Individual differences in maternal care as a predictor for phenotypic variation later in life
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CHAPTER 5

Individual Differences in Maternal Care Correlate with DNA Methylation Status of the Brain-Derived Neurotrophic Factor Exon IV Promoter

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Abstract

Attenuated levels of brain-derived neurotrophic factor (BDNF) have been implicated in the pathophysiology of depression and post-traumatic stress disorder. Importantly, vulnerability for these diseases was shown to be largely affected by the early life environment, and so is brain BDNF expression. Rats that received a Low amount of maternal care in early life display lower levels of hippocampal BDNF mRNA expression than offspring of high caring mothers. Additionally, animals subjected to early life adversity have been reported to exhibit increased DNA methylation levels of BDNF promoter regions. We here tested whether the percentage of licking and grooming (%LG) received by individual rat pups from their mother correlates with BDNF exon IV promoter methylation in adulthood. We found a significant positive correlation between %LG and the degree of methylation of this promoter in the hippocampus. However, methylation state in these animals did not correlate with total hippocampal BDNF mRNA expression levels, which might be due to the differential regulation of the BDNF gene by its various promoters. Overall, we conclude that early life variation in the amount of maternal care received by individual rats is related to long-lasting epigenetic changes in the BDNF exon IV promoter.
Introduction

In humans, the adult phenotype not only depends on genetic background, but also on environmental factors and experiences in childhood. For example, early life adversity was found to augment the risk of developing psychopathology later in life, including depression and post-traumatic stress disorder (PTSD) (Heim and Nemeroff, 2001; Schore, 2002; Bremner, 2003; McEwen, 2003; Nemeroff, 2004a, b). One of the factors thought to be implicated in the pathogenesis of depression is the neurotrophic system (Altar, 1999; Vaidya and Duman, 2001; Calabrese et al., 2009), and particularly alterations in the expression of brain-derived neurotrophic factor (BDNF), a neurotrophic factor that plays a role in neuronal development, hippocampal cell morphology and synaptic plasticity (Kang and Schuman, 1995; Henderson, 1996; Bartrup et al., 1997; McAllister et al., 1999; Hennigan et al., 2007).

Patients suffering from major depression and suicide victims show decreased BDNF levels in serum and brain respectively (Karege et al., 2002; Dwivedi et al., 2003; Sen et al., 2008), and a similar reduction has been found in PTSD patients (Dell’osso et al., 2009). Likewise, animals subjected to chronic adult stress (considered to be a rodent model for depression), to early life adversity or low levels of maternal care exhibited low levels of BDNF mRNA compared to controls (Smith et al., 1995; Liu et al., 2000b; Roceri et al., 2002; Fumagalli et al., 2004; Duman and Monteggia, 2006; Roth et al., 2009; Macri et al., 2010). Moreover, antidepressant treatment of these animals restored the levels of BDNF (Nestler et al., 2002; Xu et al., 2004; Duman and Monteggia, 2006; Castren et al., 2007).

Long-lasting alterations in gene expression are often underlain by epigenetic processes: modifications to the chromatin (DNA and histones) without affecting the sequence of the DNA. In general, DNA methylation and both histone deacetylation and histone methylation are associated with an inactive state of the chromatin and thus transcriptional repression. Indeed, previous studies have shown that decreases in brain BDNF mRNA levels after adult chronic defeat stress or early life adverse experience are epigenetically regulated and involve increased DNA and histone methylation (Tsankova et al., 2006; Roth et al., 2009).

Using a subtle model of maternal care in rats, in which we assessed the effects of specific licking and grooming (LG) behavior directed towards each individual pup, we found that LG scores of individual pups predict hippocampal BDNF mRNA expression in adulthood (Van Hasselt et al., 2011); animals that received relatively high amounts of LG from their mother showed an increased expression level compared to animals with lower LG scores. We wondered if these effects of differential individual maternal care on adult hippocampal BDNF mRNA expression might also be regulated by epigenetic modifications, occurring early in life and persisting throughout adolescence into adulthood. We here assessed the DNA methylation status of the BDNF exon IV (former exon III; Aid et al., 2007) promoter, since exon IV-containing transcripts are abundantly...
present in the hippocampus during post-natal development (Timmusk and Metsis, 1994; Sathanoori et al., 2004; Aid et al., 2007), and dynamic methylation of this promoter has been suggested as a putative mechanism mediating BDNF gene expression during development (Dennis and Levitt, 2005; Aid et al., 2007; Roth et al., 2009).

