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Brain region-specific methylation in the promoter of the murine oxytocin receptor gene is involved in its expression regulation

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Summary Oxytocin is a nine amino acid neuropeptide that is known to play a critical role in fetal expulsion and breast-feeding, and has been recently implicated in mammalian social behavior. The actions of both central and peripheral oxytocin are mediated through the oxytocin receptor (Oxtr), which is encoded by a single gene. In contrast to the highly conserved expression of oxytocin in specific hypothalamic nuclei, the expression of its receptor in the brain is highly diverse among different mammalian species or even within individuals of the same species. The diversity in the pattern of brain Oxtr expression among mammals is thought to contribute to the broad range of social systems and organizations. Yet, the mechanisms underlying this diversity are poorly understood. DNA methylation is a major epigenetic mechanism that regulates gene transcription, and has been linked to reduced expression levels of the Oxtr in individuals with autism. Here we hypothesize that DNA methylation is involved in the expression regulation of Oxtr in the mouse brain. By combining bisulfite DNA conversion and Next-Generation Sequencing we found that specific CpG sites are differentially methylated between distinct brain regions expressing different levels of Oxtr mRNA. Some of these CpG sites are located within putative binding sites of transcription factors known to regulate Oxtr expression, including estrogen receptor α (ERα) and SP1. Specifically, methylation of the SP1 site was found to positively correlate with Oxtr expression. Furthermore, we revealed that the methylation levels of these sites in the various brain regions predict the relationship between ERα and Oxtr mRNA levels. Collectively, our results suggest that brain region-specific expression of the mouse Oxtr gene is epigenetically regulated by DNA methylation of its promoter.
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1. Introduction

In the past two decades, a growing body of evidence pointed to a central role of neuropeptide oxytocin (Oxt) in mammalian social behavior (Lee et al., 2009). This applies to a wide range of relationships, including parental and affiliative behaviors (Insel, 2010), as well as aggression and fear (Lukas et al., 2011; Pagani et al., 2011). Oxt, mostly known for its role in labor and suckling, is produced in the hypothalamus and released either to the periphery via the pituitary gland, or within the brain (Lee et al., 2009). In humans, its intranasal administration improves multiple aspects of human social cognition (Meyer-Lindenberg et al., 2011) and augments social emotions (Feldman, 2012). All actions of Oxt are mediated by the oxytocin receptor (Oxtr), a G-protein coupled receptor encoded by a single gene (Gimpl and Fahrenholz, 2001). Polymorphism of the human *OXTR* was found by several independent studies to be genetically associated with autism spectrum disorders (ASD) (Wu et al., 2005; Jacob et al., 2007; Lerer et al., 2007), and administration of Oxt to individuals with ASD alleviated impairments in social cognition (Bartz and Hollander, 2008).

In sharp contrast to the highly conserved expression of Oxt in specific hypothalamic nuclei (Lee et al., 2009), the expression of its corresponding receptor in the brain is highly diverse among different mammalian species (Gimpl and Fahrenholz, 2001) or even within individuals of the same species (Ross et al., 2009). The heterogeneity in Oxtr expression in the brain is thought to contribute to the diversity of social behavior and social organization among mammals (Young, 1999). Nonetheless, the mechanisms that underlie this heterogeneity are poorly understood. The regulation of Oxtr expression was intensively studied in peripheral tissues such as the uterus, where it is significantly affected by gonadal steroid hormones (Ivell and Walther, 1999), and positively correlates with estrogen levels (Larcher et al., 1995). The mechanism by which estrogen regulates *Oxtr* transcription is elusive, partly because the *Oxtr* promoters of several mammalian species, including those of humans, lack a full (palindromic) estrogen response element (ERE) (Ivell and Walther, 1999; Ivell et al., 2001). Interestingly, in the ovine species, an indirect effect of estrogen on *Oxtr* transcription was found to be mediated by SP1 binding elements (Fleming et al., 2006).

One of the major biological processes that generate species- and experience-dependent diversity in gene expression is DNA methylation (Champagne, 2010); a reversible epigenetic process in which a methyl group is added covalently to a cytosine residue immediately followed by a guanine (CpG). These CpG dinucleotides are well known to be underrepresented in the genome of many vertebrates, and to frequently occur in small clusters known as CpG islands. Hypermethylation of these islands in the vicinity of genes is often associated with gene inactivation (Suzuki and Bird, 2008). The expression of the human OXTR was previously shown to decrease by DNA methylation in liver cells (Kusui et al., 2001). Furthermore, hypermethylation of the *OXTR gene* was found to be associated with decreased levels of its mRNA in the temporal cortex tissue of individuals with ASD (Gregory et al., 2009). We recently showed that transcription of the mouse *Oxtr* is regulated by DNA methylation of specific sites in its promoter. Moreover, we found that changes in the methylation of the *Oxtr* promoter around parturition time correlates with modulation of the gene transcription in a tissue-specific manner (Mamrut et al., 2013). Here we hypothesize that differential DNA methylation of the *Oxtr* promoter is involved in its region-specific expression in the brain. To challenge this hypothesis we examined the relationship between the level of transcription and the methylation of specific CpG sites in the *Oxtr* promoter within various regions of the mouse brain.

2. Materials and methods

2.1. Animals

Male and female C57BL/6 mice, 12—16 weeks of age (20—25 g, purchased from Harlan, Israel) were housed under diurnal lighting conditions and allowed food and tap water ad libitum. All experimental protocols were approved by the Animal Care and Use Committee of the University of Haifa. Five males and five females were used for the initial analysis of the cerebellum and olfactory bulb, whereas 6 females were used for the later analysis of multiple brain regions.

