR. See the Supplement for details. Nucleotide 204321 (NT204321), located in the 3′ UTR, was polymorphic (minor allele frequency, .33) and was used for the detailed allelic imbalance study.

Allelic Imbalance

Subjects were euthanized with carbon dioxide. Brains were frozen in crushed dry ice and stored at −80°C. Nucleic acids for the allelic imbalance assay were isolated from microdissected brain tissues using the QIAGEN mRNA/DNA Micro Kit. For details, see the Supplement.

Long-Range Polymerase Chain Reactions for Target Enrichment of 70 kb Surrounding *Oxtr*

DNA was isolated from previously sectioned brains stored at −80°C with the QIAGEN DNeasy Kit. All polymerase chain reactions were performed using the QIAGEN LongRange PCR Kit. There were 10 loci 6.6–10 kb amplified. For details, see the Supplement.

Amplicon Library Preparation

Sequencing library preparation and sequencing analyses were performed by the Yerkes Nonhuman Primate Genomics Core (Atlanta, Georgia). Polymerase chain reaction amplicons from each animal were pooled and cleaned using Solid Phase

Reversible Immobilization beads (Beckman Coulter, Brea, California). Libraries were generated using the Illumina Nextera XT DNA Library Prep Kit (Illumina, Inc., San Diego, California), and dual barcoding and sequencing primers were added according to the manufacturer protocol. Libraries were validated by microelectrophoresis, quantified, pooled, and clustered on Illumina TruSeq Cluster Kit v3. The clustered flow cell was sequenced on an Illumina HiSeq 1000 in 100-base single-read reactions.

Amplicon Sequencing Analysis

Sequencing reads were mapped to *Microtus ochrogaster* target 220 haplotype 2 genomic scaffold (DP001215.2) using the Burrows-Wheeler Aligner (bwa version 0.7.10) (52). The aligned reads were processed with the DNAseq variant analysis workflow of the Genome Analysis Toolkit (GATK version 3.2.2) (53), including marking duplicate reads and local realignment around insertions/deletions. Variants were called on a per-sample basis and combined to produce a joint variant call file.

OXTR Autoradiography

OXTR autoradiography was performed as previously reported (36). For details, see the Supplement.

In Situ Hybridization

Sense and antisense ^{35}S-uridine triphosphate–labeled RNA probes for prairie vole *Oxtr* mRNA were generated as described previously (54). For details, see the Supplement.

Partner Preference Test

The partner preference was performed as previously described (55). For details, see the Supplement.

Statistical Analysis

All statistical analyses were performed in the R statistical software package version 3.1.1 (R Project for Statistical Computing, Vienna, Austria), unless stated otherwise. Associations between genetic information and brain data were examined using linear regression with Bonferroni corrections for multiple comparisons. Regarding the factor analyses, to determine how many factors to extract, we used the nFactors package in R, and the factor extraction decisions were based on the eigenvalues-greater-than-one rule, parallel analysis, the optimal coordinates method, and acceleration factor. Exploratory factor analyses were performed with the factanal( ) function, using "varimax" as the rotation method. Processing of next-generation sequencing data was performed in VCFtools, a program package designed for working with variant cell format files from sequencing projects (56). For associations between individual markers in the *Oxtr* sequence and autoradiography expression data, linear regressions were performed using PLINK/SEQ, an open-source library for working with genetic variation data (https://atgu.mgh.harvard.edu/plinkseq/). For further details see the Supplement.

RESULTS

As expected based on previous experiments, the NAc and CP exhibited more individual variation in OXTR density than other brain regions (Figure 1A–C). Furthermore, OXTR binding density appeared to be correlated with *Oxtr* mRNA levels based on in situ hybridization signal (Figure 1A). To test the hypothesis that the high variability in OXTR density within the NAc was due to the influence of putative *cis*-REs, we first assayed for allelic imbalance. We sequenced the transcribed region of the *Oxtr* in a small sample of voles to identify any SNP in our prairie vole colony with a relatively high minor allele frequency. One SNP in the 3′ UTR (minor allele frequency 33%), heretofore referred to as NT204321 based on the position of this nucleotide on a sequenced prairie vole bacterial artificial clone (DP001215.2) (57), was identified using this method. In heterozygous animals for this SNP, we found significant allelic imbalance in NAc (complementary DNA [cDNA], 3.16; genomic DNA [gDNA] threshold, 1.11), CP (cDNA, 5.24; gDNA threshold, 1.13) and, to a lesser degree, amygdala (cDNA, 1.19; gDNA threshold, 1.14). The allelic imbalance was pronounced in the two striatal subregions, NAc and CP, with the T-allele transcript being three to five times more prevalent than the C-allele in the same animals in these regions (Figure 1D). These data strongly suggest that *cis*-REs linked to NT204321 generate individual variation in expression of *Oxtr* in select brain tissues.

