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Natural variation in maternal care and cross-tissue patterns of oxytocin receptor gene methylation in rats

Annaliese K. Beery[1,2,*], **Lisa M. McEwen**[3], **Julia L MacIsaac**[3], **Darlene D. Francis**[2,4], and **Michael S. Kobor**[3]

[1] Department of Psychology, Neuroscience Program, Smith College, Northampton, MA, USA

[2] Robert Wood Johnson Health and Society Scholars Program, University of California, Berkeley and San Francisco, CA, USA

[3] Centre for Molecular Medicine and Therapeutics, Department of Medical Genetics, University of British Columbia, Vancouver, BC, Canada

[4] School of Public Health, University of California, Berkeley, CA, USA

Abstract

Since the first report of maternal care effects on DNA methylation in rats, epigenetic modifications of the genome in response to life experience have become the subject of intense focus across many disciplines. Oxytocin receptor expression varies in response to early experience, and both oxytocin signaling and methylation status of the oxytocin receptor gene (*Oxtr*) in blood have been related to disordered social behavior. It is unknown whether *Oxtr* methylation varies in response to early life experience, and whether currently employed peripheral measures of *Oxtr* methylation reflect variation in the brain. We examined the effects of early life rearing experience via natural variation in maternal licking and grooming during the first week of life on behavior, physiology, gene expression, and epigenetic regulation of *Oxtr* across blood and brain tissues (mononucleocytes, hippocampus, striatum, and hypothalamus). Rats reared by "high" licking-grooming (HL) and "low" licking-grooming (LL) rat dams exhibited differences across study outcomes: LL offspring were more active in behavioral arenas, exhibited lower body mass in adulthood, and showed reduced corticosterone responsivity to a stressor. *Oxtr* methylation was significantly lower at multiple CpGs in the blood of LL versus HL rats, but no differences were found in the brain. Across groups, *Oxtr* transcript levels in the hypothalamus were associated with reduced corticosterone secretion in response to stress, congruent with the role of oxytocin signaling in this region. Methylation of specific CpGs at a high or low level was consistent across tissues, especially within the brain. However, individual variation in methylation relative to these global patterns was not consistent across tissues. These results suggest that blood *Oxtr* methylation may reflect early experience of maternal care, and that *Oxtr* methylation across tissues is highly

[*]**Corresponding author:** Annaliese K. Beery, Smith College, Clark Science Center, 44 College Lane, Northampton, MA 01063, Phone: (413) 585-3918, Fax: (413) 585-3786, abeery@smith.edu.

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concordant for specific CpGs, but that inferences across tissues are not supported for individual variation in *Oxtr* methylation.

Keywords

cross-tissue; epigenetic; DNA methylation; *Oxtr*; oxytocin; oxytocin receptor; natural variation; maternal care; anxiety behavior; concordance

1. INTRODUCTION

Research across many disciplines and organisms has identified a crucial role for the physical and social environments of early life as determinants of later development, health, and behavior (e.g. Meaney, 2001; Suomi, 1999; Hertzman and Boyce, 2010; McEwen, 2012). Experience of parental care in particular, shapes numerous developmental outcomes in offspring. In rats, decades of research have characterized effects of maternal care on stress-reactivity and anxiety-like behaviors, mediated in part through the tactile stimulation of licking and grooming pups during the early postnatal period (Francis and Meaney, 1999; Gonzalez et al., 2001). Dams display natural variation in grooming frequency, which allows for comparisons between pups exposed to high licking (HL) and low licking (LL) in the absence of external manipulation (Liu et al., 1997; Caldji et al., 1998; Francis et al., 1999). In recent years there has been a great expansion in the known physiological and behavioral outcomes of early maternal care beyond anxiety, including effects on social behaviors. Natural variation in maternal care alters adult social behavior in both male and female rats, with greater social interaction times in the offspring of HL dams (Starr-Phillips and Beery, 2014). Early maternal care also affects play behavior, both in juveniles and adults (Parent and Meaney, 2008; van Hasselt et al., 2012; Parent et al., 2013).

Oxytocin (OT) is a neuropeptide that plays a role in many aspects of both anxiety and social behavior including anxiety, fear, depression, social buffering of stress, maternal behavior, individual recognition, trust, empathic accuracy, and social attachment formation (Neumann and Landgraf, 2012; Knobloch et al., 2012; Guzmán et al., 2013; Kirsch et al., 2005; Smith and Wang, 2014; Beery and Kaufer, 2015; Shahrokh et al., 2010; Guastella and Macleod, 2012; Carter et al., 2008; Ross and Young, 2009). While oxytocin production and release patterns within the brain are highly conserved across species, the distribution and density of oxytocin receptors (OTR) is highly variable between species and plastic within species, suggesting that the regulation of oxytocin receptor location and abundance may be an important mechanism underlying variation in behavior (Young, 1999; Insel and Young, 2000; Beery et al., 2008; Donaldson and Young, 2008; Phelps et al., 2010; Anacker and Beery, 2013). Oxytocin receptor density has been associated with behavioral variation in many brain regions including olfactory bulb, striatum, septum, hypothalamus, hippocampus, and amygdala (e.g. Yu et al., 1996; Bale et al., 2001; Beery and Zucker, 2010; Ophir et al., 2012; Dölen et al., 2013; Lukas et al., 2013), as well as other regions that are part of the extended limbic system or social behavior network (Newman, 1999; Goodson, 2005; O'Connell and Hofmann, 2011).

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Maternal care impacts multiple aspects of oxytocin circuitry, with early life social environment associated with altered OT and/or OTR profiles in mice, rats, and voles (reviewed in Bales and Perkeybile, 2012; Veenema, 2012). Oxytocin is released in pups following grooming-like tactile stimulation (Lenz and Sengelaub, 2010), and variation in maternal care is associated with OTR density. Specifically, natural variation in maternal care has been associated with changes in OTR density in the bed nucleus of the stria terminalis and central amygdala of female offspring (Francis et al., 2002), and maternal separation has been linked to changes in OTR in the hypothalamus, lateral septum, and caudoputamen in male rats (Lukas et al., 2010). While many questions remain, the potential for early life events to interact with oxytocin circuitry and later social behavior is evident.

Epigenetic modifications of the genome that alter the activity of specific genes represent a path by which experience may influence later physiology and behavior. One such modification is DNA methylation, in which a methyl group is added to the 5’ carbon of a cytosine that is typically adjacent to a guanine nucleotide — referred to as a CpG. Such regulation has been associated with long term differences in glucocorticoid receptor density by maternal care; these differences appear to be maintained throughout the life-course at least in part by variation in the extent of glucocorticoid receptor gene (*Nr3c1*) expression (Liu et al., 1997; Weaver et al., 2001; van Hasselt et al., 2012), and differential DNA methylation of its promoter (Weaver et al., 2004; Pan et al., 2014) or neighboring regions (McGowan et al., 2011).