**Materials & Methods**

*MATERNAL CARE*

All experimental procedures were approved by the animal ethical and welfare committee of the University of Amsterdam. The animals used in this study were bred in-house as described previously (Van Hasselt et al., 2011) and kept on a 12h light/dark schedule (lights on at 8:00 hrs) at a room temperature of 20-22°C and 40-60% humidity during the entire experiment. Food was available *ad libitum*. Adult male and female outbred Long Evans rats were obtained from Harlan (Indianapolis, USA). After habituation to the animal facility one male and two females were housed together for one week to allow mating. Females were then housed in pairs for one week, and subsequently separated and put in large observation cages until weaning of their litters on PND21.

Maternal care observations were performed exactly as in our previous studies (Van Hasselt et al., 2011), from post-natal day 1 (PND1) to PND7. Before the start of observations on PND1 litters were culled to 8, consisting preferably of 4 males and 4 females. Every morning, until weaning on PND21, each pup was uniquely marked with a non-toxic, non-scenting surgical Codman marker (Johnson & Johnson, Brunswick, NY) to enable pup identification. Observations were done five times per day for one hour, twice during the dark period (7:00 and 20:00 hrs) and three times in the light (10:00, 13:00 and 17:00 hrs). Maternal behavior, particularly licking and grooming (LG) towards individual pups within each litter, was scored every 3 minutes, adding up to 700 observations in a week. We were able to distinguish which pup underwent LG in about 60% of the cases, and because this percentage differed slightly between litters we used the following equation to correct for this: (% individual LG observed) /(% total LG identified) * 100%.

At weaning, on PND21, all pups were ear-punched for later identification and group-housed with their same-sex siblings until PND57, when they entered the experiment.

*SODIUM BISULFITE MAPPING*

Sodium bisulfite mapping was performed as described previously (Frommer et al., 1992; Clark et al., 1994). After decapitation, brains were rapidly removed from the skull, snap-frozen and stored at -80°C until use. The whole hippocampus was dissected out and genomic DNA was extracted from the tissue using the GenElute Mammalian Genomic DNA Purification Kit (Sigma-Aldrich, Canada). After EcoR1 digestion and additional
Chapter 5

phenol-chloroform DNA extraction, 1.0μg of hippocampal genomic DNA was treated with sodium bisulfite (EpiTect Bisulfite kit, QIAGEN, Canada; Zhang et al., 2010). Converted DNA fragments containing the BDNF exon IV promoter were amplified in consecutive outside and nested PCR reactions (outside primers: forward 5’-GTTAGAGGAGGTATTATATGATAGTTTA-3’, reverse 5’-TACTCCTATTCTCTACAAAAAATTAAAT-3’; nested primers: forward 5’-GAATTAGGGATATTATGTTTAAGGTTTT-3’, reverse 5’-AAAATCAAACATTATTTAAGTCTTC-3’). The PCR protocol included an initial 5-minute DNA denaturation cycle at 95°C, followed by 34 cycles of denaturation (1 min, 95°C), primer annealing (2.5 min, 60°C), and extension (1 min, 72°C), and a final extension step (10 min, 72°C), after which the samples were kept at 4°C. The nested PCR product was then run on a 1.2% agarose gel for 30 minutes, the band containing the desired DNA fragment was cut out and the DNA was extracted from the gel using a QIAEX II Gel Extraction kit (QIAGEN, Canada). It was then subcloned (QIAGEN PCR cloning plus kit, QIAGEN, Canada), transformed, and 20 different colonies per plate were mini-prepped (Zyppy Plasmid Miniprep Kit, Zymo Research, Canada). Finally, twenty plasmids per animal containing the ligated BDNF exon IV promoter DNA fragment were sequenced at the Genome Québec Innovation Centre (McGill University, Montréal, Québec, Canada).

Corticosterone assay
At decapitation, which was performed at the beginning of the light phase, in the nadir of the circadian corticosterone rhythm, trunk blood from each animal was collected in EDTA-coated tubes, placed on ice and centrifuged for 20 minutes at 5000 rpm. The plasma was stored at -20°C, until use for determination of circulating corticosterone levels by radio-immunoassay (RIA) (MP Biomedicals, Amsterdam, The Netherlands).

Statistical analysis
Statistical analyses were conducted using SPSS 11.0 for Windows. All correlations were tested using linear regression with %LG as the independent (predictor) variable.

Results
Maternal care
For this study, 5 dams with their litters were observed and maternal behavior was scored during the first seven days after birth. Similar to what was reported previously, relatively large variation existed in the amount of licking and grooming (LG) that each individual pup received from its mother (mean %LG=0.63, SD=0.31, n=36; Figure 1). Hippocampal BDNF exon IV methylation status was determined in 12 males (from 5 litters) that we selected based on their LG scores (along the entire range, between 0.22% and 1.29% LG),
thus representing a cross section of the cohort. In these 12 animals we did not observe a significant correlation between %LG received during the first postnatal week and basal plasma corticosterone levels in adulthood (r=0.116, p=0.720; data not shown).