2.2. Tissue processing

Mice were anesthetized with isoflurane (Abbott Laboratories, Abbott Park, IL) and sacrificed by cervical dislocation. The brains were then rapidly extracted on ice for quick isolation of the cerebellum and olfactory bulb. Both tissues, as well as the rest of the brain were immediately frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ until further processing. On the day of preparation of micro-punch samples, brains were transferred to a cryostat set at $-20\,^{\circ}C$, where sections of 250 μm were obtained and transferred to precooled glass slides. Micro-punch sampling was done as previously described by others (Elliott et al., 2010) on a frozen stage (-25 to $-35\,^{\circ}C$) using an 18-gauge microdissection needle.

2.3. RNA isolation and quantitative PCR

Total RNA was isolated from brain tissues as we described before (Mamrut et al., 2013). RNA quality was assessed using Bioanalyzer (Agilent Technologies, Santa Clara, CA) and only samples with RIN value >7.5 (range 7.8—9.6) were used. Levels of the Oxtr, SP1 and ERα mRNAs were assessed by quantitative Real-Time PCR that was carried out in triplicates on a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) using fluorescent SYBR Green technology. The hypoxanthine-guanine phosphoribosyltransferase (Hprt1) mRNA was used as endogenous control. The calibration of the Oxtr, SP1, ERα and Hprt1 mRNA amount and the examination of the primer sets efficiencies were based on the generation of standard curves using a serial of diluted cDNA.

The first analysis examined only Oxtr expression in OB and Cer samples and was performed on samples from five males and five females. The second analysis explored the expression of Oxtr, SP1 and ERα in seven brain regions in six female samples.

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2.4. DNA extraction, bisulfite treatment and Sanger sequencing

These processes were carried out as previously described (Mamrut et al., 2013).

2.5. SOLiD sequencing

cDNA Libraries were prepared using SOLiD™ Fragment Library Construction Kit (Invitrogen, Life Technologies, Carlsbad, CA), according to the SOLiD fragment library construction protocol. Size selection was performed using the E-Gel® 2% size select Agarose Gels (Invitrogen). Library fragment size and sample purification were analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Library was quantified using the KAPA ABI SOLiD library quantification Kit (KAPA Biosciences, Woburn, MA). Sequencing was done using the ABI SOLiD 3 analyzer (Applied Biosystems), according to the Applied Biosystems protocols.

2.6. Mapping of SOLiD sequencing data

Approximately 900,000 SOLiD reads of ~50 nt were mapped on the bisulfite sequence of the amplicon (400 nt in length) by the BLAST-like algorithm with one permitted mismatch per every 12-mer. The average coverage of a position across the fragment was about 20,000. The variability of the coverage was very high (SD = 110,000). Results were summarized in a table where the numbers of matching to each nucleotide (A, T, C or G) were calculated for every position of the amplicon. Error-rate normalized methylation levels were calculated for each CpG site as detailed below. Similar procedures of read mapping and error rates estimation were applied to the Sanger sequences 450 nt-long reads. The coverage of sequence positions in that case was 80—100 per sample.

2.7. Calculating error-rate normalized methylation

Error-rate normalized methylation levels were calculated for each CpG site. This original analysis, which is based on general statistical considerations (see below), was developed and used to statistically analyze DNA methylation levels of pooled bisulfite-converted DNA samples. This procedure was applied initially to the Sanger sequencing 450 nt-long reads (Fig. 2A), pooled from five males and five females, in order to compare it with the standard analysis (Fig. 1C). The coverage of sequence positions in that case was 80—100 reads per sample. The same analysis was then used for the SOLiD sequences derived of the same DNA samples (Fig. 2B) for validation. We then used the same procedure for analyzing the SOLiD sequences from the seven brain regions extracted from six females (Fig. 5A).

We assumed that two types of errors may appear in any C-position during bisulfite treatment and mapping of SOLiD/Sanger reads upon the position:

1. C is mapped by C in a read of a non-CpG position (C→C), as opposite to the expected transform: C→T (bisulfite error: probability p_{be}).