To test whether NT204321 might serve as a marker to predict overall OXTR density in the NAc, we collected expression data for 12 brain regions (n = 31) using autoradiography. Visual inspection of OXTR density across brain regions suggested that density in some regions covaried with NAc density, whereas density in other regions did not. Therefore, we used factor analysis to determine the correlation structure between OXTR density data from the 12 brain regions investigated. We hypothesize that correlations between OXTR densities from different brain regions can be explained by unobserved variables, possibly reflecting transcriptional processes giving rise to the patterns of correlation. Exploratory factor analysis is a method to identify such unobserved, latent variables. Our analysis revealed two factors together explaining most variance (58%). Factor 1 strongly reflects covariability in a set of regions involved in reward processing (NAc, CP, and olfactory tubercle), whereas factor 2 reflects covariation between cortical and subcortical regions that have relatively uniform levels of OXTR density (Table 1, Sample A). We identified similar patterns when we investigated the associations between NT204321 and OXTR expression in the 12 brain regions, with regions loading into factor 1 being more related to genotype than regions loading into factor 2 (Figure 2). We performed a second factor analysis in an additional sample (n = 85) (Table 1, Sample B), and this analysis, similar to the first one, revealed two factors explaining most of the variance. The OXTR binding in the NAc was almost perfectly correlated with the first factor, and binding in the insula was almost perfectly correlated with factor 2. Thus, further analyses including brain data focused on these two regions as representatives of factor 1 and factor 2.

The choice of NT204321 was not based on any assumption of functional importance, and we suspected that other SNPs across and outside the *Oxtr* transcribed region might be more closely associated with the *cis*-RE and better predict *Oxtr* expression. We first characterized the suite of polymorphisms across the *Oxtr* gene by sequencing 70 kb of DNA including

