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DNA methylation and childhood maltreatment: from animal models to human studies

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Abstract

Childhood maltreatment (CM) has estimated prevalence among Western societies between 10 and 15%. As CM associates with increased risk of several psychiatric disorders, early age of illness onset, increased comorbidity and negative clinical outcome, it imposes a major public health, social and economic impact. Although the clinical consequences of CM are well characterized, a major challenge remains to understand how negative early life events can affect brain function over extended periods of time. We review here both animal and human studies indicating that the epigenetic mechanism of DNA methylation is a crucial mediator of early life experiences, thereby maintaining life-long neurobiological sequelae of CM, and strongly determining psychopathological risk.

1. Introduction

Childhood maltreatment (CM) is a global problem of significant proportion that affects children of all ages, race, economic, and cultural backgrounds (Gilbert et al., 2009, Green et al., 2010, McLaughlin et al., 2010b). There are four main types of childhood maltreatment: sexual abuse, physical abuse, psychological abuse and parental neglect. In addition, other forms of early life adversity have been studied in human, including witnessing parental violence, rearing in orphanage or severe parental psychopathology (Lupien et al., 2000, Essex et al., 2002, Halligan et al., 2007). While obtaining reliable estimates of the prevalence of childhood maltreatment is challenging, community studies suggest rates for all forms of maltreatment averaging around 10 to 15% (KESSLER et al., 1997, Holmes and Slap, 1998, Edwards et al., 2003, Gilbert et al., 2009).

There exists a strong relationship between CM and negative mental health outcomes (Mullen et al., 1996, Collishaw et al., 2007, McLaughlin et al., 2010b). Maltreatment during early development is among the strongest predictors of psychiatric pathology and severity of clinical course, including early onset of illness, poor treatment response, increased comorbidity and chronic health care utilization (KESSLER et al., 1997, Widom, 1999, Lansford et al., 2002, Edwards et al., 2003, Ystgaard et al., 2004, Evans et al., 2005, Collishaw et al., 2007, Widom et al., 2007a, b, Widom et al., 2007c, Afifi et al., 2008, Gilbert et al., 2009, McLaughlin et al., 2010a). It is strongly associated during adulthood

with obesity, personality disorders, depression, substance use disorders, aggressive and violent behaviours (Widom, 1989, Zingraff et al., 1993, Smith and Thornberry, 1995, Magdol et al., 1998, Stouthamer-Loeber et al., 2001, Thornberry et al., 2001, Bevan and Higgins, 2002, Fagan, 2005, Loeber et al., 2005, Lansford et al., 2007), as well as suicidal behaviors (Brezo et al., 2008, Fergusson et al., 2008, Wanner et al., 2012). In comparison, the association of CM with psychosis appears weaker (Gilbert et al., 2009).

Close family members are the main source of support during development and are essential to provide healthy attachment patterns, appropriate emotional regulation to environmental stimuli, and stress resilience (Malatesta, 1988, Cole et al., 1994). Therefore, the experience of repetitive acts of abuse by parental figures, caregivers or other close relatives signals a hostile and unreliable environment that may trigger brain adaptations in key response systems. These changes may then induce the development of personality and cognitive traits, which in turn increase the risk of psychopathology (Turecki et al, TIN 2012). Epigenetic processes are now emerging as crucial mediators of such long-term biological embedding of CM (Turecki et al., 2011, Hertzman, 2012).

Epigenetics refers to the collective chemical and physical processes that program the genome to express its genes in a time- and cell-dependent manner. These mechanisms are capable of conveying information through meiotic and mitotic divisions in the absence of a change in the DNA sequence. The epigenome is responsive to developmental, physiological and environmental cues. As such, epigenetics explains how the environment regulates the genome, and are well suited to mediate the effects of early environmental factors, potentially throughout the lifespan. Epigenetics includes post-translational modifications of histone proteins (the core components of chromatin, see reviews by Hooker and colleagues, and Akbarian and colleagues, in this *Neuroscience* special issue), non-coding RNAs (most notably micro-RNAs), and DNA methylation. The present review will focus on DNA methylation, the epigenetic mark that received by far the most interest in the field of CM.

Overall, the plethora of adverse mental health consequences associated with the experience of CM strongly suggests the involvement of several neurotransmitter systems and brain regions. In addition, complementary human and animal studies clearly indicate that negative early life experiences affect homologous neurobiological substrates across species, potentially through similar epigenetic mechanisms. Therefore, we will discuss here both animal models and human studies of CM. In rodents, several groups have now reported convincing evidence for the crucial role of epigenetic processes in mediating maladaptive neurobiological and behavioural consequences of the early life environment and environmental adversity. In humans, studies of brain post-mortem tissues have started unraveling epigenetic alterations associated with CM, which strikingly resemble previous animal findings. Hopefully, studying CM-induced DNA methylation changes in peripheral tissues of living subjects may lead in the future to the identification of epigenetic bio-markers, with potentially major clinical implications.