Since the report of postnatal variation in DNA methylation in rats in response to maternal care (Weaver et al., 2004), it has become increasingly clear that methylation can be dynamic after birth and throughout the lifespan (Fraga et al., 2005; Siegmund et al., 2007; Christensen et al., 2009; Miller et al., 2010). The oxytocin receptor is differentially but highly methylated across a wide variety of tissues (Kimura et al., 2003), and may be a good candidate for regulation by experience-dependent methylation. Expression of the oxytocin receptor gene (humans: *OXTR*, rodents: *Oxtr*) is sensitive to DNA methylation patterns; experimentally induced methylation in a CpG rich region ~1kb upstream of the *OXTR* translation initiation site labeled “MT2” by Kusui et al. (2001) has been shown to suppress gene transcription in human and mouse tissues (Kusui et al., 2001; Mamrut et al., 2013). The function of *OXTR* methylation has also been explored in a few studies. Hypermethylation of multiple CpGs within the *OXTR* promoter was identified in blood samples from affected individuals within a human family with autism (Gregory et al., 2009), and DNA methylation of a single CpG in this promoter region in peripheral blood mononuclear cells (PBMCs) has since been associated with altered neural activity in multiple brain regions in functional MRI scans (Jack et al., 2012; Puglia et al., 2015). *OXTR* methylation has been associated with emotional traits and circulating oxytocin in humans (Dadds et al., 2014). Methylation of one *OXTR* CpG in human blood was associated with a diagnosis of social anxiety disorder and correlated with stress reactivity in the Trier Social Stress test (Ziegler et al., 2015), and another recent study found that *OXTR* methylation varied with both clinical depression and *OXTR* genotype (Reiner et al., 2015). These findings suggest that methylation of the oxytocin receptor gene and neighboring regions are good candidates for investigation within the realm of the prolonged impacts of early maternal care.

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While multiple studies have begun to examine DNA methylation in the brain or peripheral tissues and their associations with life experience, we still know relatively little about how specific these associations are to tissue type. There has been intense interest in the interpretation of DNA methylation assays in readily available tissues such as blood. While blood methylation may be an important biomarker for outcomes such as cancers (Langevin et al., 2012), it is less clear if it will be a useful and relevant indicator of epigenetic changes related to brain and behavior. Methylation varies across cell types (Lam et al., 2012; Reinius et al., 2012), and blood cell composition may vary across and within individuals. Many methylation patterns are also distinct across tissue lineages, often with greater cell-type specific variation than inter-individual variation. Both concordance and discordance across tissues have been widely reported (e.g. Iyer et al., 2010; Liberman et al., 2012; Davies et al., 2012; Jiang et al., 2015; Farré et al., 2015). A few studies have reported on methylation of *OXTR/Oxtr* in multiple tissues, demonstrating greater methylation in liver than in uterine myometrium (Kusui et al., 2001; Kimura et al., 2003), CpG specific methylation patterns in uterine and mammary tissue (Mamrut et al., 2013), and variation in methylation across brain regions (Harony-Nicolas et al., 2014). In the latter study, cross-tissue correlations of methylation levels in olfactory bulb and cerebellum were performed by CpG, and no significant correlation was found. In all behavioral epigenetic studies of human *OXTR* methylation to date, blood samples have been used as the tissue source, and a key unresolved question is whether blood measures of *OXTR* methylation are associated with methylation in brain regions (Kumsta et al., 2013). We address this question in rats, and interrogate whether potential cross-tissue correlations are useful predictors at an individual level.

The present study characterizes *Oxtr* promoter methylation and gene regulation in rats born to and reared by rat dams exhibiting natural variation in maternal care in order to a) assess the impacts of early life experience in the form of maternal care on *Oxtr* methylation and b) examine these methylation patterns across multiple tissues of interest either for their ease of sampling or connections to behavior. *Oxtr* methylation was assessed in a CpG island ~1.2 kb upstream of the coding region; this sequence was chosen because it encompasses a region of high conservation across vertebrates, as well as much of the MT2 region associated with *in vitro Oxtr* expression (Kusui et al., 2001). We assessed several anxiety-like behaviors and physiological outcomes in these offspring in order to document effects of maternal care. We contrast *Oxtr* methylation profiles by maternal care experience, and compare methylation across tissue types — including blood and three limbic system brain regions: hippocampus, striatum, and hypothalamus — to gain a better understanding of the tissue specificity of variability in *Oxtr* methylation. In rats, oxytocin receptors are present and relate to behavior in all three of these brain tissues, particularly the nucleus accumbens within the ventral striatum, the dorsal hippocampus, and the ventromedial hypothalamus (Tribollet et al., 1992; Starr-Phillips and Beery, 2014; Dumais et al., 2013). Finally, we relate *Oxtr* mRNA expression to methylation and corticosterone (CORT) secretion.

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2. MATERIALS AND METHODS

(a) Animal subjects

Long-Evans rats were bred locally from individuals obtained from Charles River (Wilmington, MA). Rats were maintained on a 12:12 light:dark cycle with lights off at 19:00 and housed in transparent plastic cages (48 × 27 × 20 cm) on Tek-Fresh bedding (Harlan Teklad, Madison, WI). Food (Purina Rat Chow, Purina Mills, St. Louis, MO) and tap water were available *ad libitum*. Ambient temperature was 20 ± 2°C and humidity was 50 ± 5%. Litters were designated high-licking or low-licking as described below. From 144 total offspring, 38 males (18 HL, 20 LL), and 36 females (18 HL, 18 LL) from 9 litters were followed in this study. Offspring were pair-housed at weaning on postnatal day 22 (PND22; the day of parturition was designated PND0). All procedures were approved by the Animal Care and Use Committee of the University of California, Berkeley and conformed to NIH guidelines.

(b) Experimental design and timeline

Rats reared in HL or LL litters were weighed at weaning, and weekly from 10 to 16 weeks of age. Behavioral testing took place at 13-14 weeks of age on the open field test, light-dark box, and elevated plus maze (described below), with at least one day between tests. Blood sampling for corticosterone took place at 15 weeks of age. Rats were otherwise unmanipulated. At week 16, rats were sacrificed for organ weight determination and tissue collection for DNA/RNA extraction as described below. CORT assays and DNA/RNA extraction were performed on males; female tissues were saved for another study.

DNA methylation was measured across a ~450 base pair region of the *Oxtr* promoter spanning 25 CpGs within a CpG island (figure 1A; sections e and f, below). Sequenom sequencing was the principal method used to measure methylation for all samples in peripheral blood mononuclear cells and hippocampus; 12 “units” within this sequence of either single CpGs or pairs were considered for analysis (see section e below). In a series of follow-up assays, the same region of 25 CpGs captured by the Sequenom assay was assessed by pyrosequencing in a subset of the same tissue samples (specific methods described below). Pyrosequencing was conducted on genomic DNA from hippocampus, striatum (caudoputamen and nucleus accumbens), and hypothalamus. Hippocampal samples were used to validate the similarity of outputs of the two sequencing methods, and hippocampus, striatum, and hypothalamic samples were used to examine cross-tissue similarity of multiple brain regions involved in anxiety and social behavior.

(c) Maternal care characterization

Maternal behavior was observed for 7 days beginning on the day following parturition (PND1) (Champagne et al., 2003; Francis et al., 1999; Liu et al., 1997). The behavior of each dam was monitored for 5 h from 6:00-8:00, 12:00-13:00, and 18:00-20:00 following a scan-sampling procedure. Dams were observed every 2min during the observation session (for 180 daily and 1260 total observations). Outcomes recorded were pup licking/grooming, self-grooming, and location of the dam (on/off the nest). Nursing posture was described in a narrative. Maternal licking scores were calculated as the % of total observations in which the

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dam was observed grooming her pups. HL and LL dams were defined as ≥1SD above or below the mean (Champagne et al., 2003).

(d) Behavioral tests

Behavioral tests each lasted 5 min and were video recorded without the presence of an experimenter in the room. Tests were scored without knowledge of treatment group using custom software (A. Beery, available on request).