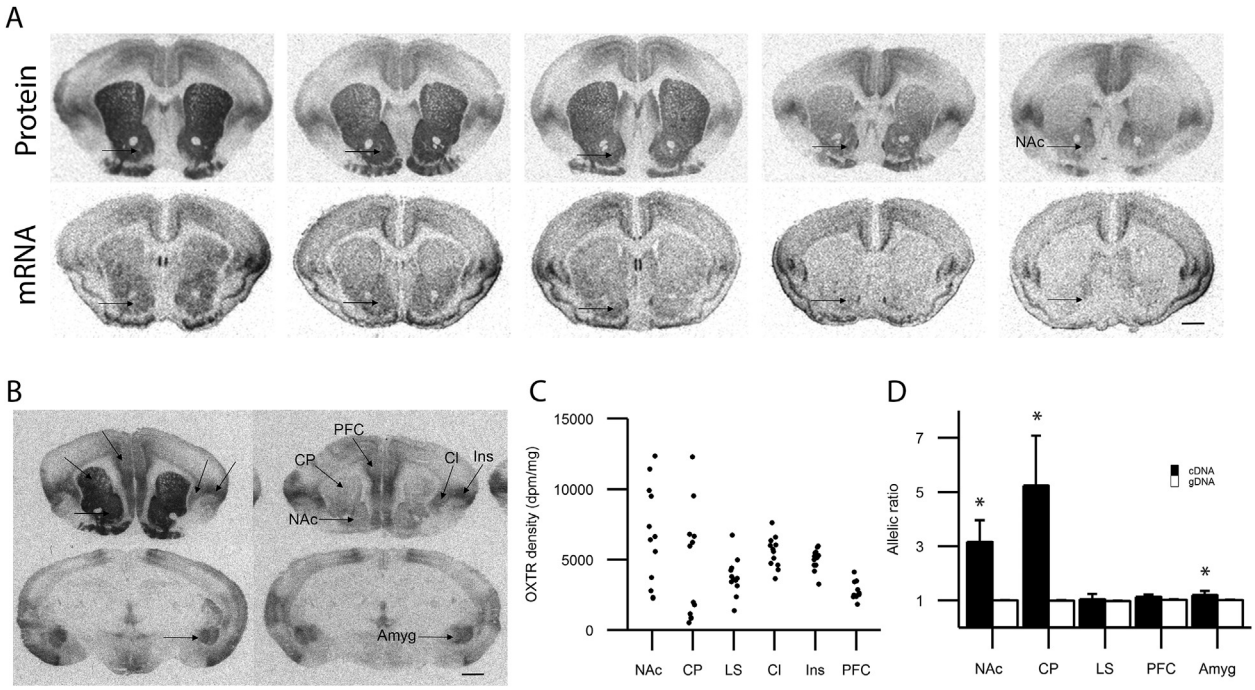

Figure 1. Region-specific variation in neural expression of the prairie vole *Oxtr* gene is caused by *cis*-regulatory elements. **(A)** An illustration that oxytocin receptor (OXTR) protein density (visualized by receptor autoradiography) and *Oxtr* messenger RNA (mRNA) levels (visualized by in situ hybridization) vary in the striatum, including the nucleus accumbens. **(B)** In contrast to the individual differences in nucleus accumbens and caudate putamen OXTR density, other brain regions show less variation. Scale bar = 100 μm. **(C)** Quantification of individual variation in OXTR density. Each dot represents OXTR density (dpm/mg) for an individual prairie vole (n = 12, males). **(D)** Allelic imbalance was calculated as the average allelic ratio (%T/%C) for complementary DNA (cDNA) and genomic DNA (gDNA) from animals heterozygous at nucleotide 204321 (n = 8). A significant allelic imbalance was detected in the cDNA derived from the nucleus accumbens, caudate putamen, and amygdala, but not in cDNA derived from the prefrontal cortex or lateral septum. The striatal regions had very high allelic imbalance, with threefold to fivefold differences between alleles. The gDNA T and C alleles are amplified at equal levels in all tissues. *cDNA allelic ratio is significantly greater than a threshold calculated by the mean of the gDNA allelic ratio + 3 gDNA standard deviations. Data are expressed as mean ± SEM. Except for caudate putamen gDNA (n = 6), n = 7. Amyg, amygdala; Cl, claustrum; CP, caudate putamen; Ins, insula; LS, dorsal lateral septum; NAc, nucleus accumbens; PFC, prefrontal cortex.

Table 1. Two Factors Encompass the Covariation in OXTR Density Among Brain Regions

Sample A			Sample B		
Region	Factor 1	Factor 2	Region	Factor 1	Factor 2
NAc	.99	.14	NAc	.99	.04
Tu	.82	.09	CP	.92	.1
CP	.82	.18	Tu	.91	−.01
CeA	.69	.3	LS	.56	.14
OB	.56	.5	Ins	−.06	.99
LS	.56	.15	Cl	.07	.75
Cl	.17	.88	PFC	.51	.6
Ins	.12	.85			
BLA	.04	.71			
PFC	.5	.51			
VMH	.3	.47			
AON	.28	.41			

Values in the table represent factor correlations. Results are shown for two autoradiography samples. Sample A: 12 regions analyzed, n = 31; and Sample B: 7 regions analyzed, n = 85.

AON, anterior olfactory nucleus; BLA, basal lateral amygdala; CeA, central amygdala; Cl, claustrum; CP, caudate putamen; Ins, insula; LS, dorsal lateral septum; NAc, nucleus accumbens; OB, olfactory bulb; OXTR, oxytocin receptor; PFC, prefrontal cortex; Tu, olfactory tubercle; VMH, ventral medial hypothalamus.

and surrounding the gene. Among the 45 voles we sequenced, we identified 967 SNPs with a read depth no lower than 100 reads, with a quality score of at least 1000, and for which all were variable in our sample. Figure 3A shows a quantile-quantile plot for the association between the SNPs in the *Oxtr* sequence and NAc OXTR binding density. As can be seen in Figure 3A, many of the SNPs in our set were strongly associated with NAc OXTR density. We were primarily interested in identifying variants that could potentially be functional, and therefore we focused on the SNPs with the lowest p values (and largest effect sizes). As a result of linkage disequilibrium, 15 SNPs spanning a 30-kb region were associated with NAc OXTR density with the same minimal p value ($p = 1.06 \times 10^{-15}$, adjusted R^2 = .78), which is a remarkable effect size for a genotype-phenotype relationship.