2. DNA methylation: a major epigenetic actor

DNA methylation refers to the covalent addition of a methyl group in position 5' to a cytosine residue (5-mC), in particular when a cytosine is followed by a guanine (CpG dinucleotide). DNA methylation at sequences other than CpG, as well as other chemical modifications of the DNA molecule (such as 5-formyl-cytosine or 5-carboxy-cytosine), have been described (Lister et al., 2009, Yu et al., 2012, Varley et al., 2013) but will not be discussed further: their abundance in mammalian genomes is low (Ito et al., 2011), in particular in somatic cells (Ziller et al., 2011); the dynamics and functional impact of these marks only begins to be appreciated (Shen et al., 2013, Song et al., 2013), and their potential role in behavioral phenotypes such as those associated with CM has yet to be studied.

DNA methylation is controlled by a family of DNA methyl-transferase proteins (DNMT). DNMT1, DNMT3A and DNMT3B all three contribute to the maintenance of DNA methylation patterns through mitotic divisions, while DNMT3A and DNMT3B are responsible for acquisition of *de novo* methylation (Jones, 2012). Active mechanisms of DNA demethylation have long remained controversial, but are now clearly documented, notably through excision base repair processes, or the conversion to 5-hydroxyl-methyl-cytosine (5-hmC) (Kriaucionis and Heintz, 2009). As will be discussed below, both DNA methylation and demethylation mechanisms are likely recruited by early life unfavorable experiences.

Around 70–80% of CpGs are methylated in the genome (see (Hoffmann and Spengler, 2012) in this special issue, and (Jones, 2012)). This epigenetic mark globally associates with decreased transcriptional activity (Jones, 2012), and the strength of this general rule has been recently confirmed in the brain at the genome-wide level (Labonte et al., 2012a), although there are documented exceptions (such as the CRH-R2 gene, see below). Most work on DNA methylation has focused on CpG islands, which are defined (although this is debated (Illingworth and Bird, 2009)) as short, 1kb CpG-rich regions that are present in roughly half of the genes in vertebrate genomes. CpG islands are overrepresented in promoter regions, where methylation levels are very low, leaving surrounding DNA and transcription start site unwrapped and accessible for transcription. The functional implications of DNA methylation in other genomic regions (“shores” of CpG islands, gene bodies, intergenic regions) remain comparatively less understood. CpG methylation in gene bodies, in contrast with promoter regions, was initially associated with increased transcription in B-lymphocytes and fibroblasts (Ball et al., 2009, Lister et al., 2009). However, most recent data reveal a neuron-specific negative correlation between gene bodies CpG methylation states and gene expression (Guo et al., 2011a, Mellen et al., 2012), suggesting epigenetic regulatory mechanisms specific to brain tissue.

In the context of CM, most available studies correlate DNA methylation states with genes expression levels, potentially implicating several mechanisms. First, the methylation of certain CpG dinucleotides, notably in genes promoter regions, impairs the ability of regulatory proteins (such as transcription factors) to bind the DNA and to promote gene expression (see below a prototypical example with the GR gene promoter). Second, several proteins have been shown to specifically bind methylated DNA. This family of methyl

binding proteins (MBD) includes methyl-CpG binding protein 2 (MeCP2) and MBD1, 2, 3, 4 and 5. Distinct binding properties to DNA and partner proteins are progressively emerging for each MBD protein. For example, 5-mC binding by MeCP2, the most studied member of the MBD family, associates with the recruitment of numerous chromatin regulators (eg., HP1, Sin3A, ATRX, histone deacetylases, NCoR), with both repressive and activating effects on transcription (Chahrour et al., 2008, Guy et al., 2011, Lyst et al., 2013). Beyond 5-mC, the MeCP2 protein also interacts with A–T rich DNA (Baker et al., 2013), as well as 5-hmC (Mellen et al., 2012), suggesting several modes of MeCP2-dependent regulation of chromatin architecture (Shin et al., 2013). Further, it appears that MBD2 tightly interacts with both 5-mC and the nucleosome remodeling and deacetylase complex (NuRD complex, see (Baubec et al., 2013)), while MBD3, in contrast, poorly binds to 5-mC and 5-hmC (Mellen et al., 2012, Baubec et al., 2013). Third, complex interactions have been documented between DNMTs and histone modifying enzymes (histone methyltransferases in particular, see (Hoffmann and Spengler, 2012) in this special issue), suggesting cross-talks between DNA methylation and the “histone code”. One of the big challenges for future research will be to unravel the impact of CM on DNA methylation and multiple other epigenetic marks, and to integrate these findings into broad epigenetic organizational and regulatory domains (see below and (Klengel et al., 2012)).