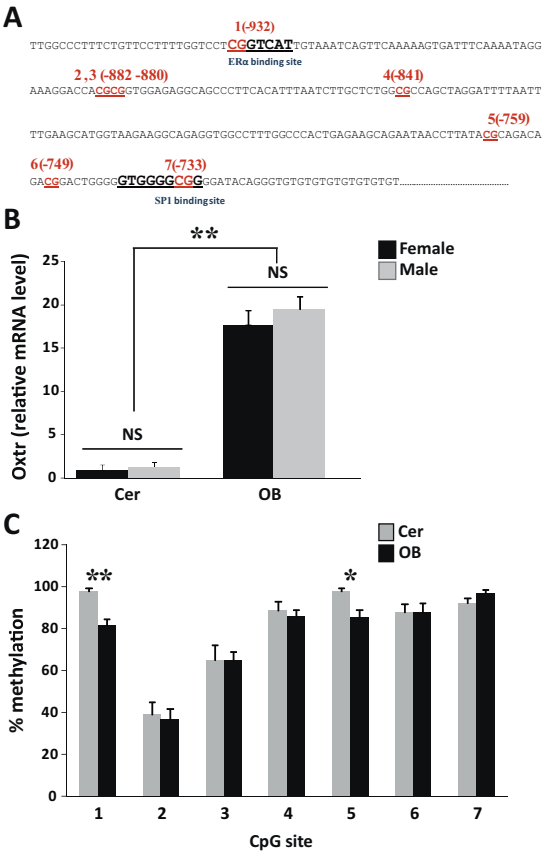

Figure 1 mRNA levels and methylation of specific CpG sites of the *Oxtr* promoter differ between the cerebellum and olfactory bulb. (A) The sequence of the PCR *amplicon*, including the positions of the seven CpG sites. The half-ERE harboring CpG site 1 and the SP1 binding sequence harboring CpG site 7 are underlined. (B) Comparison of relative Oxtr mRNA levels in cerebellum (Cer) and olfactory bulb (OB) of male ($n = 5$) and female ($n = 5$) mice. 2-way ANOVA ($F_{(3,12)} = 48.5$, $p < 0.001$) found no difference between sexes ($p > 0.1$, NS), significant difference between brain regions ($F_{(1,12)} = 144.78$, $^{**}p < 0.001$) and no interaction between factors. Bars represent mean ± SEM. (C) Comparison of methylation levels (%) at the seven CpG sites of the *Oxtr* amplicon in the OB and Cer of the same 10 mice (5 males and 5 females). No significant difference was found between males and females, while a significant difference was found between the OB and Cer (repeated mixed model ANOVA $F_{(6,96)} = 39.76$, $p < 0.001$, $n = 10$) in CpG sites 1 (paired t-test, $t_{(18)} = 4.77$, $^{**}p < 0.001$, $n = 10$) and 5 (paired t-test, $t_{(18)} = 2.7$, $^{*}p < 0.05$, $n = 10$). Bars represent mean ± SEM.

2. C is mapped by A or G in a read (C→A,G) (mapping error: probability p_e).

So, for C in the non-CpG position the probability to get matching T in a mapped read is: $p_t = (1 - p_{be})*(1 - p_e)$. If the total number of mapped reads onto a genomic C position is K, the normal (Gaussian) approximation $N(m,s)$ of the binomial distribution for the expected number of Ts in mapped reads will be with parameters:

$$m = K * p_t, \quad s = K * p_t * (1 - p_t)$$

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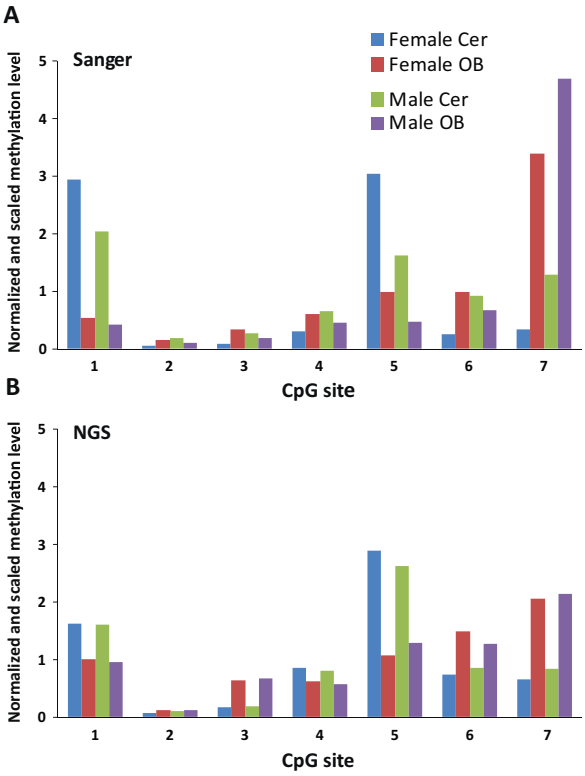

Figure 2 The differential methylation of specific CpG sites between the cerebellum and olfactory bulb is confirmed by Next-Generation Sequencing. (A) Error-rate normalized and scaled methylation analysis of the seven CpG sites in the *Oxtr* amplicon, performed for the same sequences analyzed in Fig. 1C (Sanger sequences). These sequences were pooled in groups (80–100 reads from 5 animals per group) according to sex (females vs. males) and brain region (Cer vs. OB). B) Error-rate normalized and scaled methylation analysis of the same samples, obtained by SOLiD Next-Generation Sequencing (NGS sequences).

The ratio of m (the expected number of Ts in the absence of methylation) to the observed number of Ts in the CpG site (T_0) is taken as a measure for the methylation level at the GpC site. Thus, the observed T_0 is reversely normalized by m, which is estimated from the observed rates of bisulfite and mapping errors. This error-rate normalized measure ranges between 1 to infinity and is getting higher with more methylation in the CpG site.

2.8. Regression analysis

Oxtr mRNA level in the various brain regions was considered to be the dependent variable of the regression. The error-rate normalized methylation levels of the seven CpG sites (scaled by the average per CpG site across all regions) were considered as main independent variables (main factors). We also used pair dot-products of the corresponding vectors for the main factors (''pair-interactions'') as independent variables. These pair interactions help to approximate the putative deviation from the linear dependence on the corresponding main effects when both main factors are high or both are low. In order to avoid the regression over-fitting, only regressions with two-terms and intercept were analyzed using all independent variables.

2.9. Statistical analysis

All statistical tests were performed using SPSS 19.0 (IBM, Armonk, NY). Parametric tests were used following validation of normality by Kolmogorov–Smirnov test, otherwise we used non-parametric tests. Post hoc Tuckey test was used throughout. For the analysis of differences in methylation between OB and Cer of male and female mice (Fig. 1C) we used a priori repeated mixed model ANOVA to determine if there are significant differences between males and females, distinct CpG sites and brain regions. Following the significant differences found between Cer and OB samples, we used paired t-test with corrected alpha to compare between them for each CpG site separately. For the analysis of predictors of the relationship between ERα and Oxtr expression, we used a priori screen of all terms in a short two-terms plus intercept regression, as detailed above, in order to reveal the best predictors (Fig. 6A). We then screened for the best fit regression using two of these terms (Fig. 6C).

3. Results

The mouse *Oxtr* gene is comprised of four exons and three introns (Kubota et al., 1996). The promoter of the gene contains several half-palindromic estrogen receptor elements (half EREs), a palindromic ERE and two SP1 sites (Mamrut et al., 2013). To examine the relationship between *Oxtr* promoter methylation and its expression we focused our analysis of DNA methylation on a ~400 bp region within the promoter (herein termed *amplicon*), which we have recently shown to be differentially methylated between cell lines with high and low *Oxtr* transcription levels (Mamrut et al., 2013). This amplicon region contains seven CpG sites (Fig. 1A), of which the first (−932, CpG site 1) is located within a half-ERE sequence, and the last (−733, CpG site 7) resides within an SP1 binding sequence. As we have previously shown, the methylation of these two CpG sites is critical for the effect of DNA methylation on Oxtr expression in cell lines (Mamrut et al., 2013).