To assess whether any of the 15 most associated SNPs are more likely than others to lie in a putative *cis*-RE, we investigated their homology with regions of the mouse *Oxtr* gene that overlap with signatures of functional activity occurring within neural tissues where OXTR is expressed in the mouse (58). We mapped a vole sequence containing these SNPs and surrounding sequence of ~500 bp per SNP to the mouse *Oxtr* gene. We compared our vole sequences with ENCODE tracks for markers of general transcriptional activity

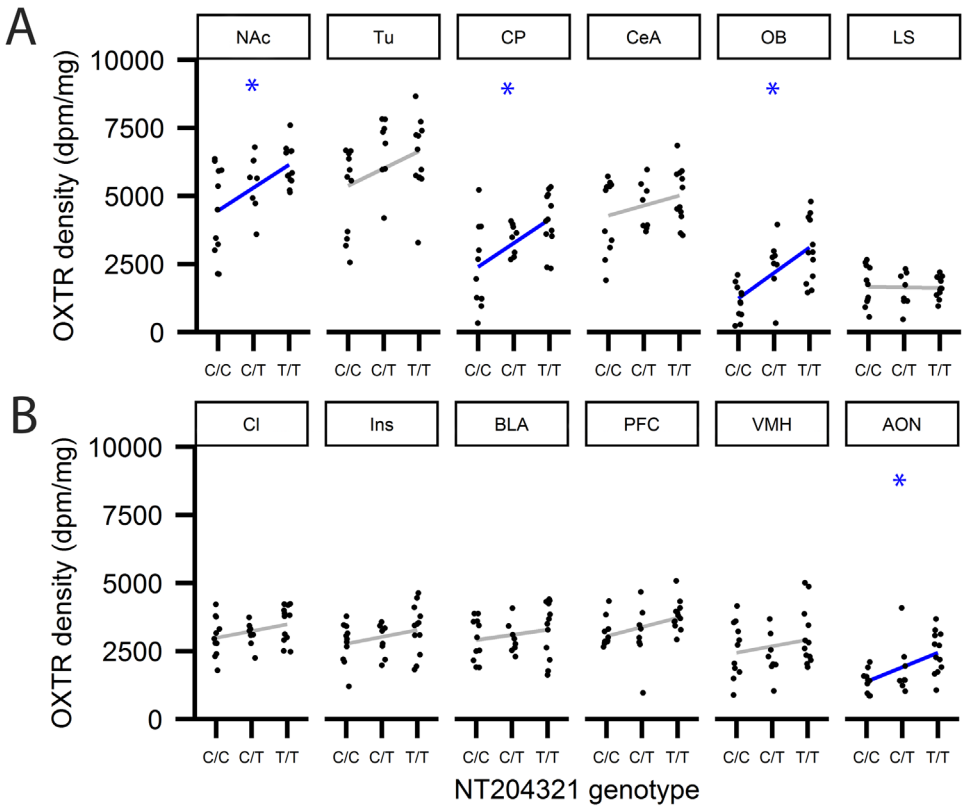

Figure 2. Oxytocin receptor (OXTR) binding density in striatal and olfactory regions is associated with nucleotide 204321 (NT204321). Sample sizes for each genotype are C/C = 11, C/T = 8, and T/T = 12. **(A)** Brain regions are sorted based on the how strongly the regions loaded on factor 1. Within factor 1 regions, OXTR binding is significantly related to genotype in nucleus accumbens, caudate putamen, and olfactory bulb. **(B)** Brain regions are sorted based on how strongly the regions loaded on factor 2. In the factor 2 grouping, only the anterior olfactory nucleus was significantly associated with genotype. Associations were investigated using simple linear regression. $^*p < .004$ (α corrected for 12 comparisons). Data are shown as individual OXTR density (dpm/mg) with trend line for the linear regression. AON, anterior olfactory nucleus; BLA, basal lateral amygdala; CeA, central amygdala; Cl, claustrum; CP, caudate putamen; Ins, insula; LS, dorsal lateral septum; NAc, nucleus accumbens; OB, olfactory bulb; PFC, prefrontal cortex; Tu, olfactory tubercle; VMH, ventral medial hypothalamus.

such as deoxyribonuclease hypersensitivity, single methyl modification of lysine 4 of histone H3, and acetyl modification of lysine 27 of histone H3 as well as binding of the transcription factor CCCTC-binding factor (CTCF), which can act as a canonical transcription factor or as an organizer of genomic architecture (59). Only one SNP overlapped with strong signatures of transcriptional function, a SNP occurring at nucleotide 213739 (NT213739; minor allele frequency, .32)