DNA methylation was initially perceived as a stable epigenetic mark that could be reprogrammed only at specific developmental time points, or disrupted by pathological epigenetic instability (e.g., in cancer cells). In contrast, it is now clear that DNA methylation is a dynamic mechanism that can rapidly respond to environmental factors, in physiological as well as pathological processes, even in post-mitotic neuronal cells (Borrelli et al., 2008, Guo et al., 2011a). Moreover, compared to other organs, the mechanisms associated with DNA methylation appear highly active in the mammalian brain, where 5-hmC is particularly abundant (Kriaucionis and Heintz, 2009), and where MBD proteins are strongly expressed and necessary for proper brain and behavioural development (Amir et al., 1999, Zhao et al., 2003, Skene et al., 2010). From an evolutionary point of view, one might speculate that the brain, being the specialized organ for sensing internal and environmental stimuli, has evolved so as to be particularly prone to epigenetic plasticity.

Important questions remain opened in the emerging field of neurobehavioral epigenetics. First, there is a debate as to whether 5-hmC solely represents a transient state in the process of demethylation, or whether this mark associates with specific functional consequences (Guo et al., 2011b). The latter hypothesis implies that 3 epigenetic states are possible at CpGs (unmethylated cytosine, 5-mC or 5-hmC), thus increasing the encoding capacities of DNA. Interestingly, MeCP2 has been shown recently to bind both 5-mC and 5-hmC, and the later critically depends on a specific amino-acid, R133 (Mellen et al., 2012). While 5-mC associates with decreased transcriptional activity, 5-hmC associates with the opposite effect. How MeCP2 distinguishes these 2 covalent modifications of the DNA to recruit divergent molecular pathways with opposed effects on transcription is currently unknown, in particular on a structural basis. Second, the extremely high diversity of cell types encountered in the brain may well associate with a similar diversity in epigenomes. Epigenetic mechanisms controlling transcriptional activity may be cell-type, and even gene-specific (Mellen et al., 2012). Developing new methodologies (such as the visualization of histone modifications at

single genomic loci with single-cell resolution (see (Gomez et al., 2013)) will be required to assess epigenetic heterogeneity across brain regions, cell types and genomic loci. As discussed below, few labs have addressed this issue by applying fluorescence-assisted cell sorting to human post-mortem tissues, notably in the study of CM (Labonte et al., 2012a, Shulha et al., 2012, Labonte et al., 2013). Third, post-translational modifications at MBD proteins, as a function of physiological and pathological processes, may have profound effects on their binding properties, the genome's expression and brain function (Ebert et al., 2013). Unraveling these mechanisms may have important therapeutic implications in the future.

3. Animal models of early life parental and social environments

Here, we provide an overview of the rapidly growing body of literature addressing the relation between early life environment and behavioral phenotypes in adulthood, focusing on animal studies that directly document epigenetic mechanisms. Other studies and models are only briefly mentioned to show that early life adversity widely affects the brain, suggesting numerous avenues for future epigenetic studies.

The animal models used to investigate the impact of environment on behavior implicitly refer to the old concept of biological embedding (Hertzman, 1999), whereby early life experiences and environmental factors, together with individual factors, determine developmental trajectories and lifespan health outcomes. As summarized recently (Hertzman, 2012), 4 characteristics define biological embedding: life experience must translate into neurobiological adaptations; these adaptations are variable according to the intensity or the quality of early life experiences; such variable effects must be stable and long-term; and these effects must influence behavioural patterns or mental health outcomes over the lifetime. Epigenetic mechanisms obviously represent only one step in the complex brain adaptations to CM, that also likely include structural and functional changes in synaptic plasticity and connectivity patterns, among others.

Maternal care

Maternal care in rodents, mostly studied in rats, has been investigated by easily quantifiable behaviours (licking and grooming, LG, and arch back-nursing) that are characterized by high inter-individual variability, even in laboratory animals. In the pioneering model developed by Meaney and colleagues, maternal behaviours are observed daily (60 min a day) during the first 6 to 10 days of life, defining high- and low-LG mothers (Liu et al., 1997). Consistent with the requirements of biological embedding, these variations in maternal care have strong effects on endocrine and behavioural responses: offspring from high-LG mothers show in adulthood a blunted hypothalamic-pituitary-adrenal (HPA) axis reactivity to stressful experiences (such as restraint) (Meaney et al., 1996, Liu et al., 1997) and decreased anxiety-like behaviours (in the open-field and novelty-suppressed feeding tests, (Caldji et al., 1998)).