Light-dark box—Rats were placed in the dark portion of a chamber consisting of an enclosed black Plexiglas box (38 × 38 × 20 cm) connected to a clear Plexiglas chamber with no lid (38 × 38 × 38 cm). Time spent in the light relative to the dark portion of the box was recorded, as well as latency to exit the dark box and activity within the apparatus.

Open-Field—Exploratory behavior was assessed in a novel, circular open arena 152 cm in diameter for 5 min. Time spent in a zone within 15 cm of the wall vs. the center was determined, as well as latency to enter the center and movements between zones of the apparatus.

Elevated Plus-Maz: The elevated plus-maze was constructed of black Plexiglas with two open arms and two enclosed arms (10 cm wide, 112 cm long), elevated to a height of 50 cm. Rats were placed in a dark arm facing the center. Latency to enter the light arm, ratio of time in the light:dark arms, and movements within the maze were scored.

(e) Corticosterone hormone immunoassay

Plasma corticosterone concentration was assayed by enzyme immunoassay (Assay Designs/ Enzo Life Sciences Corticosterone EIA, ADI-900-097; sensitivity 27 pg/ml) as previously described (Beery et al., 2012). Briefly, blood was collected from a tail nick within 1 minute of cage disturbance prior to restraint, following 20 min restraint stress, and at 30 min intervals for four additional recovery measurements. Samples were centrifuged at 4°C for 20 min and plasma was stored at −80°C until the time of assay. Samples were thawed, centrifuged, diluted 1:20 with assay buffer, and aliquotted into 96-well plates in duplicate. 6 standards ranging from 32-20,000 pg/ml and reference samples were assayed in duplicate on each plate. Mean intra-assay variation was 4% and inter-assay variation was 8%.

(f) Tissue collection and DNA extraction

~7ml blood was collected via cardiac stick, mixed 1:1 with PBS, placed on ice, and separated on a Ficoll gradient within 1 hour of collection. Samples were layered on 12 ml Ficoll, centrifuged at 500 RCF for 30 min, and the white cell layer was collected. Cells were washed twice with PBS, aliquotted into multiple samples and pelleted. Aliquots were stored at −80° until DNA and RNA extraction.

Brains were removed, placed on crushed dry ice, and bisected. Frozen brains were stored at −80°C. Left halves were microdissected and the hippocampus, striatum (nucleus accumbens and caudoputamen), and hypothalamus of each brain was stored in RNAlater at −80°C. Left

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and right adrenal glands were collected from all animals and paired testis weight was determined in males.

Genomic DNA was extracted using the AllPrep DNA/RNA kit (Qiagen, Inc.) according to manufacturer instructions. Samples were purified if needed with the Clean and Concentrate kit (Zymo Research).

(g) DNA Methylation analysis of Oxtr by Sequenom

Oxtr methylation was assessed across a 434 bp sequence ~1.2 kb upstream of the coding region. This sequence encompasses a CpG island, a region of high conservation across vertebrates, and 25 CpGs—including 8 within the MT2 region previously associated with *in vitro Oxtr* expression (figure 1A). Primers were designed using Sequenom EpiDesigner (see supplemental Table S1 for all primers and numbered CpGs) to capture the sequence from 207717398 to 207717832 on chromosome 4 (March 2012 RGSC 5.0/rn5 assembly, UCSC Genome Browser). Samples were bisulfite converted with the EZ DNA Methylation Kit (Zymo Research) and eluted into 100mL of HyPure water (HyClone). 20ng of bisulfite converted DNA was prepared for analysis on a Sequenom MALDI-TOF mass spectrometer by PCR amplification and T cleavage transcription reactions. The Sequenom platform assesses DNA methylation of fragments based on the mass of the unmethylated versus methylated versions of fragments generated by enzymes with base specific cleavage patterns. The resulting fragments may have one or more CpGs contained within depending on the specific sequence. Units (fragments containing one or more CpGs) were excluded if they were high or low mass fragments or had overlapping peaks (CpG #3, #8, #10, and #18), or spanned ≥3 CpGs (CpG #11-13, CpG #20-23). This resulted in 12 units containing one or two CpGs for analysis (figure 1B). Assays were run in triplicate alongside methylated and demethylated rat control DNA. *Oxtr* promoter methylation was assessed by Sequenom in PBMCs and hippocampus.

(h) DNA Methylation analysis of Oxtr by Pyrosequencing

PyroMark Assay Design 2.0 (Qiagen, Inc.) software was used to design the bisulfite pyrosequencing assay covering the ~450 BP/25 CpG region assayed in the Sequenom assays, using four sequencing primers (supplemental Table S2). DNA was subjected to bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research). HotstarTaq DNA polymerase kit (Qiagen, Inc.) was used to amplify the *Oxtr* target region using the biotinylated primer set with the following PCR conditions: 15 minutes at 95°C, 45 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 30s, and a 5 minute 72°C extension step. Streptavidin-coated sepharose beads were bound to the biotinylated-strand of the PCR product and then washed and denatured to yield single-stranded DNA. Sequencing primers were introduced to allow for pyrosequencing (Pyromark™ Q96 MD pyrosequencer, Qiagen, Inc.).

(i) qPCR

RNA was extracted from study samples using the AllPrep DNA/RNA kit (Qiagen, Inc.) according to manufacturer instructions. Contaminating genomic DNA was eliminated with genomic wipeout buffer (Qiagen, Inc.). One μg of RNA was reverse transcribed with the

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QuantiTect Reverse Transcription Kit (Qiagen, Inc.) using random primers according to manufacturer protocol. The complementary DNA was analyzed using a Rotor-Gene 6000 (Corbett Research) and PerfeCTa SYBR Green FastMix (Quanta Biosciences). Ct values of duplicates or triplicates were averaged and used to calculate relative amounts of transcripts, normalized to β-actin (*Actb*) transcript.

(j) Statistics and data analysis

Sequenom data were analyzed using Mass ARRAY EpiTyper version 1.0 and the BioConductor package for R (The R project for statistical computing). Methylation values were assessed in triplicate and averaged for each sample. Pyrosequencing methylation values were assessed in singlicate, and analyzed with Pyro Q-CpG software (Qiagen) to generate quantitative methylation levels of the targeted CpG dinucleotides of interest. Values identified by the software as failing validation were excluded from analysis. qPCR values for *Oxtr* were analyzed as fold-change relative to β-actin (*Actb*) expression in duplicate for each sample. When values were discrepant between technical duplicates for either *Oxtr* or *Actb* by more than 0.25 SDs, subsequent runs were done with triplicates and the average of those values was used in further data analysis.

Differences between HL and LL groups were assessed by 2-way ANOVA including sex and maternal care for outcomes measured for both sexes, or by *t*-tests assuming unequal variance (Welch's *t*-test) for outcomes measured in males. Correlations between continuous variables are reported with Pearson's *r*, or Spearman's rho (r_s) for non-parametric data. Comparisons across multiple brain regions were conducted by one-way ANOVA followed by Tukey's HSD; all other tests are described in the text. Corrections for multiple comparisons are reported using false discovery rate (Benjamini and Hochberg, 1995), expected number of false positives, and aggregate probabilities if appropriate, depending on the number of findings reported. The main study outcomes (*Oxtr* methylation and transcript levels) were examined for litter effects, which were not found. Statistical analyses were performed with JMP 8.0.2 (SAS Institute, Cary, NC) and R. Results were considered significant if the two-tailed *p*-value was <0.05.