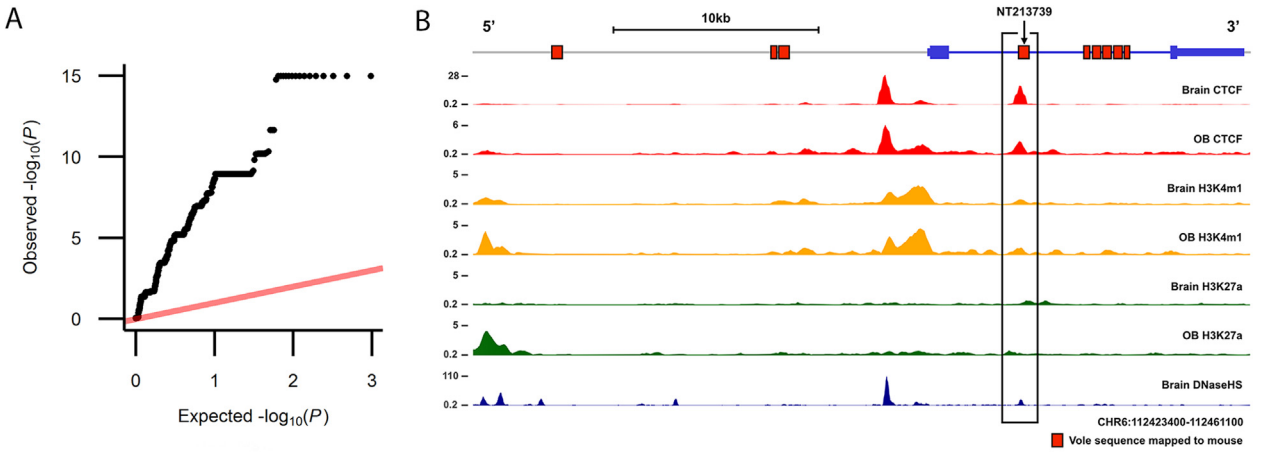

Figure 3. Identification of nucleotide 213739 (NT213739) as a marker of nucleus accumbens oxytocin receptor (OXTR) density. **(A)** Quantile-quantile plot of the associations between the 967 *Oxtr* markers and nucleus accumbens OXTR density. Each dot represents the $-\log p$ of the association between a particular single nucleotide polymorphism (SNP) with OXTR density placed in ascending order. **(B)** Schematic of the mouse *Oxtr* gene with accompanying functional data from the ENCODE project. The prairie vole sequences containing the SNPs showing the strongest association with nucleus accumbens OXTR density that also map to mouse *Oxtr* sequence. Approximately 500 bp per SNP of prairie vole sequence surrounding each SNP was aligned to the mouse genome as indicated by red rectangles. Chromatin immunoprecipitation followed by sequencing signal or deoxyribonuclease I hypersensitive sites signal is shown. Brain, whole brain; CTCF, CCCTC-binding factor (red); DNaseHS, deoxyribonuclease I hypersensitive sites (dark blue); H3K27a, acetyl modification of lysine 27 of histone H3 (green); H3K4m1, single methyl modification of lysine 4 of histone H3 (orange); OB, olfactory bulb.