Maternal care effects on the hippocampus (and the dentate gyrus in particular) have been studied extensively in this paradigm, uncovering several epigenetic adaptations, most notably in the glucocorticoid receptor (GR) gene (Weaver et al., 2004). Compared to the offspring of low-LG mothers, the offspring of high-LG mothers show increased

hippocampal GR expression, which accounts for a strengthened negative feedback onto the HPA axis and decreased reactivity to stress. Importantly, maternal LG correlates with an epigenetic modification of a neuron-specific exon 1₇ glucocorticoid receptor promoter (Weaver et al., 2004): increased maternal care associates in high-LG offspring with decreased methylation levels in this promoter region, and increased hippocampal GR expression. Critical to the hippocampal GR expression level is a particular CpG site where the transcription factor NGFI-A binds, and this site is highly differentially methylated between offspring of high- and low-LG mothers (Weaver et al., 2004). These important data reveal a critical pathophysiological pathway, whereby a change in DNA methylation induced by early life experience at a specific genomic site impacts on subsequent binding of transcription regulatory proteins. An analogy can be drawn between naturally occurring variations in maternal rodent behaviours and maternal care in humans (Hane and Fox, 2006). Most importantly, the same epigenetic mechanism at the GR gene has been documented in the human hippocampus as a function of CM (see below).

Maternal care also affects synaptic plasticity in the hippocampus. Compared with offspring from low-LG mothers, offspring from high-LG mothers show increased hippocampal long-term potentiation (Bagot et al., 2009, Bagot et al., 2012a) and depression (Bagot et al., 2012b). These 2 proposed cellular substrates of learning and memory processes critically rely on glutamatergic transmission. Accordingly, the complex organization of glutamate signaling highly depend on the quality of maternal care, as revealed by increased expression of ionotropic (NMDA GluN2A, GluN2B and GluN1, see (Bagot et al., 2012a)) and metabotropic (mGluR1, see (Bagot et al., 2012b)) glutamate receptor sub-units in low- and high-LG offspring, respectively. As for the GR gene, DNA methylation changes account for some of these regulations: increased mGluR1 expression in high-LG offspring associated with decreased DNA methylation in the mGluR1 gene (as well as an increase in histone post-translational modifications classically responsible for increased transcriptional activity). Whether the increased expression of ionotropic sub-units in low-LG offspring similarly relies on decreased DNA methylation has not been assessed, to our knowledge. Finally, in addition to the excitatory transmission, maternal care also epigenetically programs inhibitory GABAergic transmission in the hippocampus (Zhang et al., 2010). DNA methylation was increased in low-LG offspring in the promoter region of the GAD1 gene, the rate-limiting enzyme of GABA synthesis, which again led to decreased occupancy by NGFI-A and decreased gene expression. The behavioural significance of decreased hippocampal GABAergic transmission following early-life adversity remains to be investigated. Altogether, these series of groundbreaking studies reveal that maternal care widely reprograms the hippocampal epigenome, with changes in both DNA methylation and histone post-translational modifications.

Another major feature of this model is the transmission of maternal behaviours across generations, so that female offspring from high-LG mothers also show increased maternal behaviours when adult (Francis et al., 1999). This trans-generational transmission is independent of genetic factors, as evidenced by cross-fostering studies: the biological offspring of Low-LG mothers reared by High-LG mothers resembled the normal offspring of High LG-ABN mothers. The trans-generational transmission also associates in the high-LG lineage with increased expression and function of estrogen and oxytocin receptors in the

medial preoptic area of the hypothalamus (Champagne et al., 2001). Furthermore, maternal behaviours are not fixed throughout life: in high-LG mothers, gestational stress decreases licking and grooming behaviours to levels similar to low-LG mothers (Champagne and Meaney, 2006). Importantly, such gestational stress not only decreased maternal care, but also reversed, back to low-LG mothers' levels, the increased expression of oxytocin receptors in several brain regions (BNST, MPO, central amygdala). These data strongly support the regulation of hypothalamic hormonal signaling (oxytocin, estrogen) by maternal care and, similar to GR data, DNA methylation changes have been reported for these genes. In the MPO, the alpha subtype of the estrogen receptor gene's promoter show increased DNA methylation in low- compared to high-LG offspring, along with decreased binding of the transcriptional activator Stat5b (Champagne et al., 2006).

These results reveal that complex phenotypic traits and associated epigenetic marks can be transmitted together over successive generations. Most recent data suggest that such non-genetic inheritance may rely on epigenetic factors (such as micro-RNA in the germ-line, see (Rodgers et al., 2013)) or behavioural transmission (see below). The relative contributions of these 2 mechanisms remain to be addressed in the rat model of maternal care.

