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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 °C until further processing. On the day of preparation of micro-punch samples, brains were transferred to a cryostat set at −20 °C, where sections of 250 μm were obtained and transferred to pre-cooled glass slides. Micro-punch sampling was done as previously described by others (Elliott et al., 2010) on a frozen stage (−25 to −35 °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.