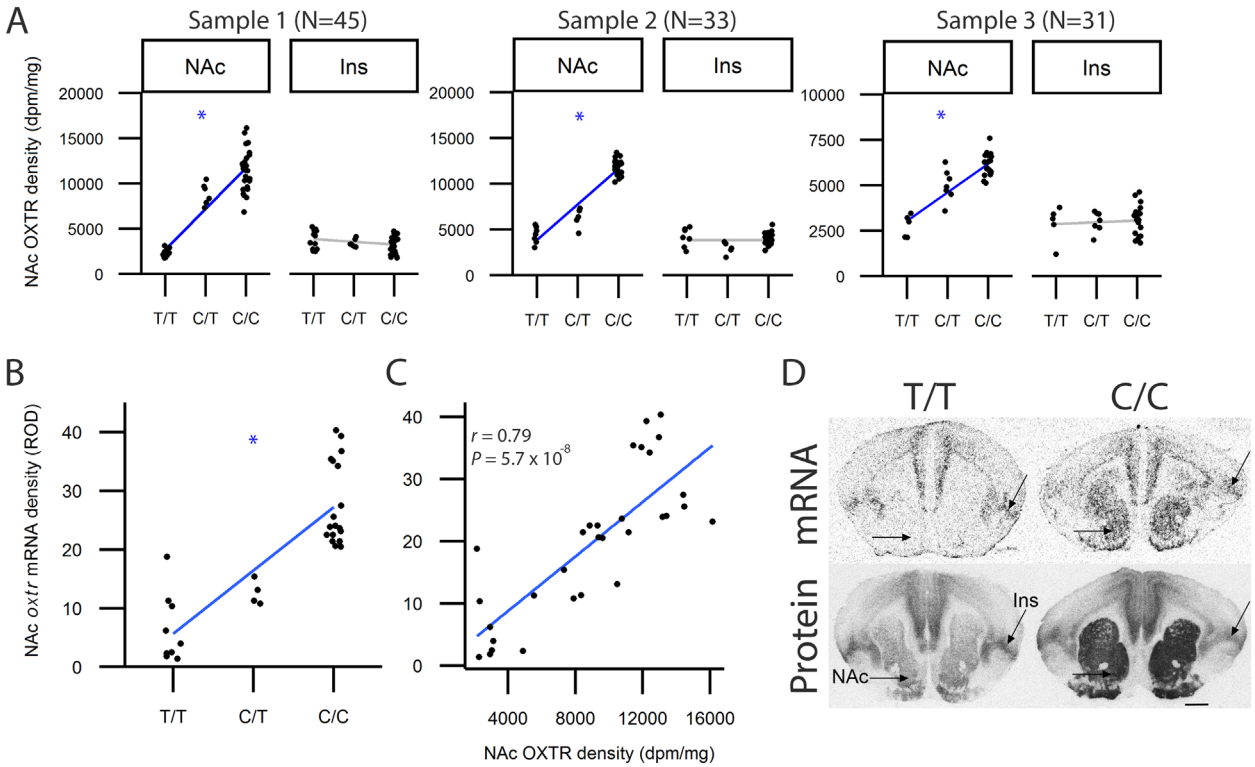

Figure 4. Nucleotide 213739 (NT213739) genotype robustly predicts *Oxtr* expression. **(A)** Nucleus accumbens oxytocin receptor (OXTR) density was associated with NT213739 genotype across three independent samples (sample 1, adjusted R^2 = .81; sample 2, adjusted R^2 = .90; sample 3, adjusted R^2 = .74). OXTR density in the insula was not associated with NT213739. **(B, C)** Nucleus accumbens *Oxtr* messenger RNA (mRNA) density was significantly associated with NT213739 genotype (n = 31, adjusted R^2 = .69). **(C)** Nucleus accumbens *Oxtr* mRNA density is significantly correlated with OXTR protein binding density (n = 31). **(D)** Representative images highlighting the differences between NT213739 genotypes in *Oxtr* expression within the striatum, particularly the nucleus accumbens. Scale bar = 100 μm. *$p < 1 \times 10^{-8}$. Data are shown as individual OXTR density (dpm/mg) or individual mRNA density (relative optical density [ROD]) with trend line for the linear regressions. Ins, insula; NAc, Nucleus accumbens.

(Figure 3B). The sequence containing NT213739 overlapped peaks of deoxyribonuclease hypersensitivity and CTCF binding within the large intron, a region proposed to contain *cis*-REs in humans (20,60,61). Based on this evidence, we chose to investigate the predictive power of NT213739 further in two additional samples.

After genotyping additional voles (sample 2, n = 33; sample 3, n = 31) for NT213739, we confirmed the SNP was robustly associated with OXTR density in the NAc but not in the insula (Figure 4A). In both the second and the third independent samples investigated, our findings from the sequenced sample were very closely replicated. In all our relatively small samples, NT213739 was strongly associated with NAc expression (all p values $< 4 \times 10^{-10}$). Similarly, the effect size of this association was very large in all samples (adjusted $R^2 \geq 74\%$), strongly suggesting that NT213739 explains at least 74% of the variance in NAc expression of OXTR in prairie voles. The effect was marked, such that OXTR density values in the NAc between homozygous animals of the two genotypes did not overlap at all, whereas heterozygous animals displayed an intermediate phenotype.

To confirm that the association between NT213739 and receptor density is mediated through mRNA levels, we performed in situ hybridization on adjacent sections of brains from 31 individuals. We found that NT213739 is also significantly associated with *Oxtr* mRNA levels in the NAc (Figure 4B) and that mRNA levels and OXTR density were significantly correlated (Figure 4C, D). Together, these data suggest that NT213739 is strongly associated with transcriptional variation of the *Oxtr* gene and tightly linked to a *cis*-RE.