3. RESULTS

(a) Maternal care was associated with variation in physiology and behavior

HL and LL offspring consistently manifested significant variation across the three behavioral tests. LL rats exhibited greater activity in the light-dark box ($p = 0.01$, figure 2A) and emerged earlier ($p < 0.04$). Across groups, greater activity level predicted earlier emergence time in the light-dark box ($p < 0.0001$, $r = 0.81$). LL rats also exhibited earlier emergence in the elevated plus maze ($p < 0.02$), and greater activity in the open field test ($p < 0.02$). No group differences were present in percent of time spent in the open on any test.

Baseline corticosterone secretion was equivalent between HL and LL male offspring (females not tested) but differed in response to 20 minute restraint at 30, 60, 90, and 120 minutes after the initiation of restraint, as well as in area under the curve (AUC) ($p < 0.03$, figure 2C). Rats with higher CORT AUC showed markedly higher latency to enter the

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center of the open field ($p < 0.0001$, $r = 0.63$), and greater activity in the elevated plus maze ($p < 0.02$, $r = 0.35$). They also trended towards less time in the center of the open field ($p = 0.07$, $r = 0.25$) and less time in the open arm of the elevated plus maze ($p = 0.07$, $r = 0.25$).

Body weight was equivalent between HL and LL rats at weaning, but diverged in adulthood in both sexes (figure 2B; $p = 0.006$ effect of maternal care in two-way ANOVA at week 16). At week 16, HL female rats weighed 316±5g, while LL females weighed 297±8g. HL males weighed 578±12g and LL males weighed 527±18g. Uterine mass showed a trend towards higher mass in HL relative to LL females (0.69±0.038 high, 0.60±0.033 low, $p = 0.08$) despite lack of association with terminal body weight ($p = 0.29$, r=.20). Paired testis weight tended to be greater in HL males (paired testis weight: 3.98±0.05 versus 3.72±0.12, $p = 0.06$), but this effect was likely due to the strong correlation between testis weight and body weight ($p < 0.0001$, r =0.67). Terminal measurements of adrenal mass did not differ between groups in either sex.

(b) DNA methylation of Oxtr *varies with maternal care*

Oxtr methylation was assessed in a CpG island ~1.2 kb upstream of the coding region, and mean methylation across individuals ranged from 0-50% depending on CpG and tissue (figure 1B). Sequenom *Oxtr* methylation in PBMCs exhibited significant variation with maternal care in 4 of 12 units examined (figure 3A), and methylation was greater in HL offspring than in LL offspring in each case: CpG 5 (3.1±0.39 HL versus 1.86±0.37 LL; $p = 0.03$), CpG 9 (1.2±0.24 HL versus 0.5±0.14 LL; $p = 0.01$), CpG 14&15 (7.9±0.57 HL versus 6.4±0.36 LL; $p = 0.04$), and CpG 25 (17.42±0.77 HL versus 15.31±0.51 LL; $p = 0.03$). The probability of 4 CpGs out of 12 varying at this level is $p = 0.002$, or $p = 0.0003$ for all four findings in the same direction. No single high/low difference retains significance after correction for multiple comparisons using false discovery rate (FDR), thus we cannot detect which of the four differences are reliable, despite a strong aggregate signal.

In hippocampus, one unit differed significantly based on maternal care (CpG 6&7; 2.07±036 HL versus 3.13±035 LL; $p = 0.04$; figure 3B). Such a result would be expected by chance ~45% of the time with 12 comparisons, and is thus not meaningful.

Finally, variation in PBMC methylation was compared to stress-induced corticosterone response in the same individuals. Higher CORT AUC was associated with lower DNA methylation, most strongly for CpG 24 (p=0.006, r =.58), becoming a trend at p=0.07 after correction for multiple comparisons by FDR.

(c) Oxtr methylation concordance between tissues across individuals

Pyrosequencing assays—Cross-tissue methylation comparisons (in addition to those performed for PBMCs and hippocampus using Sequenom) were conducted by pyrosequencing in three brain regions: hippocampus, hypothalamus, and striatum. Hippocampal samples previously analyzed by Sequenom were re-analyzed by pyrosequencing for validation, and values obtained were significantly correlated across sequencing methods ($p < 0.0001$, r=.63). Mean methylation across subjects was assessed in each neural tissue, with significant differences by brain region, CpG, and brain region by

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CpG interactions (2-way ANOVA; $p < 0.0001$ all effects; figure 4A). The overall pattern of high and low methylation across the sequence exhibited both similarities and differences relative to the sequence in PBMCs (figure 3A). Concordance of particular CpGs across brain tissues was extremely high (striatum versus hippocampus: $p < 0.0001$, $r = .95$; hypothalamus versus hippocampus: $p < 0.0001$, $r = .94$; n=25 CpGs, figure 4B).

Sequenom assays—Mean % methylation of all individuals for each CpG containing unit within the *Oxtr* amplicon in peripheral (PBMC) samples was positively correlated with mean % methylation of the same units in central (hippocampal) samples ($p = 0.05$, $r = .50$, figure 4C), such that a CpG with low overall methylation in one tissue was likely to have low methylation in another tissue.

(d Oxtr methylation discordance across tissues within individuals

Concordance across tissues within individuals was examined for each CpG by calculating the correlation coefficients between hippocampus and hypothalamus methylation values from all individuals measured in both tissues (figure 5A). These two brain regions had the greatest number of samples yielding high quality data for both regions (n=21-28 matched samples depending on CpG). All correlation coefficients could be classified as indicative of "weak" or "no" correlation, and none had significant *p*-values associated with them despite a 72% chance (0.95^{25}) of at least one false positive and a 36% chance of 2 or more. The same procedure was applied to hippocampus and striatum with a smaller data set (n=12-14 matched samples per CpG; CpGs 7 and 8 were excluded for low matched sample sizes; figure 5B). Two correlations were individually significant (CpG 1: $r = .75$, $p = 0.004$, FDR corrected $p = 0.08$; CpG 11: $r = .66$, $p = 0.02$, FDR corrected $p = 0.23$), however this result could also be expected by chance given the number of comparisons. Correlation coefficients for single comparisons of mean methylation across the sequence by individual were also calculated but were not significant (hypothalamus to hippocampus: $p = .36$, $r = .30$; striatum to hippocampus: $p = .38$, $r = -.21$).

A parallel analysis was performed with Sequenom data from individuals measured in blood and hippocampus. Correlation coefficients ranged from no (0.03) to moderate (.46) correlation for each CpG, with only one individually significant correlation (CpG 9; $r = .46$, $p = 0.02$, FDR corrected $p = .22$), as could be expected by chance. Thus, individual methylation in PBMCs was also not demonstrably predictive of methylation in hippocampus. Across all comparisons, individually significant correlations (prior to correction) for particular CpGs in any given pair of tissues did not correspond to those found in any other tissue pairings.

(e) Oxtr expression, corticosterone secretion, and DNA methylation

Oxtr expression was quantified via qPCR as fold-change relative to *Actb*. Oxtr expression varied across brain regions ($p < 0.0001$, one-way ANOVA, figure 6A), with significant *post-hoc* differences detected between striatum and hippocampus ($p < 0.0001$, Tukey's HSD), and between hypothalamus and hippocampus ($p < 0.0001$, Tukey's HSD). Expression of *Oxtr* in RNA samples extracted from PBMCs was too low to characterize reliably.

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Hypothalamic oxytocin signaling has been previously related to reduced corticosterone levels, so *Oxtr* expression levels in the hypothalamus were compared to CORT area under the curve in response to restraint in all samples with both data types available (n=18). Greater hypothalamic *Oxtr* expression was associated with lower CORT AUC (p=0.05, r=.46) across samples (figure 6B).

