



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



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ABSTRACT

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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* DNA 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* DNA methylation was significantly lower at multiple CpGs in the blood of LL versus HL males, 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 DNA methylation relative to these global patterns was not consistent across tissues. These results suggest that blood *Oxtr* DNA methylation may reflect early experience of maternal care, and that *Oxtr* methylation across tissues is highly concordant for specific CpGs, but that inferences across tissues are not supported for individual variation in *Oxtr* methylation.

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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 considerable 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., 2012a, 2012b; 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

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Young, 2009). While oxytocin production and release patterns within the brain are largely conserved across species, the distributions and densities of oxytocin receptors (OTRs) are 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).

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., 2012a, 2012b), 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 DNA 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 ~1 kb 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 behavioral 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* DNA 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.

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 measures in readily available tissues such as blood. While blood DNA methylation values may provide an important biomarker for outcomes such as cancers (Langevin et al., 2012), it is less clear if they will be useful and relevant indicators of epigenetic changes related to brain and behavior. DNA methylation varies across cell types (Lam et al., 2012; Reinius et al., 2012), and blood cell composition may vary across and within individuals. Many DNA 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 specifically on the methylation of *OXTR/Oxtr* DNA in multiple tissues. These studies have described greater DNA methylation in the liver than in the 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 of 7 CpGs in the olfactory bulb and cerebellum were performed, 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. Our goals were to a) assess the impacts of early life experience on *Oxtr* methylation, and b) examine DNA methylation patterns across multiple tissues that are of interest either for their ease of sampling or for 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 DNA methylation across tissue types – including PBMCs 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 related 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 DNA methylation and corticosterone (CORT) secretion.

Materials and methods

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%.

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