Because NT213739 robustly predicted NAc OXTR density, and NAc OXTR signaling is important for regulating partner preference formation in prairie voles, we sought to determine whether NT213739 would influence mating-induced partner preference formation, a measure of social attachment that involves social information processing and social reinforcement. Males of varying genotype were housed with a female for 6 hours and then tested in the partner preference test. There was a significant effect of genotype on partner preference formation, with C/C voles spending significantly more time huddling with the partner than the stranger, whereas C/T and T/T males did not display a partner preference (Figure 5).

DISCUSSION

We demonstrate in the present study that genetic variation in the *Oxtr* exerts robust control over individual diversity in *Oxtr* expression and OXTR density in the prairie vole brain and that this influence on expression occurs in a region-specific manner. Allelic imbalance is strongest in the striatum, and

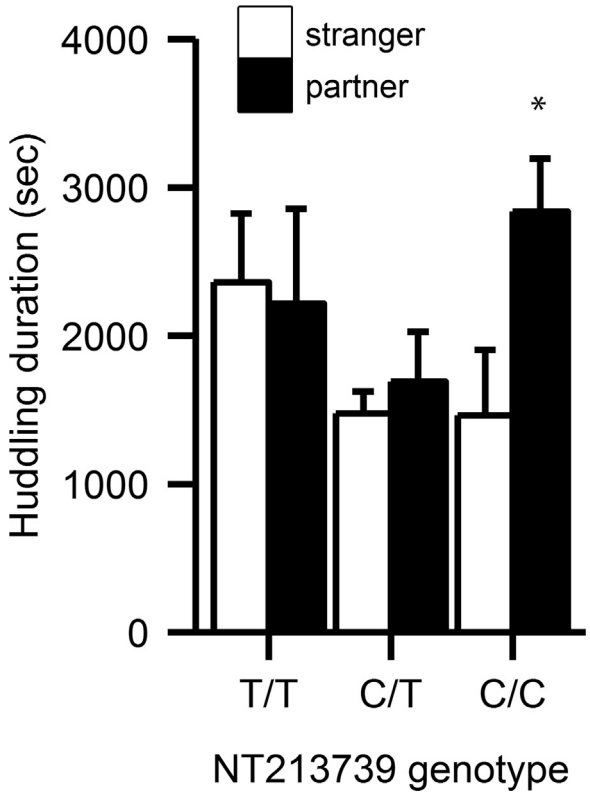

Figure 5. Nucleotide 213739 (NT213739) genotype influences partner preference formation in male prairie voles. The effect of genotype on behavior was investigated using two-way analysis of variance. The interaction of genotype × stimulus on huddling duration was significant ($F_{1,136}$ = 4.45, p = .037). Male animals with a C/C genotype (n = 39) spent significantly more time huddling with the partner than the stranger, whereas animals with a C/T (n = 13) or T/T (n = 18) genotype did not. *Indicates a partner preference—mean partner huddling time is significantly greater than mean stranger huddling time (t test, $p < .01$). Data are expressed as mean ± SEM.

genotype-OXTR associations are most robust in this region. Exploratory factor analysis identified a cluster of striatal-olfactory regions with correlated OXTR density. One intriguing interpretation of this finding is that the unobserved latent variable represented by factor 1 may reflect a set of transcriptional regulators with maximal effect on *Oxtr* expression activity in prairie vole olfactory–reward processing regions. Whatever transcription factor leads to the covariation appears to interact with the *cis*-REs associated with NT213739 to generate the high variation in expression observed in these regions but not other regions. In this manner, *cis*-REs appear to contribute exquisite control over OXTR and through this process influence behavioral diversity in prairie voles.

Little is known about the molecular mechanisms regulating brain region–specific *Oxtr* expression in any species. Prairie voles and montane voles display species-specific patterns of OXTR expression. Comparisons of ~1500 bp of 5′ flanking regions from the *Oxtr* gene reveal only a few SNPs and 99% homology between the species (62). Transgenic mice carrying a reporter gene driven by 5 kb of the prairie vole *Oxtr* 5′ flanking sequence express the reporter in the brain in a pattern resembling prairie voles (63), suggesting the sequence may suffice for some aspects of brain region–specific expression. DNA methylation of *Oxtr* differs between brain regions in rodents (64,65). DNA methylation of *OXTR* in humans may be mediated by an intronic SNP (66). In some rodent brain regions, OXTR expression is regulated by gonadal steroids in a species-specific manner. For example, testosterone increases OXTR in the hypothalamus of rats but decreases OXTR in mice (67,68). A better understanding of the molecular mechanisms underlying species differences and individual variation in *Oxtr* transcription in the brain is needed and may inform our understanding of how genetic variation in human OXTR relates to psychiatric phenotypes or responses to OXT-based therapies.