At a sequence-wide level, higher methylation levels matched up with reduced transcript. Within the brain, hypothalamic samples were the most methylated and exhibited the least transcript expression, followed by striatum, and then hippocampus, although absolute differences in % methylation by tissue were small. Blood samples exhibited the highest degree of methylation and undetectable transcript.

DNA methylation levels were also compared to *Oxtr* mRNA expression within each brain region. *Oxtr* fold-change in the hippocampus was compared to methylation of each Sequenom unit, and was positively correlated with hippocampal methylation of CpG 19 (p = 0.006, r= .50), yielding a FDR corrected p-value 0.07. Mean methylation across all sites assayed in hippocampus was positively but not significantly associated with *Oxtr* fold-change (p = 0.28, r=.21), and there were no group differences in hippocampal *Oxtr* expression by maternal care. Expression was also quantified in a smaller set of striatum tissue available from study subjects (n=16 samples). *Oxtr* fold-change in the striatum appeared positively correlated with pyrosequencing values for striatum methylation for two CpGs (CpG 2: p=0.035, r=.53; CpG 16: p=0.032, r=.55), but two or more differences at the p<0.05 threshold would be expected by chance 36% of the time with 25 tests, and these values do not survive FDR correction (both becoming p=.43). No correlations between methylation and expression were evident in hypothalamus (n=26 samples). Thus methylation of *specific* CpGs may have a positive relationship with gene expression, but this is difficult to detect.

DISCUSSION

Natural variation in early maternal care was associated with both physiological and behavioral differences in adult offspring. Previous studies have demonstrated cross-generational effects of early experience on activity measures (Denenberg and Whimbey, 1963), which we found across several different behavioral testing setups, with LL offspring exhibiting greater activity. Interestingly, LL offspring of both sexes also exhibited lower body weights in adulthood, despite the absence of differences at weaning, which may be related to their distinct activity profiles. Rats subjected to three weeks of daily maternal separation have been shown to weigh less than controls, despite normal feeding behavior under baseline conditions (Iwasaki et al., 2000), which may parallel the present findings.

High- and low-licking offspring had similar basal corticosterone levels, but LL offspring mounted a reduced CORT response to restraint and downregulated CORT levels sooner, similar to rats born to prenatally stressed dams (Burton et al., 2007), and to female rats receiving the lowest levels of licking within LL litters (Pan et al., 2014). Chronic stress exposure in adulthood is also associated with faster downregulation of CORT levels in response to restraint in this rat strain (Beery et al., 2012). Thus HL and LL rats in this study

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appear to have important differences in their stress-reactivity that were correlated with differences in activity behavior.

Terminal measures of reproductive traits including uterine and testicular mass both showed trends towards higher mass in HL versus LL rats. Prior studies have shown earlier onset of fertility, increased sexual attractivity, and more proceptive behaviors in female LL offspring (Cameron et al., 2008a, 2008b; Sakhai et al., 2011), which appears opposite the present findings. However our measures reflect organ weight differences taken in later adulthood, and in the case of males these differences may be mainly reflective of differences in body weight. Together, these findings on activity, body weight, corticosterone secretion and reproductive structures add to a growing body of known persistent effects of maternal care on later life outcomes, documented across an ever-increasing variety of domains (e.g. Caldji et al., 1998; Francis et al., 1999; Zhang et al., 2005; Parent and Meaney, 2008; Walker et al., 2008; Beery and Francis, 2011; Starr-Phillips and Beery, 2014).

Oxtr methylation, gene expression, and maternal care: PBMC findings

Oxtr promoter methylation varied with maternal care (high- versus low-licking offspring) in PBMCs. Methylation was increased at four of 12 sites examined in adult HL offspring relative to LL offspring. The aggregate probability of all four of these changes was 0.0003, however any one specific difference might not be reliable. These differences were of small absolute magnitude, with total differences in methylation ranging from ~1-3%, although in some cases this indicated a large percent difference between LL and HL groups (from 13% to 144% change). These small differences may reflect the inclusion of multiple cell types in PBMC samples that are not affected by manipulations, or it may reflect a lack of biological signficance.

Oxytocin receptor transcript has been detected in PBMCs in some species (Ndiaye et al., 2008; Nicholson et al., 2004), and OT causes functional changes in bovine T lymphocytes and human PBMCs (Ndiaye et al., 2008; Macciò et al., 2010), but PBMCs are generally considered to be an OXTR non-expressing tissue (Kimura et al., 2003). In the present study, *Oxtr* transcript level was too low to reliably characterize, even in concentrated samples. It is possible that *Oxtr* is expressed in blood cells but that mRNA persistence is very low. However, very low to non-existent transcript levels imply that PBMC *Oxtr* methylation differences between HL and LL are more likely to serve as a marker of prior experience than to act as functional contributor to physiological or behavioral regulation via altered gene expression.

Ziegler et al. (Ziegler et al., 2015) found that mean methylation across 12 *OXTR* CpGs in a human population was negatively associated with stress-induced salivary cortisol levels; in our study, mean methylation was weakly negatively correlated with PBMC *Oxtr* methylation in rats, with a strong negative relationship for CpG #24 that became a trend following correction for multiple comparisons. If *Oxtr* methylation in blood cells is associated with reduced cort in additional studies, this will highlight the need to determine whether it is part of a functional pathway, or simply a biomarker of experience. Finally, prior research has shown that there can be important and substantial sex differences in

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methylation (e.g. Kurian et al., 2010; Liu et al., 2010; McCarthy et al., 2014), and *Oxtr* methylation levels should be examined in females.

Oxtr methylation, gene expression, and maternal care: neural findings

Oxytocin receptors across the three brain regions measured for methylation and transcript expression have previously been associated with a variety of social, sexual, and anxiety behaviors. OTR in the nucleus accumbens has been associated with reward-mediated affiliative behavior and parental care (e.g. Aragona and Wang, 2009; Olazábal and Young, 2006), and receptors in the ventromedial hypothalamus have been implicated in sexual behavior and vary with reproductive status (Bale et al., 2001; Young et al., 1997; Dumais et al., 2013). Oxytocin within the hypothalamus also plays an important functional role in the inhibition of the stress-axis (Neumann et al., 2000). Oxytocin receptors in the hippocampus have been less well studied, but oxytocin activity at OTR in this region modulates interneurons and enhances cortical information transfer (Owen et al., 2013). Additional research suggests that hippocampal OTR density changes seasonally with behavior, and may be involved in spatial aspects of social behavior (Beery et al., 2014; Ophir et al., 2012).

In contrast to group differences by maternal care found in PBMCs, only 1 of 12 comparisons in hippocampus yielded a difference between HL and LL groups, with an adjusted (unmeaningful) *p*-value of .46. This was surprising, because we expected that *Oxtr* methylation differences by maternal care would be more likely in neural than peripheral tissue. It remains possible that *Oxtr* methylation was altered by maternal care in other brain regions, including those measured in this study, as sample sizes and methodology (single sequence reads) used in the pyrosequencing portion of this study were insufficient to detect group differences.

Oxtr transcript levels were readily measurable in all three neural tissues and were highest in the hippocampus, followed by the striatum, with the lowest levels in the hypothalamus. Low *Oxtr* transcript within the hypothalamus likely reflects the heterogeneity of this tissue, with some nuclei expressing OTR at a high level in rats (e.g. ventromedial hypothalamus) relative to others.

Prior research has established an important role for hypothalamic OT signaling in the suppression of cort secretion (Neumann et al., 2000; Smith and Wang, 2014). In the present study, higher levels of *Oxtr* transcript within the hypothalamus were correlated with reduced cort area under the curve in response to restraint stress, suggesting that increased ability to receive an OT signal might play a role in this downregulation.