The main goal of the present study was to determine whether *Oxtr* transcription is associated with differential methylation of its promoter in various regions of the mouse brain. To approach this question, we began by analyzing the cerebellum (Cer) and olfactory bulb (OB) because they are easily accessible for extraction, and were previously shown to exhibit marked differences in Oxtr expression (Gould and Zingg, 2003). As depicted in Fig. 1B, Oxtr mRNA levels were significantly higher in the OB compared to Cer of both male ($n = 5$) and female ($n = 5$) animals (2-way ANOVA, $F_{(3,12)} = 48.5$, $p < 0.001$), with no apparent differences between sexes ($p > 0.1$). We next analyzed the methylation pattern of all seven CpG sites located within the *Oxtr* amplicon of the OB and the Cer of all animals and found a conserved pattern of methylation between all male and female mice (Fig. 1C). The methylation level was CpG site-specific (repeated mixed model ANOVA $F_{(6,96)} = 39.76$, $p < 0.001$, $n = 10$), with site 2 exhibiting the lowest levels (<40%), site 3 — moderate levels (<70%) and the rest

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exhibiting high methylation levels (>80%). No significant differences were found between males and females in any of the CpG sites ($p > 0.1$), therefore we analyzed the differences between the OB and Cer of all animals together. The statistical analysis confirmed the existence of significant differences between the OB and the Cer in two of the CpG sites, namely sites 1 (paired t-test, $t_{(18)} = 4.77$, $p < 0.001$, $n = 10$) and 5 (paired t-test, $t_{(18)} = 2.7$, $p < 0.05$, $n = 10$). In both sites higher methylation levels were observed in the Cer, which expresses low levels of Oxtr mRNA, as compared to the highly expressing OB.

The analysis presented in Fig. 1C represents the results of 20–25 sequences per tissue per animal. To further validate these data we performed another type of analysis, which is more sensitive to differences between groups. For this, reads from the same brain region of all animals of the same sex were pooled together and assigned into four groups: male OB, male Cer, female OB and female Cer, with 80–100 reads per group (Sanger sequencing). We then calculated the error-rate normalized methylation level (see Section 2) for each of the seven sites, and scaled it to the average value across all CpG sites. As shown in Fig. 2A, profound differences were found between the OB and Cer of both females and males in sites 1 and 5. Surprisingly, site 7, which did not show significant differences in the standard analysis (Fig. 1C) exhibited large differences between the two brain regions of both sexes. However, these differences were in the opposite direction to sites 1 and 5, with the OB showing elevated methylation levels. To confirm these findings on a much larger scale, we performed Next-Generation Sequencing (NGS, ~200,000 reads per group) on the same DNA samples and analyzed the data using the same method. As shown in Fig. 2B, methylation levels of both sexes were indeed higher in CpG sites 1 and 5, and lower in CpG site 7 in the Cer compared to the OB. The fact that the same relationships between the OB and Cer methylation were replicated between the independent samples of males and females across all CpG sites strongly upheld our analysis. Overall, these data confirm that the OB and the Cer display significant differences in the methylation levels of specific CpG sites.

Given our findings in the OB and Cer, we next sought to examine the relationship between DNA methylation and transcription of the *Oxtr* in multiple regions of the mouse brain. To that end we used micro-punching to isolate the following areas from six other female mice: anterior olfactory nucleus (AON), caudate-putamen (CPu), medial amygdala (MeA), cerebral cortex (CTX) and ventromedial hypothalamus (VMH). The isolation of the OB and Cer was done as explained above. Tissues from one side (randomized) of the brain of all animals were used for measuring DNA methylation using NGS, while the other side was used for quantitative PCR analysis of SP1, ERα and Oxtr mRNA levels. As shown in Fig. 3A, SP1 mRNA levels were relatively homogeneous (<5 fold differences) in all brain regions with the Cer and OB both showing the highest levels. In contrast, ERα (Fig. 3B) and Oxtr (Fig. 3C) mRNA levels varied greatly (>25 fold differences) between the distinct brain regions. Interestingly, Oxtr mRNA levels were elevated in areas directly associated with the olfactory system (i.e. AON, OB and MeA), while ERα mRNA levels were highest in areas associated with social behavior (i.e. MeA and VMH). Since both transcription factors (ERα and SP1) were shown to regulate Oxtr expression in other tissues, we examined if their expression