In summary, poor rat maternal care has been associated so far with a disrupted pattern of DNA methylation in several genes across 2 brain regions: the hippocampus and the hypothalamus (see Figure 1). Many other neuromodulatory systems are likely impacted by natural variations in maternal behaviors. Within this line, hippocampal transcriptomes show differences for hundreds of transcripts between low- and high-LG offsprings (Weaver et al., 2006). Expression of DNMT1, one of the enzymes responsible for DNA methylation maintenance, is increased in the hippocampus of low-LG offspring (Zhang et al., 2010), suggesting that levels of maternal care may affect DNA methylation at numerous genomic sites. Accordingly, recent evidence indicate that maternal care affects DNA methylation across wide genomic regions, with clustered regions of epigenetic changes (McGowan et al., 2011). Future studies will have to determine how complex life experiences translate into broad, yet spatially restricted and specific epigenetic reprogramming. Beyond the hippocampus, high-LG offspring, compared with low-LG offspring, show (i) increased inhibitory GABA_A receptors in locus coeruleus, medial prefrontal cortex, basolateral and central amygdala; (ii) increased α 2-adrenoreceptor in the locus coeruleus; (iii) decreased CRH-receptor in the locus coeruleus and nucleus of the tractus solitarius (Caldji et al., 1998, Caldji et al., 2003). Whether epigenetic mechanisms also mediate these brain-wide molecular adaptations remains to be explored.

Similar naturally occurring variations in maternal behaviours have been comparatively poorly studied in mice. How these behaviours, and epigenetic programming in the offspring, may be affected by genetic manipulations is a promising avenue for future research in this species. For example, KO mice for DNMTs or MBD proteins (Martin Caballero et al., 2009) are available, and KO mice for the alpha subtype of the estrogen receptor show significant impairments in maternal care and often display "infanticide" behaviors (Ogawa et al., 1998).

Roth et al recently developed another model of maternal care, trying to directly trigger maternal abusive behaviors. In this model, pups are repeatedly exposed (30mn per day, post-

natal days 1 to 7) to stressed, non-biological mothers (Roth and Sullivan, 2005, Roth et al., 2009). Upon limited stress (short habituation to the pup exposure chamber, limited clean aspen shavings), rat mothers express various abusive behaviours (stepping, dropping, dragging, pushing away/actively avoiding, and rough handling), which induce intense pup vocalizations. Maternal abusive behaviours increased DNA methylation of the BDNF gene in the PFC, and consistently decreased BDNF expression (Roth et al., 2009). Both were reversed by chronic intra-cerebro-ventricular treatment with zebularine, a DNMT inhibitor. Considering the wealth of data on the role of BDNF in anxiety and depression in both animal models and human (Castren and Rantamaki, 2010), epigenetic dysregulation of this neurotrophic factor is likely to affect emotional maturation. Future studies should explore whether epigenetic adaptations previously demonstrated as a function of natural variations in maternal care also occur in this model of abusive maternal behaviors. Such studies would have the potential (i) to uncover epigenetic adaptations specific to various forms of early life experiences, and (ii) to reproduce behavioural and molecular findings across different laboratories, an important step in the validation of animal models of complex human traits. It is important to note that “epigenetic” causality is extremely challenging to demonstrate, as we currently have no experimental tool to specifically manipulate epigenetic marks at precise genomic locations. It is possible that the combined effects of widespread aberrant methylation patterns induced by early life adversity at multiple brain sites, as summarized here, are required to mediate maladaptive behavioural responses into adulthood.

Maternal deprivation

Early separation (separation from the mother and littermates) and maternal deprivation (separation from the mother, but not from littermates) represent additional animal models of early life adversity that have been extensively studied in rodents. Results have revealed the implication of numerous brain systems (Pryce and Feldon, 2003, Champagne and Curley, 2009), such as the HPA axis, in a clear overlap with aforementioned studies of maternal care and behaviours. The activity of the HPA axis, and ACTH release from the pituitary gland, are controlled centrally by various hypothalamic peptides, including CRH and AVP in the hypothalamic paraventricular nucleus, PVN. In mice, maternal deprivation (placement in a cleaned cage, devoid of maternal odor, during 3 h each day from post-natal days 1 to 10) produces increased stress-induced corticosteroid secretion, increased despair-like behavior and a memory deficit (Murgatroyd et al., 2009). These deficits are mediated notably through AVP signaling, as they were partly reversed by systemic treatment with the AVP V1b receptor antagonist. Interestingly, maternal separation triggers successive epigenetic adaptations at the AVP genomic locus (Murgatroyd et al., 2009). In 10 days-old mice, immediately after the stressful experience of separation, increased neuronal activation in the PVN promoted MeCP2 phosphorylation. As a result of this post-translational modification, MeCP2 occupancy of an enhancer element located in the AVP 3'UTR was decreased, leading to increased AVP expression. Later on, in 6-week-old mice, hypomethylation of the AVP promoter was detected, accounting for sustained, increased AVP expression. These elegant experiments strongly suggest that early-life adversity may trigger distinct epigenetic processes at various genomic sites (e.g., the 3'UTR enhancer and promoter region in the AVP gene) to maintain aberrant genomic expression (e.g., increased AVP expression) and phenotypic traits.