We found that prairie vole *Oxtr* expression is strongly influenced by polymorphic *cis*-REs that include or are associated with NT213739, a SNP in the intron of the gene. We focused our attention on NT213739 because, of 15 SNPs in perfect linkage disequilibrium with one another, only NT213739 mapped to a site in the mouse *Oxtr* intron with robust evidence of transcriptional activity. Such comparisons should be made with the understanding that transcription factor binding sites may differ among species (69). We were particularly interested in the proximity of the mapped prairie vole sequence to a putative CTCF binding site, as CTCF binding peaks are found in the intron as well as near the promoter of both the mouse *Oxtr* (Figure 3B) and the human *OXTR* (20). One role of CTCF is to act as a spatial organizer, aiding DNA looping to permit *cis*-RE-promoter interactions required for gene transcription (59). Polymorphic *cis*-REs have been shown to influence CTCF binding (70,71) and DNA looping (72). In the human *OXTR* third intron, the CTCF peak is found near a SNP, rs237887, that was predicted to be near a *cis*-RE and associated with face recognition abilities (20) and ASD diagnosis (21). Additionally, the human intron may contain other *cis*-REs (60) and accumulates rare SNPs in cases of ASD (61). If the large intron of *Oxtr* contains *cis*-REs in multiple species, spatial organization of DNA by factors such as CTCF could offer a potentially general regulatory mechanism required for proper function of species or region-specific *cis*-REs. Although this evidence provides a potential molecular mechanism by which NT213739 could lead to region-specific differential transcription, our current genotype-phenotype relationships do not implicate NT213739 above any of the other SNPs in perfect linkage disequilibrium with it spanning the 30 kb. Future studies using large samples of more genetically diverse animals, including wild-caught specimens, may be needed to break this haplotype structure to identify which SNP is most likely functionally contributing to OXTR density variation. Furthermore, biochemical analyses, including chromatin immunoprecipitation followed by sequencing, chromosome conformation capture (73), and in vitro transcription assays could be used to investigate interactions between CTCF binding, DNA looping, and *Oxtr* regulation.

One of the most remarkable findings of our study is the amount of OXTR density variance explained by genetic polymorphism. Our data suggest that NT213739, or any of the SNPs in perfect linkage disequilibrium with it, explain 74% of the variation in NAc OXTR. Behavioral genetic studies typically report that 1%–10% of the variance in behavioral phenotype is explained by candidate gene polymorphisms. We presume

that OXTR density is biologically more proximate to genotype than behavior, and in typical behavioral genetic studies, many more variables are contributing to behavioral variation. Thus, direct measures of brain phenotype are likely to yield stronger effect sizes than reported in behavioral studies.

Prairie voles strongly express OXTR in regions of the MLR, a conserved neural network (74) involved in reward processing and implicated in numerous human psychiatric disorders, including depression (75), addiction (76), and schizophrenia (77). In a key region of the MLR, the NAc, OXTR activation is necessary for prairie vole partner preference formation (34), and OXTR variation mediates individual differences in pair bonding behaviors (36–38). In the present study, we confirm a genetic role for naturally occurring OXTR density differences that contribute to individual variation in social behavior (35,39). In addition to region-specific effects, OXTR signaling enhances functional connectivity within a network of regions during prairie vole pair bond formation (78). Research in humans found that intronic *OXTR* variants predicted individual differences in functional connectivity between brain regions during the processing of social information (79,80). These results highlight a potentially conserved role for OXTR in social cognition between species despite likely differences in sites of expression. The prairie vole may prove a useful model to understand how individual differences in OXTR expression in key regions lead to variation in network connectivity.

A recent meta-analysis supports the conclusion that genetic variation in *OXTR* is associated with diagnosis of ASD (21), and other authors have reported associations with endophenotypes of ASD (20,81,82), including structural variability in brain regions relevant to social cognition (79,83). Such findings in humans call for a better understanding of molecular mechanisms regulating OXTR variation (21,84). The present study suggests that these genotype-phenotype relationships may be mediated by polymorphisms in *cis*-REs in the *OXTR* gene that influence OXTR density in a brain region–specific manner.

In conclusion, we used the prairie vole model to demonstrate for the first time that a single SNP can predict most variance in OXTR expression in specific brain regions. Further studies to identify functional mechanisms leading to this difference in *Oxtr* transcriptional activity may provide exciting insights into the precise genetic mechanism generating OXTR mediated diversity in social behavior, including human psychopathology.

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