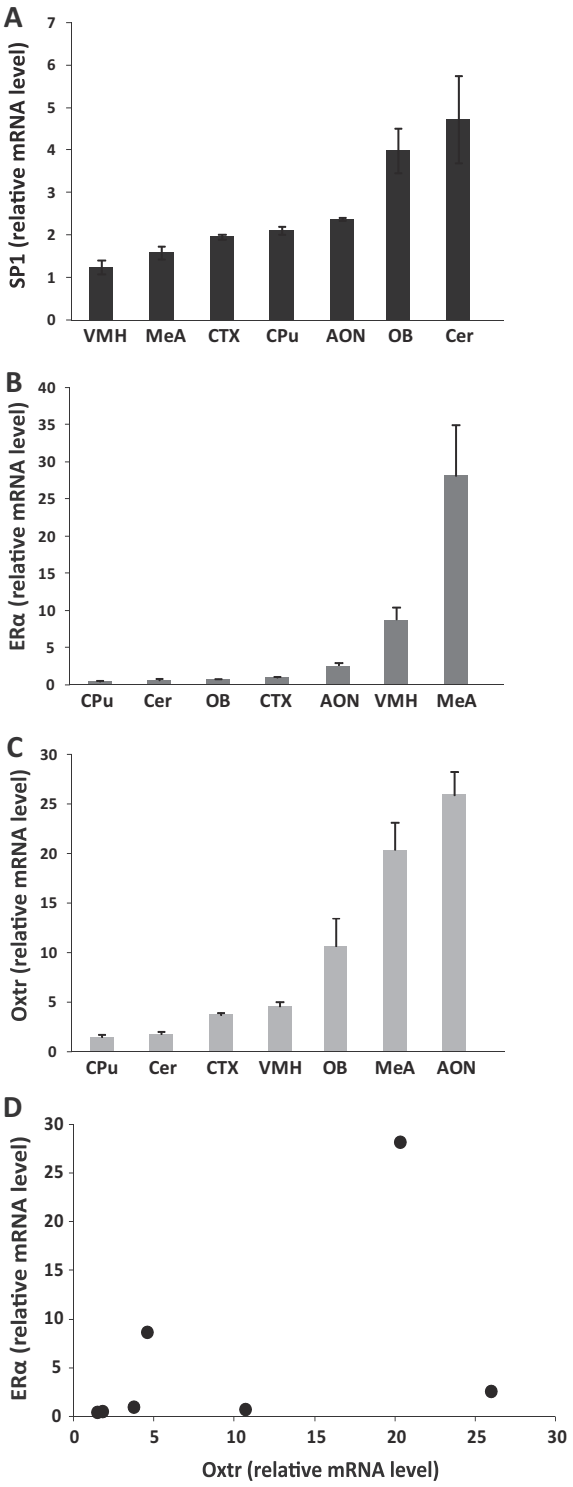

Figure 3 The mRNA levels of SP1, ERα and Oxtr in the various brain regions. (A) SP1 mRNA levels measured at seven brain regions of six female mice including the olfactory bulb (OB), cerebellum (Cer), anterior olfactory nucleus (AON), caudate-putamen (CPu), medial amygdala (MeA), cerebral cortex (CTX) and ventromedial hypothalamus (VMH). Bars represent mean ± SEM. (B) Same for ERα. (C) Same for Oxtr. (D) No correlation ($p > 0.1$, Spearman's) was found between the Oxtr and ERα mRNA levels (shown above in (C) and (B), respectively) at the seven brain regions.

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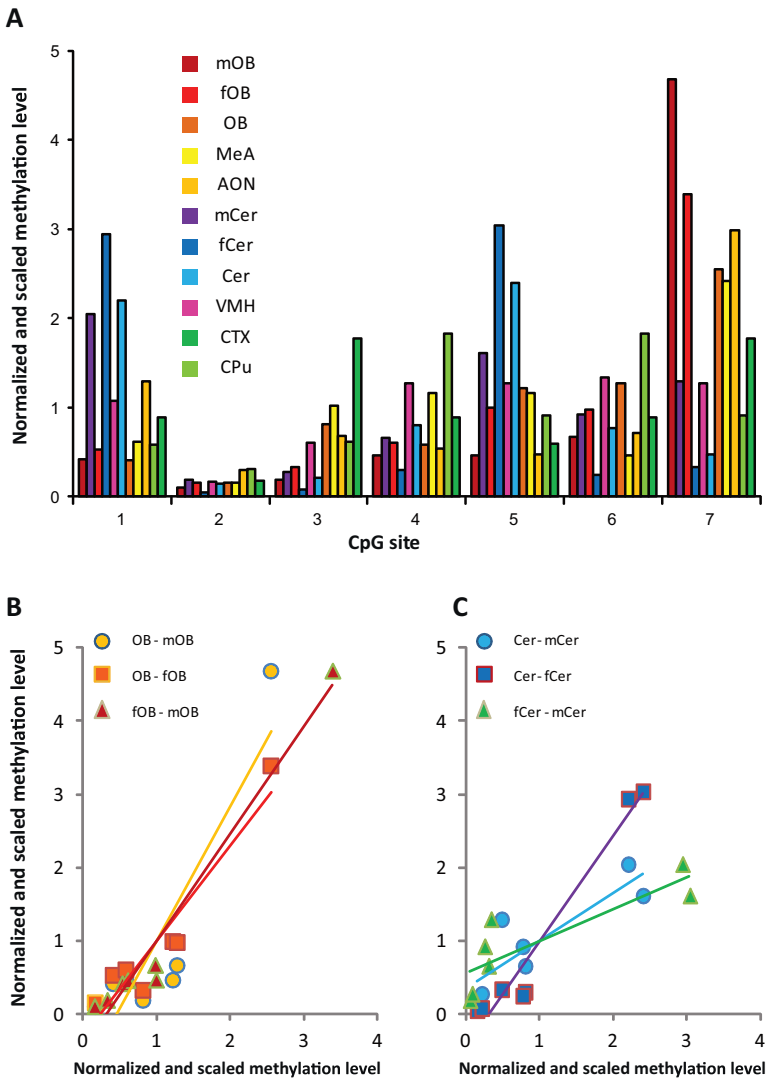

Figure 4 Brain-region specific pattern of DNA methylation in the *Oxtr* promoter. (A) Error-rate normalized and scaled methylation analysis of the seven CpG sites in the *Oxtr* amplicon, across all eleven DNA samples, of seven brain regions analyzed using NGS. Note that samples mOB, mCer and fOB, fCEr represent the OB and Cer of the 5 males and five females, respectively, that were analyzed in Fig. 2, whereas all other samples represent the seven brain areas of the six females of the later analysis. (B) Correlations between the methylation patterns among the three OB samples. Each pair of correlated samples is marked by a different symbol. All samples were significantly correlated with each other ($p < 0.05$, Spearman's, Supplementary Table 1). (C) Similarly to (B), correlations among the Cer samples. All samples were significantly correlated with each other ($p < 0.05$, Spearman's, Supplementary Table 1).

correlates with Oxtr expression in the brain. We found no significant correlation ($p > 0.1$, Pearson) between the mRNA levels of the Oxtr and ERα (Fig. 3D) or SP1 (not shown) in the seven brain regions.