To further potentiate the effects of early life experiences, Franklin et al recently combined maternal separation with maternal stress during weaning. The study of long-term effects of maternal stress is justified by the recognition that in humans, mothers suffering from stress-related psychopathology expose their progeny to an increased risk of negative mental health outcomes in adulthood (Lupien et al., 2000, Essex et al., 2002, Halligan et al., 2007). In this paradigm, « unpredictable maternal separation combined with unpredictable maternal stress » (MSUS) is applied from postnatal day 1 to 14 (Franklin et al., 2010), and behavioural consequences are assessed in the offspring of stressed mothers (F1), as well as in next generations of adult mice: F2, which did not experience MSUS, and F3, whose germ-line has not been exposed to MSUS. In this model, increased despair-like behavior and anhedonia were observed in F1 male, as well as increased despair in F2 females, and F3 males. Trying to dig into these intriguing sex switches across successive generations, the authors characterized DNA methylation changes induced by MSUS in the germ cells of F1 and F2 male mice, and in the cortex of F2 female mice. In F1 males' sperm, increased DNA methylation was found in CpG islands of MeCP2 and CB1 (the main endocannabinoid receptor in the brain) genes, while a decrease was observed for the CRH receptor-2 (CRH-R2) gene. In F2 females, the same epigenetic pattern was found in the cortex that correlated with decreased expression of MeCP2 and CB1. Interestingly, cortical expression of CRH-R2 was also decreased (contrasting with increased DNA methylation), indicating that functional consequences of DNA methylation may vary across genomic locations. Of note is that a similar trans-generational transmission of behavioural deficits has been recently documented in the chronic social defeat paradigm, applied to adult mice (Dietz et al., 2011). To test the contribution of epigenetic adaptations in the germ-line to trans-generational inheritance, *in vitro* fertilization (IVF) was used to inseminate female mice with sperm from stressed mice and controls. As acknowledged by the authors, the epigenetic impact of the IVF procedure, and its potential behavioural consequences, are unknown. Nevertheless, results indicated that only a small subset of the inherited behavioural phenotype was recapitulated by IVF. In this model therefore, in contrast with the MSUS, epigenetic adaptations in the germ-line seem to poorly contribute to trans-generational transmission, which may mostly implicate behavioural transmission during mating (reminiscent of the aforementioned behavioural transmission in the rat model of maternal care). Early life (e.g. MSUS), but not adulthood (e.g. social defeat), may represent a sensitive period for epigenetic changes to occur in the germ-line and to be transmitted in the offspring. The interested reader will find another very recent report of the perpetuation of phenotypic traits through epigenetic changes in the germ-line in the addiction field (Vassoler et al., 2013). In the context of early life adversity, future studies are required to assess the relative contributions of epigenetic adaptations caused by behavioural interactions, or transmitted through the germ-line, in the trans-generational perpetuation of phenotypic traits.

Finally, several comments arise from the comparison of mice and rat models. First, early life experience may bi-directionally regulate DNA methylation, as both decreases (AVP, CRH-R2) and increases (GR, GAD1 and the alpha subtype estrogen receptor, see above) in methylation levels have now been documented. Results from human studies also suggest cell-type specific epigenetic adaptations (see below). Therefore, systemic treatment with compounds globally increasing or decreasing DNA methylation levels (such as DNMT

inhibitors) may have limited therapeutic potential, as they could (i) prevent only a subset of CM-induced adaptations, while potentially worsening the others, and (ii) have deleterious effects on cell types unaffected by CM. Second, at least 2 epigenetic mechanisms may mediate a persistent hyper-reactivity of the HPA axis following early life stress, with increased AVP expression in the hypothalamus and decreased hippocampal GR-dependent negative feedback. Third, it is likely that epigenetic regulatory mechanisms differ across mammalian species, or across genetic backgrounds, and according to the type of experimental setting considered. Within this line, a recent study used a slightly different model of maternal deprivation (the mother was removed from the litter on postnatal day 9 for 24 h) to show strain- and sex-specific epigenetic alterations, notably in GR and AVP genes (Kember et al., 2012). In humans, recent data start to unravel similar interactions between single nucleotide polymorphisms and DNA methylation in relation to life experiences (see (Oertel et al., 2012) for an example on addiction, and below for an example directly relevant to CM).