DNA methylation and transcript abundance

Increased methylation of the *OXTR/Oxtr* gene has been associated with reduced gene expression, as has been reported for many genes (Kusui et al., 2001; Mamrut et al., 2013). Thus one might expect reduced oxytocin receptor gene expression in the blood of HL offspring. Important exceptions to this negative relationship between *OXTR* methylation and gene expression exist, however, with high methylation and gene expression in mammary tissues (Mamrut et al., 2013) and at specific CpG sites (Harony-Nicolas et al., 2014), thus an

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inverse relationship between methylation and expression cannot be assumed and was investigated in this study.

Oxtr transcript levels relative to the control gene *Actb* were measured by qPCR in RNA extracted from PBMCs, and related to methylation. Tissues that expressed more transcript also had lower methylation levels, with the greatest methylation and least *Oxtr* transcript in PBMCs. However we also found tentative support for a positive association between methylation and transcript levels at particular CpGs. In hippocampus, increased methylation was associated with increased *Oxtr* mRNA relative to *Actb*, most strongly for one CpG (#19) but with a similar pattern across the sequence analyzed. Two potential correlations between methylation and expression were found in striatum, both also in the positive direction. The complexities involved in predicting expression from methylation have been echoed by others (Mamrut et al., 2013). Lam et al. (2012) found that in a human cohort, only a minority of individual CpG sites had significant correlations with mRNA levels across individuals, similar to the present findings. They also found that, in addition to the canonical negative relationship between methylation and gene expression, increased methylation with increased mRNA expression was observed for a substantial number of genes. This has been specifically described for *OXTR* in some tissues, and for some CpGs (Harony-Nicolas et al., 2014; Mamrut et al., 2013). One possible explanation for the positive correlation found between methylation and gene expression in some studies is that methylation measured following bisulfite conversion of DNA includes 5-hydroxy methylation, typically associated with transcriptional activation (Branco et al., 2012; Hackett et al., 2013), although potentially a stable modification (Bachman et al., 2014). Additional complexity in the relationship between methylation and transcription is also becoming apparent, for example the methyl-CpG binding protein MeCP2 may be more associated with transcriptional activation than repression (Chahrour et al., 2008).

Methylation patterns across tissues

Methylation levels differed by tissue and were highest in blood. Across the sequence, regions of high and low methylation were similar across tissues such that specific CpGs that tended to be more or less methylated in one tissue were also more or less methylated in others (figures 3A, 3B, 4A). These correlations were strongest between brain regions, with hippocampal methylation measures at each CpG strongly correlated with methylation of the same CpGs in striatum and hypothalamus (figure 4B) – two brain regions in which oxytocin receptors are known to play important roles in a variety of social behaviors (Goodson, 2005; Anacker and Beery, 2013). Similarly, peripheral methylation in PBMCs was moderately correlated with brain methylation levels in hippocampus (figure 4C). Farré et al. (2015) found that blood methylation values are significantly more variable than brain values, in part because of heterogeneity of cell types in the blood.

These correlations across tissues by CpG in the present study contrast with the lack of significant correlation in *Oxtr* methylation by CpG across two brain regions reported in Harony-Nicolas et al. (2014). Their findings may reflect greater methylation differences between neural tissues of disparate developmental origins (olfactory bulb and cerebellum), the assessment of CpGs in a different region of the *Oxtr* gene, or reduced power to find

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significant correlations based on the smaller number of CpGs (7) and subjects assessed in their study.

Despite consistent patterns of high and low methylation across CpGs (and resulting correlation across tissues), individual variation in one tissue was not demonstrably predictive of variation in another (with comparisons made in blood vs. hippocampus, hypothalamus vs. hippocampus, and striatum versus hippocampus). Correlation coefficients were as low as or lower than expected by chance even between brain regions, indicating that individual variability did not have detectable predictive power across tissues.

Conclusions

We demonstrate for the first time that early life experience is associated with subtle methylation differences in the *Oxtr* gene in blood, together with changes in body-weight, activity, and stress-reactivity. *Oxtr* expression was measurable across all brain regions, and hypothalamic transcript levels were correlated with reduced CORT secretion, as expected based on the suppressive role of OT signaling in this region. *Oxtr* expression was not detectable in PBMCs. Together these findings suggest that *Oxtr* in mononuclear cells may be a target of experience-dependent DNA methylation without reflecting functional connections to expression.

The ability to use PBMC methylation as an indicator of methylation in other tissues has been the subject of much interest. Epigenetic studies in humans regularly sample only blood (*OXTR* examples: Gregory et al., 2009; Unternaehrer et al., 2012; Puglia et al., 2015; Ziegler et al., 2015; Reiner et al., 2015) or other peripheral tissues, while most behaviorally relevant epigenetic changes have been documented in the brain (e.g. Weaver et al., 2004; Roth et al., 2009; Miller et al., 2010). Some studies have suggested that one tissue may be a good proxy for another (Houde et al., 2014; Iyer et al., 2010; Liberman et al., 2012; Stenz et al., 2014). The present study highlights a crucial distinction between tissue concordance of patterns of methylation across a region of the genome (very high in these samples) and the ability to use individual variation in methylation in one tissue to make inferences about another (not supported in this study).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Rats were reared by dams exhibiting high or low maternal care
- Physiological, behavioral, and epigenetic outcomes were assessed in offspring
- *Oxtr* promoter methylation varied with maternal care in peripheral blood samples
- Methylation patterns over the target region were highly correlated across neural tissues
- Nonetheless, individual variation in methylation was uncorrelated across tissues

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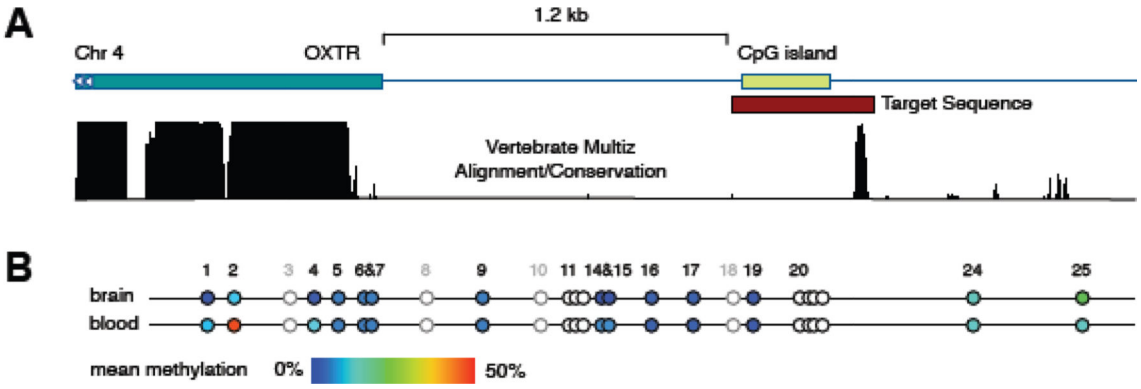

Figure 1.

Location and components of the target sequence within the *Oxtr* promoter in rats. (A) The *Oxtr* gene lies on the reverse (-) strand on chromosome 4. The target region begins approximately 1.2kb upstream and encompasses a CpG island and region of high conservation across vertebrates (alignment data from UCSC genome browser). (B) Epigram of CpGs within the target region, color-coded by mean % methylation in each tissue. Methylation was generally higher in PBMCs than in the brain. Open gray circles represents units providing no data in Sequenom assays because of peak mass; open black circles depict data not analyzed because of >2 CpGs clustered in one unit.