The error-rate normalized methylation levels of all samples, including the male and female Cer and OB (mCer, fCer, mOB, and fOB) that were analyzed previously (see Fig. 2) are shown in Fig. 4A. As apparent, vast differences exist between the various samples especially in CpG sites 1, 5 and 7. Specifically the data point to a negative relationship between methylation in sites 1 and 5 compared to site 7, meaning that brain areas which display high levels of methylation in CpG 7 (OB, AON and MeA), present low levels of CpG 1 and 5 methylation and vice versa.

To validate that these patterns faithfully represent brain-region specific methylation we examined the correlations across all CpG sites within the three independent samples of Cer tissue (mCer, fCer, and Cer) and OB tissues (mOB, fOB, and OB). We found statistically significant ($p < 0.05$, Spearman's) high correlation (Supplementary Table 1) among all OB samples (Fig. 4B) and among all Cer samples (Fig. 4C). In contrast, no correlation ($p > 0.1$) was found between any of the Cer and OB samples, confirming that the observed methylation values reflect consistent brain region-specific DNA methylation patterns of the *Oxtr* promoter.

We then examined if the methylation at any of the CpG sites correlates with the relative expression of Oxtr in the various brain regions (Supplementary Table 2). Of the seven CpG sites in the *Oxtr* amplicon, sites 1–6 showed no correlation with Oxtr expression ($p > 0.1$, Spearman's). In contrast, CpG site 7, which is located within the SP1 binding sequence,

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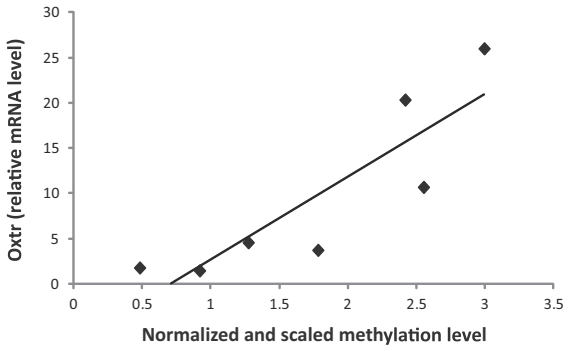

Figure 5 Correlation of Oxtr mRNA levels at the various brain regions with the methylation level at CpG site 7. The relative Oxtr mRNA levels across the seven examined brain areas from six females shows a significant correlation with the error-rate normalized and scaled methylation level of CpG site 7 ($p = 0.01$, $R = 0.876$, Spearman's). This correlation is even higher if methylation levels are used prior to scaling ($p < 0.005$, $R = 0.929$, Spearman's).

showed high correlation with Oxtr expression ($p = 0.01$, $R = 0.876$, Fig. 5).

Our analysis (Fig. 3) showed no correlation between the levels of ERα and Otxr mRNA. However, since Oxtr is known to be regulated by the ERα, we hypothesized that they may be linked by the methylation levels at the *Oxtr* promoter. To find out if we could fit the data with a linear regression, we used the ratio between the Oxtr and ERα relative mRNA levels as the dependent variable, and the error-rate normalized methylation levels of all CpG sites and their pair interactions (e.g. s1—s2) as the independent variables. Using short two-term plus intercept regressions we first identified which of the CpG sites had the most robust impact on the Oxtr: ERα mRNA ratio. As shown in 6A, one single CpG site (s2), and four pair-interactions (s1—s2, s1—s7, s2—s7, s3—s5) clearly deviate from all the others by their high average (>0.55) and low SD (<0.15) of adjusted *R*-squared values, and are thus significant predictors of the transcription ratio. A further analysis of the regression terms (Fig. 6B), revealed that whereas s3—s5 negatively contribute to the transcription ratio, the remaining terms (s2 and the s1—s2, s1—s7, s2—s7 pairs) are positively associated with it, suggesting that methylation of sites 1, 2 and 7 positively contributes to the effect of ERα on *Oxtr* transcription.

Finally, we examined how well these terms predict the relationship between *ERα* and *Oxtr* transcription, over all brain regions. For that we applied the following regression: −2.9 + 18.68*(s1—s2) + 6.25*(s2—s7), which uses only two pair interactions between sites 1, 2 and 7. As shown in Fig. 6C, the results of this regression (black bars) were highly similar to the experimentally obtained ERα-normalized Oxtr mRNA level (gray bars) across brain regions. The fit of regression equation to observations is highly significant (*F* statistics: 65.1 on 2 and 24 DF, $p < 2.02E{-}10$). Thus, the methylation levels of only a few CpG sites (1, 2 and 7) are sufficient to predict the relationship between the highly variable ERα and Oxtr mRNA levels across various brain regions.

4. Discussion

In the present study we showed that the promoter region of the murine *Oxtr* is differentially methylated between distinct brain-regions expressing profoundly different levels of the receptor. Using NGS we found that the differences in methylation between these areas were localized to particular CpG sites on the promoter. We then used bioinformatics to show that the differences between brain regions in methylation of only a few of the CpG sites are sufficient to account for the association between the expression of ERα and Oxtr. Two of these sites, 1 and 7, are located within putative binding sites of ERα and SP1, respectively, both of which are known transcription regulators of the *Oxtr* (Ivell and Walther, 1999; Fleming et al., 2006). Furthermore, the methylation of CpG site 7 was shown to be positively correlated with Oxtr mRNA level at various brain regions. Together, these results support a role for DNA methylation in regulating the spatial distribution of Oxtr in the mouse brain.