Other animal models of early social life

Over the last decade, numerous additional rodent paradigms, summarized elsewhere (Pryce and Feldon, 2003, Kentner et al., 2010), have been developed to assess the biological embedding of early life experiences: early weaning (George et al., 2010); communal nesting (Curley et al., 2009); paternal (Seidel et al., 2011) or bi-parental (Becker et al., 2007, Braun et al., 2011, Braun et al., 2012) deprivation in the bi-parental rodent *Octodon degus*; and even interactions between paternal social experiences and maternal behaviours (Mashoodh et al., 2012). In primates also, adverse early social life associates with strong, life-long consequences in the brain (Stevens et al., 2009). The large numbers of cerebral adaptations that have been identified in these models provide important avenues for further analyses of epigenetic consequences of early life adversity.

Nevertheless, it is necessary to acknowledge that rodent models of CM are limited. Notably, these models may present face validity when modeling parental neglect and physical abuse, while psychological and sexual abuses obviously remain beyond their reach. However, the link between CM and negative mental health outcome is widely thought to be the result of the associated psychological trauma, rather than a specific type of abuse. This is consistent with the fact that there seems to be no or very little specific effect of type of maltreatment on negative mental health outcomes (Gilbert et al., 2009). Appreciating whether various forms of maltreatment may recruit specific epigenetic mechanisms, potentially depending on their severity, is a difficult task that is just starting in animal models and in human (see below).

In addition, animal studies reveal profound, comparable effects of parental care across a variety of mammalian species, suggesting that some of these changes, and the underlying epigenetic underpinnings, may be conserved across phylogeny. Therefore, available evidence indicates that the study of CM in human is likely to benefit from the use and the on-going development of appropriate animal models, stressing the need for increased translational research efforts. Future avenues may include the use of rodent models of depression (chronic social defeat, unpredictable chronic mild stress) to the study of parental psychopathology as a form of early life adversity. Finally, while this review focuses on post-

natal forms of stress, a vast literature has documented the effects of prenatal stress in animals and humans, notably implicating DNA methylation (see below relevant data for the GR, and (Weinstock, 2008)). Socioeconomic factors are also under intense investigations (Lam et al., 2012, Tehranifar et al., 2013). To get an integrated view of the biological embedding of perinatal experiences, the later data are important, given the loosely defined correspondence between pre- and post-natal developmental periods across the phylogeny and across brain regions (Heim and Binder, 2012).

4. Human studies of child abuse

Studies using post-mortem brain tissues

Building on (i) the animal findings suggesting hippocampal GR expression and DNA methylation changes associated with early-life environmental variation, and (ii) the association between dysfunction of the HPA axis and CM in humans (Heim et al., 2000, Frodl and O'Keane, 2013), our group provided the first evidence for an effect of early-life adversity on the human epigenome (McGowan et al., 2009). At the transcriptomic level, expression of the GR gene was decreased in suicide completers with a history of CM compared to non-abused suicide completers. In the latter group, the level of expression of the GR gene was similar to the control group, composed of subjects with no history of CM, and who died of sudden death. Further, we examined DNA methylation in the exon 1_F of the GR gene, the human homologue to the rat exon 1₇. Increased methylation was detected in abused suicide completers at 2 discrete CpG sites in the promoter region of the exon 1_F. Additional *in vitro* experiments revealed that the genomic locus where differential methylation associated with CM was observed had promoter activity that was potentiated by NGFI-A binding and blocked by patch DNA methylation. Therefore, CM associates with increased DNA methylation at specific sites in the GR promoter, thereby limiting NGFI-A binding and decreasing GR expression in the hippocampus. Interestingly, in non-abused suicide completers, although expression of the GR gene was similar to controls, increased DNA methylation was also detectable at 3 CpG sites nearby those associated with CM (see Fig. 2). The relevance of such transcriptionally silent methylation changes remains unknown; however, it is tempting to speculate that they may mediate aberrant “epigenetic meta-plasticity” (see below) in the regulation of the HPA axis that could potentially contribute to psychopathology and suicide independently from a history of CM.

This work was subsequently expanded to other transcripts of the GR, focusing on those that are highly expressed in the hippocampus: 1_B, 1_C, and 1_H (Labonte et al., 2012b). Similar to the GR exon 1_F, the expression of all 3 variants was decreased in suicide completers with a history of CM compared to both the control group and to suicide completers with no history of CM. Therefore, decreased GR expression and increased HPA axis reactivity following CM likely stem from the combined epigenetic repression of a series of GR transcripts. Interestingly, the pattern of CM-induced DNA methylation changes appeared more complex than previously described for the exon 1_F (see Figure 2). For exons 1_B and 1_C promoters, increased methylation was measured at specific CpG sites and shown to negatively correlate with the expression of the corresponding GR transcript, in accordance with the 1_F model. Surprisingly, however, decreased methylation was detected at other CpG sites for both 1_B

and 1_C, while decreased levels of DNA methylation were measured for 1_H, at the level of individual CpGs sites and of the whole promoter region. Whether these bi-directional changes in DNA methylation levels reflect cell-type specific epigenetic response to CM in the hippocampus, or CpG site-specific effects of methylation on transcriptional activity, remains to be investigated. In sharp contrast to these widespread effects on the hippocampus, no change in DNA methylation or in GR expression was detectable in the cingulate cortex, another important site for the regulation of the HPA axis, revealing brain region-specific epigenetic adaptation to CM. Finally, this study also documented increased DNA methylation at specific CpG sites of exons 1_B, 1_C and 1_F promoters in non-abused suicide completers, while expression of these transcripts was unchanged. Again, the functional impact of these silent methylation changes, if any, remains to be determined for the regulation of the HPA axis.