Figure 1. Within-litter variation in %LG. Substantial differences exist between individual pups within each litter in the amount of licking and grooming they receive from their mother. Each column represents a litter, each data point represents a pup (males: black squares; females: grey triangles). Error bars depict one SD above and below the litter mean.

DNA methylation of the BDNF gene
Hypomethylation of CpG dinucleotides in gene regulatory sites is generally associated with an increased transcription of that particular gene (Razin, 1998). For example, in High versus Low LG rats, the relatively demethylated state of the exon 17 GR promoter was accompanied by increased levels of hippocampal GR expression (Weaver et al., 2004). In a previous study, we showed that total BDNF (exon IX) mRNA expression in the hippocampus correlated positively with individual %LG in pubertal and young-adult male rats (Van Hasselt et al., 2011). Therefore, we now questioned if this difference in expression was regulated by long-lasting differences in hippocampal DNA methylation status of the BDNF exon IV promoter in response to maternal care background.

Figure 2. BDNF exon IV methylation status. In 8-week old male offspring, %LG showed a significant positive correlation with the percentage of methylated cytosines in the exon IV promoter region of the BDNF gene (n=12, r=0.603, p=0.04).

Quite surprisingly, we found a significant positive correlation between the amount of licking and grooming received early in life and the number of methylated CpG sites in the exon IV promoter region of the BDNF gene (n=12, r=0.603, p=0.04; Figure 2). This indicates that animals with higher %LG scores showed an increased exon IV methylation pattern, whereas animals that received lower amounts of maternal care have a lower number of methylated cytosines in this promoter, a finding that is seemingly in contrast
with the BDNF mRNA expression pattern in these animals (Van Hasselt et al., 2011). There was no correlation between basal plasma CORT levels and exon IV methylation status (n=12, r=0.120, p=0.709).

**Discussion**

In this study, we aimed to determine possible differences in methylation status of the BDNF exon IV promoter in the hippocampus of adult male rats with varying individual LG scores. In general, elevated levels of DNA methylation are related to a decrease in gene transcription, although this is not always the case (Weber et al., 2007).

Increased exon IV promoter methylation has been reported to correspond to lower BDNF exon IV mRNA levels in mouse brain (Martinowich et al., 2003; Dennis and Levitt, 2005), and in the post-mortem brain of suicide victims (Keller et al., 2010). Previously we reported that in individually characterized animals of a different cohort total BDNF mRNA expression correlated positively with %LG (Van Hasselt et al., 2011). We here report a significant positive correlation between %LG and the percentage of methylated cytosines. Thus, in contrast to most findings, we observed that the correlation between our variable of interest (in this case %LG received early in life) and methylation of the rat hippocampus exon IV promoter in adulthood is in the same direction as the correlation between %LG and total BDNF mRNA expression.

This deviant observation might be explained by the fact that the mRNA expression study was done on BDNF exon IX (the coding exon, thus representing total BDNF mRNA expression), whereas here we studied methylation of the promoter of only one BDNF splice variant, the exon IV transcript. The methylation state of one promoter certainly does not necessarily predict mRNA expression of the body of the gene (Szyf, 2009). We chose to study this particular promoter since it has been shown that BDNF expression upon neuronal activation is mainly controlled by the exon IV promoter (Tao et al., 2002; Chen et al., 2003). Disruption of this activity-dependent promoter IV-driven expression results in depression-like behavior in mice (Sakata et al., 2010) and the mood-stabilizing actions of certain anti-depressant drugs are exerted through activation of BDNF promoter IV (Yasuda et al., 2009). In human prefrontal cortex, mRNA expression of the exon IV transcript predicts total BDNF protein expression (Wong et al., 2009), suggesting that the two are related, at least to a certain extent. It is possible that in our study this relationship was obscured by concurrent differential regulation of other BDNF transcripts by maternal care or other mediating factors, e.g. exon VI (former exon IV), which is known to be affected by corticosterone (Hansson et al., 2006).

Furthermore, DNA methylation is not the only chromatin modification that affects the level of gene transcription. For example post-translational modifications to histones, such as methylation or acetylation, are also strongly involved in determining the state of
the chromatin and therefore the accessibility of the DNA for transcription factors (Meaney and Ferguson-Smith, 2010). This will also affect mRNA levels. The occurrence of any of these epigenetic marks has not been studied in this model yet.

To conclude, in the present study we aimed to unravel possible epigenetic modifications that might underlie the differential BDNF mRNA expression in the hippocampus of animals individually characterized for maternal care. Interestingly, maternal LG did correlate significantly with BNDF exon IV promoter methylation state, but this was not reflected in total hippocampal BNDF mRNA expression in similar animals. DNA methylation, histone modifications and mRNA expression of the many different splice variants of BDNF should be explored in more detail in order to elucidate the connection between these factors.

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