The bulk of the analysis in the present study was performed in females. Due to its well-known role in maternal functions (i.e. parturition and nursing) the oxytocinergic system is generally considered sex-specific (Carter, 2007). Nevertheless, sex-dependent differences in *Oxtr* CNS expression reported for rats and mice were limited to the spinal cord and hypothalamus (Uhl-Bronner et al., 2005). In accordance with these results, our initial analysis showed no differences between male and female mice in either Oxtr mRNA abundance or its promoter methylation in the OB and Cer. This enabled us to combine all males and female subjects into a single cohort of ten animals, which provided a proper sample size for statistical comparisons. The differences that were found in methylation levels using this analysis were relatively small and did not exceed 20% at most. This is in contrast to the >10 fold differences found in Oxtr mRNA abundance between the same brain regions in the real-time PCR analysis. It should be noted, however, that while the analysis of expression is blind to the Oxtr non-expressing cells in the tissue, the analysis of methylation levels equally considers the genomes of all the cells in the analyzed brain region, most of which may not express Oxtr. Thus, large differences in methylation, which are limited to the restricted populations of Oxtr-expressing cells, are highly diluted by a much larger population of irrelevant cells. Therefore, some of these differences that seemed negligible using standard analyses, were exposed only after using the error-rate normalization analysis, which is highly sensitive to small differences.

The analysis of methylation in specific CpG sites is usually done by standard (Sanger) sequencing following bisulfite DNA conversion, which converts any unmethylated cytosine to thymidine. This is typically done using ~20 reads per sample, which is labor intensive and yields low statistical power. Here we present a new methodology based upon NGS that yields >10,000 reads per sample with much less effort. Nonetheless, NGS sequencing per animal is not cost effective, hence we pooled together equal amounts of DNA from several animals and analyzed them as a group using the error-rate normalization, to achieve statistical significance. This was initially done for the analysis of OB and Cer samples (five males and five females) also analyzed using the standard Sanger sequencing. By comparing the results obtained from

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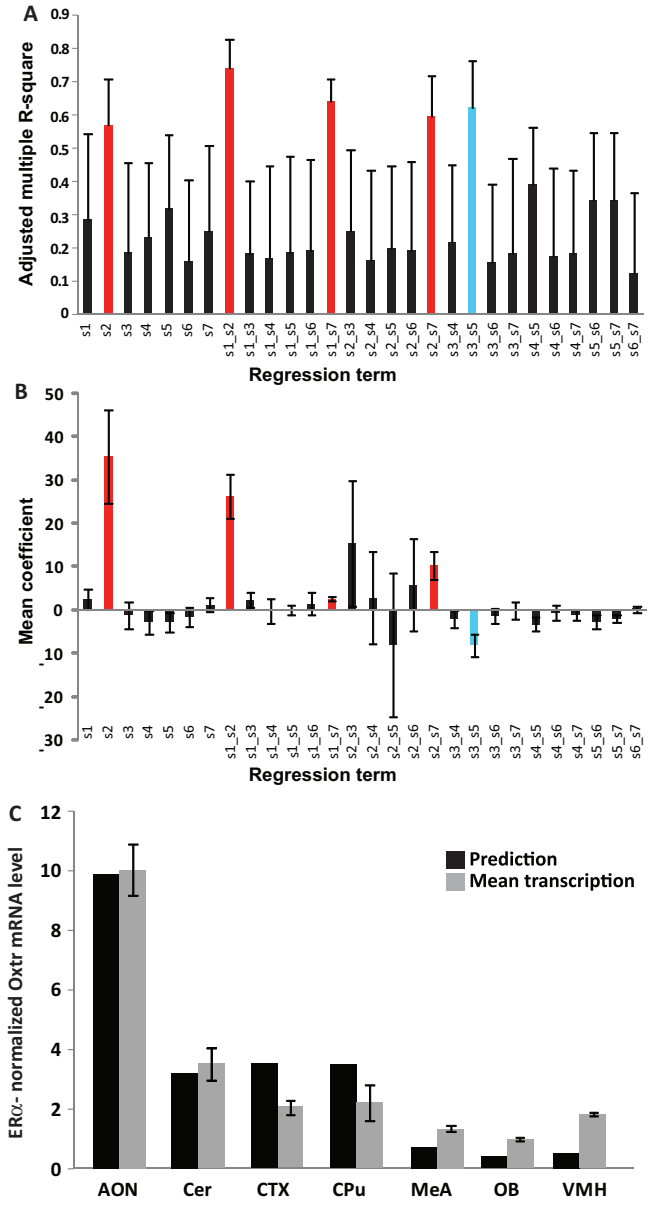

Figure 6 The methylation levels of specific CpG sites at the Oxtr promoter account for the relationship between Oxtr and ERα mRNA levels. (A) Adjusted multiple *R*-square across all brain regions as a function of each term used as independent variables in the regression. These terms represent the error-rate normalized and scaled methylation levels of all CpG sites (e.g. s1, s2) and their pair interactions (e.g. s1—s2). The ratio between the Oxtr and ERα relative mRNA levels was used as the dependent variable for each brain region. Five terms (marked as red and blue bars) clearly deviate from all the others by their high average (>0.55) and low SD (<0.15) of adjusted *R*-squared values. Therefore, these five regression terms show the highest predictive power. (B) The mean coefficient of regression associated with each term across all regressions, as a function of each term. As apparent, one of the aforementioned terms (s3—s5: blue bar) negatively contributes to the dependent variable, while the other four terms (s2, s1—s2, s1—s7 and s2—s7: red bars) are positively associated with it. (C) The ERα-normalized Oxtr mRNA level plotted for each of the seven brain areas, as observed by quantitative PCR measurements (gray bars, mean ± SEM), and as predicted by the regression equation: −2.9 + 18.68*(s1—s2) + 6.25*(s2—s7) (black bars). This regression yields a residual standard error of 1.336 on 24 degrees of freedom, multiple *R*-squared of 0.844 and adjusted *R*-squared of 0.8314. The fit of regression equation to observations is highly significant (*F* statistics: 65.1 on 2 and 24 DF, $p < 2.02E{-}10$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the same samples using both methods we showed that the error-rate normalization of NGS data is more sensitive to differences between the groups and is thus the preferred analysis for measuring changes in methylation levels. Using this methodology to analyze multiple brain regions from six females, we found a high degree of correlation among the OB samples and among the Cer samples, but no correlation between groups (Fig. 4 and Supplementary Table 1). These findings confirm that this methodology consistently identifies a brain-region specific methylation pattern of the *Oxtr* promoter.