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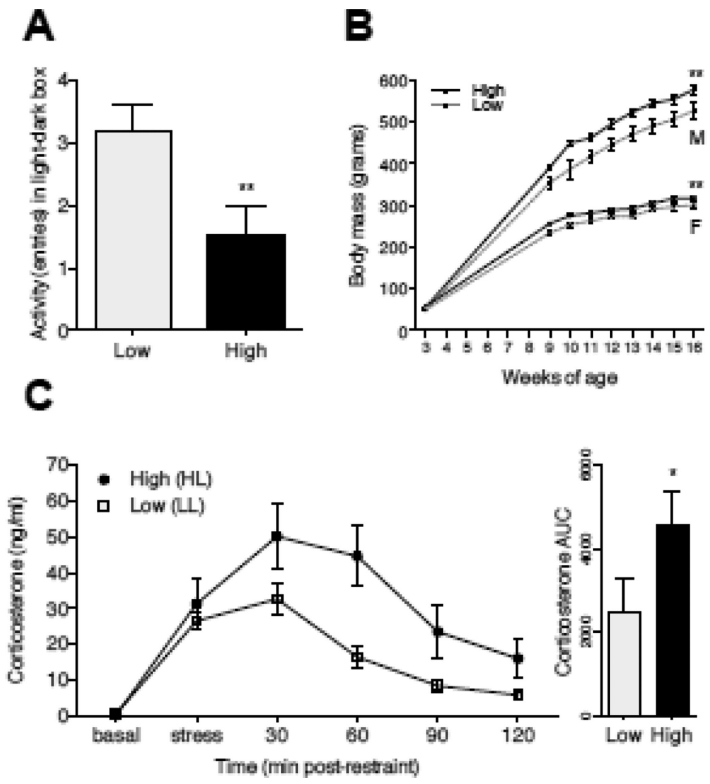

Figure 2.

Physiological and behavioral variation as a function of early maternal care. (A) LL offspring were more active in the light-dark box as well as other behavioral tests. (B) HL and LL offspring did not differ in body weight at weaning, but HL offspring of both sexes weighed more in adulthood, including at week 16 (effect of maternal care: $p < 0.01$, effect of sex: $p < 0.0001$). (C) Corticosterone response to a stressor was greater in HL offspring, with similar peak timing but greater overall CORT secretion relative to LL offspring (area under the curve, females not tested). CORT AUC was associated with anxiety-like behaviors (see text). * $p < 0.05$, ** $p < 0.01$. Error bars depict ± SEM.

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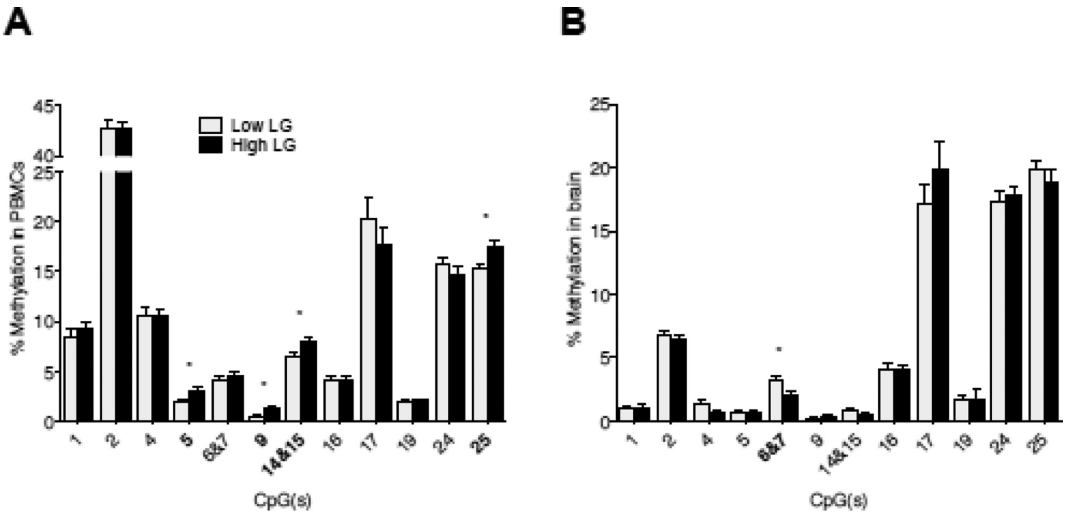

Figure 3.

Oxtr methylation across 12 Sequenom units in HL and LL offspring in peripheral blood mononuclear cells (A) or hippocampus (B). In blood, 4 of 12 units examined exhibited significantly higher methylation in HL offspring than LL offspring, representing a combined probability of 0.0003. In brain, 1 of 12 units differed with maternal care, but such a result would be expected by chance (p=.45) and is not meaningful. * = $p < 0.05$ prior to correction.

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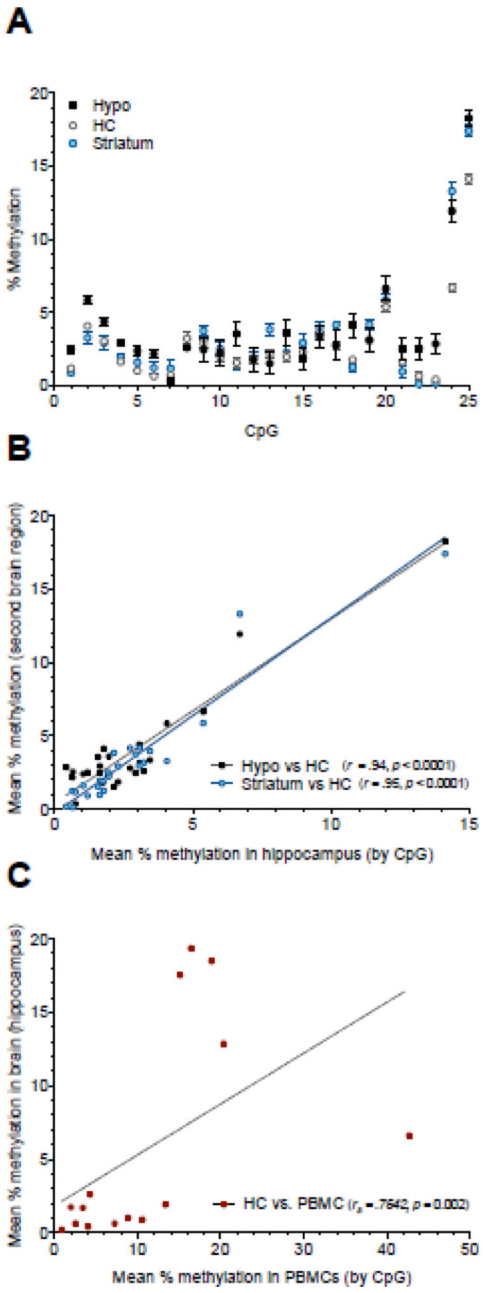

Figure 4.

Methylation measures across neural tissues. (A) Mean methylation levels were assessed by pyrosequencing across 25 CpGs in three brain tissues, including hippocampus. Methylation differed significantly by both CpG and brain region (each $p<0.0001$, 2-way ANOVA) and showed both similarities and differences in CpG methylation patterns relative to PBMCs (figure 3B). (B) Methylation levels (measured by pyrosequencing) were highly concordant between hippocampus and other brain regions (striatum and hypothalamus). (C) To a lesser extent, blood measures of methylation were associated with hippocampal methylation (both Sequenom data). Data are shown for each of 25 CpGs (4B) or 12 CpG containing units (4C), averaged across subjects.