Mammals show an extremely broad range of social systems and organizations (Kappeler et al., 2013). Yet, the social behavior of most non-mammalian and mammalian vertebrate species seems to be mediated by a common network of limbic brain structures (Goodson, 2005; Goodson and Kabelik, 2009). To explain the diversity of behaviors subserved by this common network it was suggested that depending on the social context, the activity in each neuronal population ("node") differentially relates to the activity in the other nodes of the network (Goodson and Kabelik, 2009). This modular "neural context" is thought to be regulated by several modulators, among which Oxt is pivotal (Goodson, 2013). Nonetheless, the expression of Oxt is restricted to specific hypothalamic areas, which innervate the brain in a highly conserved manner among all vertebrates (Veenema and Neumann, 2008; Lee et al., 2009). In contrast, Oxtr expression in the brain is highly diverse among mammalian species and even among conspecifics (Insel et al., 1991; Insel and Shapiro, 1992; Campbell et al., 2009; Kalamatianos et al., 2010). Correlations between brain Oxtr expression patterns and various types of social behaviors were found among several mammalian species (Young, 1999; Olazabal and Young, 2006; Ophir et al., 2012). A seminal study in different vole species showed that distinct patterns of Oxtr brain expression mediate their monogamous vs. promiscuous behavior (Ross et al., 2009). Thus, the regulation of Oxtr expression in the brain seems to be an important factor determining species- and individual-specific social behavior (Young, 1999).

Some of the most well-known regulators of Oxtr expression are gonadal steroid hormones (Ivell and Walther, 1999). Many studies show that the levels of the receptor change significantly in the course of gestation and parturition within different areas of the brain in a highly complex manner (Young et al., 1997; Bealer et al., 2006; Meddle et al., 2007). These results suggest an intricate relationship between estrogen and Oxtr expression in the mammalian brain. Our study did not find a direct correlation between Oxtr and ERα expression in the brain. Nonetheless, we showed that this relationship exists if *Oxtr* DNA methylation

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is taken into account, suggesting a pivotal role for epigenetic regulation of the Oxtr brain expression.

Epigenetic mechanisms in general, and DNA methylation in particular were shown by multiple studies to regulate many types of social behavior (Zhang and Meaney, 2010; Champagne, 2012). In particular, the involvement of Oxtr in social behavior was shown to be modulated by these processes (Francis et al., 2000, 2002). For example, mating-induced partner preference in vole females was shown to be associated with histone deacetylation-mediated upregulation of Oxtr expression (Wang et al., 2013). In humans, DNA methylation of the OXTR in blood cells predicted the neural response to ambiguous social stimuli and rapidly changed following acute psycho-social stress (Jack et al., 2012; Unternaehrer et al., 2012). Finally, hypermethylation of specific CpG sites of the OXTR gene were evident in individuals with autism (Gregory et al., 2009). We have recently reported that DNA methylation of a specific region in the murine *Oxtr* promoter regulates its expression in various cell lines (Mamrut et al., 2013). Furthermore, both the methylation pattern and expression levels change in the uterus and mammary glands during parturition. We also showed that at least two of the seven CpG sites in the promoter region are important for the effect of methylation on the Oxtr expression in cell lines. These two sites reside within putative binding sites of ERα (CpG site 1) and SP1 (CpG site 7). These observations are in agreement with the finding that SP1 sites in the ovine *Oxtr* promoter mediate ERα-mediated regulation of this gene (Fleming et al., 2006). Here we examined the methylation at the same region of the Oxtr promoter in the mouse brain and found that the same two CpG sites 1 and 7, as well as site 5, are differentially methylated between distinct brain regions. Furthermore, the fact that methylation of CpG site 7 is lower in the Cer compared to the OB, in contrast to sites 1 and 5, suggests that these differences are not due to general hypermethylation of the *Oxtr* gene promoter in the cerebellum. Interestingly, in the previous study we found no differences in Oxtr methylation between the mammary glands and uterus of virgin female mice (Mamrut et al., 2013). Thus, it seems as if the variability in methylation between specific brain regions is higher than the variability between distinct peripheral tissues, suggesting an involvement of DNA methylation in the brain-region specific pattern of Oxtr expression. This conclusion is further supported by the direct correlation we found between the methylation in CpG site 7 and Oxtr mRNA level at the various brain regions. Furthermore, using averaged estimations across all possible regressions we found that the interactions between these sites and site 2 are significantly associated with linking ERα and Oxtr mRNA levels. We speculate that the interactions of sites 1 and 7 with site 2 may be important to the chromatin remodeling process, which brings sites 1 and 7 to a close proximity and enables their functional interaction. Given that the methylation level of site 2 was the only main factor found to be positively contributing to ERα-dependent Oxtr expression and that it showed an extraordinarily low rate among all CpG sites, it is possible that its methylation is a prerequisite for *Oxtr* transcription.

In summary, we have shown that specific CpG sites of the murine Oxtr promoter are differentially methylated between various brain regions. We also found that methylation of only a few these CpG sites is sufficient to account for the relationship between ERα and Oxtr expression. Moreover, the methylation level at one of these sites, located within a putative binding sequence of the SP1 transcription factor, was positively correlated with Oxtr mRNA level at the distinct brain regions. Collectively our results suggest that experience- and region-specific expression of the mouse *Oxtr* gene in the brain is epigenetically regulated by DNA methylation of its promoter.

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Conflicts of interest statement

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2013.10.004.

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