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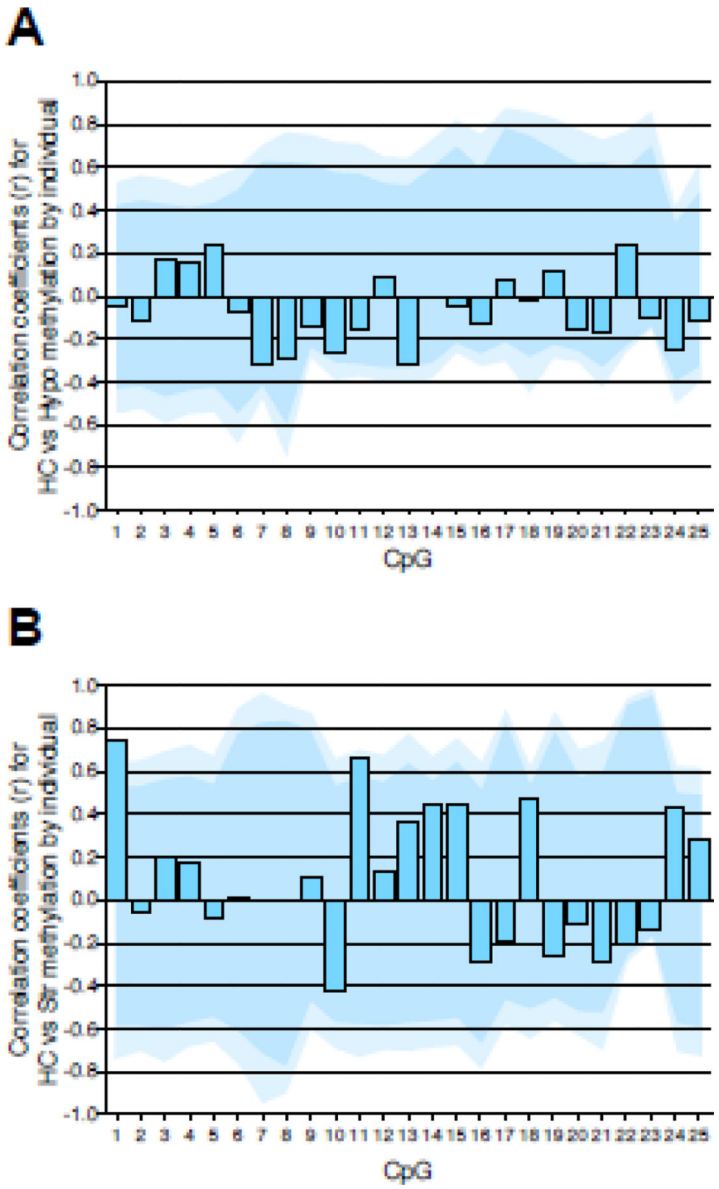

Figure 5.

Individual methylation values were not correlated across brain tissues, despite tissue concordance at the group level. For each CpG, we computed the Pearson correlation coefficient *r* between the methylation values for matched samples in pairs of brain regions (bars). Dark and light shaded regions represent 95% and 99% thresholds, respectively, of distributions of possible correlation coefficients determined from 10,000 permutations of the measured values among the individuals. These distributions represent the null hypothesis that an individual methylation value in one brain region does not help to predict the value in another region in the same animal. (A) Correlations based on pyrosequencing data for matched samples passing validation in both hippocampus (HC) and hypothalamus (Hypo). Correlations for individuals at each CpG were either weak (.2< *r* <.3) or absent (*r* <.2), and none were significant, even prior to correction for multiple comparisons. (B) Correlations for matched samples passing validation in both hippocampus and striatum (Str). Two

correlations (CpG 1 and 11) were individually significant prior to but not following correction, and this result could be expected by chance. Correlations between hippocampus and blood (described in the text) yielded similar results, and no particular CpG yielded consistently high correlation across multiple tissues.

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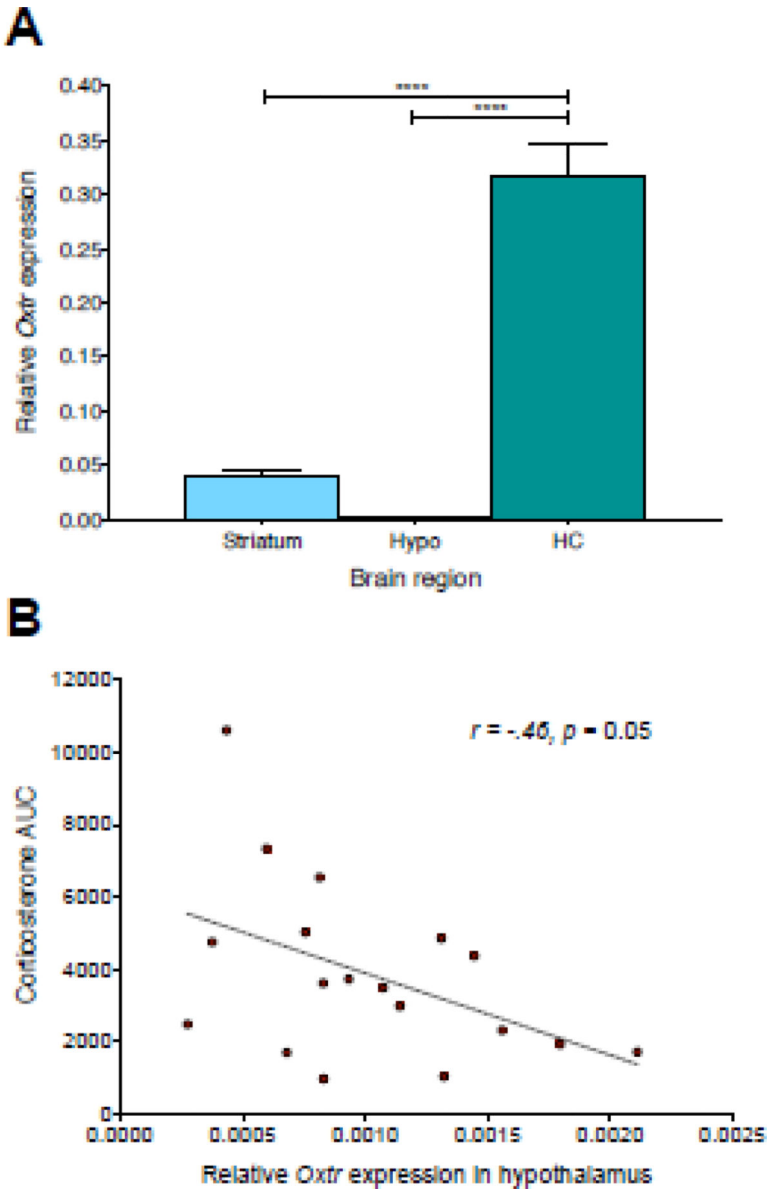

Figure 6.

Oxtr expression, quantified by qPCR as fold-change relative to *Actb*. (A) Relative Oxtr expression varied significantly by brain region with the highest expression levels in the hippocampus and the lowest (but readily detectable levels) in the hypothalamus. Transcript levels were too low to reliably assess in PBMCs (not shown). (B) Relative *Oxtr* expression was negatively correlated with corticosterone secretion in response to a stressor. **** = $p <$ 0.0001 (Tukey's HSD)