These results at the GR locus, combined with previous rat data, suggest that early-life experiences regulate life-long stress reactivity through similar epigenetic processes across species. The analysis of a 6.5 million base-pairs region centered on this genomic region further strengthened this notion (Suderman et al., 2012). Less favorable early-life environmental experiences associated with hundreds of parallel DNA methylation changes in rats and humans. These adaptations preferentially affected promoters, as evidenced in the cluster of protocadherin genes, and followed a non-random, discontinuous pattern across large genomic regions. In subsequent studies, we used more recent technologies to test the hypothesis that CM-induced epigenetic changes may not be restricted to this large, GR-centered locus, but may instead more widely affect the genome. We immuno-precipitated and quantified methylated DNA from promoter regions at the genome-wide level using MeDIP-chip (Labonte et al., 2012a). This study identified 248 hypermethylated and 114 hypomethylated promoters in suicide completers with a history of CM compared with controls, suggesting that CM widely reprograms the epigenome. Further, using an antibody against the neuron-specific Neu-N protein and fluorescence-assisted cell sorting to separate neuronal and non-neuronal cell fractions, we found that most significant DNA methylation changes mostly occurred in neurons, and were observed in genes implicated in neuronal plasticity, such as ALS2, NR1D1 and HIST2H2AB. While such analysis are challenging, these results, along with similar data on other psychiatric conditions (Schwarz et al., 2011, Shulha et al., 2012), underline the necessity to assess epigenetic adaptations at the cell-type level (Akbarian and Halene, 2013). Finally, CM also associated with increased hippocampal methylation in the promoter region of the small (40S) ribosomal sub-unit, leading again to a decreased transcriptional activity (McGowan et al., 2008). While rRNA is an essential part of the protein synthesis machinery, this finding further strengthens the notion that CM is likely to profoundly disrupt hippocampal physiology, and to affect both transcription and translation.

Studies using peripheral tissues

Following results on the role of early-life experiences in the epigenetic regulation of the hippocampal GR, several groups have recently investigated DNA methylation levels of the GR gene in peripheral samples of individuals with histories of CM or with related phenotypes. Perroud et al reported a study looking at the effects of various forms of CM on

the GR DNA methylation state from circulating leukocytes. This analysis was conducted in a large population of subjects diagnosed with borderline personality disorder, depression or post-traumatic stress disorders, all of which have been previously associated with histories of CM (Perroud et al., 2011). The authors showed that childhood sexual abuse was associated with increased GR promoter DNA methylation in the peripheral blood. In addition, the severity of child abuse, corresponding in this study to the number of different types of CM that each subject was exposed to (sexual abuse, physical abuse, emotional abuse and physical and emotional neglect, as defined by the Childhood Trauma Questionnaire) correlated positively with methylation levels. In another study, Tyrka et al investigated the DNA methylation status of the GR gene in circulating leukocytes from a cohort of adults with a history of CM (Tyrka et al., 2012). While the authors chose to focus on subjects with no psychiatric diagnosis, arguing that this design allowed to isolate the effects of CM from those associated with psychopathology, they found increased methylation of the GR gene in peripheral samples from subjects with histories of CM. Increased levels of GR methylation also associated with decreased sensitivity to dexamethasone suppression test, a marker of HPA axis hypersensitivity, suggesting a functional relationship between peripheral GR methylation and HPA axis activity. While it is unclear if these findings reflect direct or proxy relationships, they are consistent with results observed in human postmortem and animal studies (Weaver et al., 2004, McGowan et al., 2009).

Beyond these studies of post-natal stressful experiences, the epigenetic state of the GR was also evaluated in relation to maternal prenatal exposure to extreme adversity or altered maternal emotional states. Three-month old newborns exposed to depressed or anxious mothers during the third trimester of pregnancy (Oberlander et al., 2008) exhibited increased GR DNA methylation (as measured in cord blood) in the promoter region of the exon 1_F, as well as increased salivary cortisol response to a non-noxious stressor (i.e. the presentation of a novel visual stimulus). Interestingly, GR DNA methylation measured in the mother's blood did not correlate with maternal mood, suggesting that fetal blood cells are particularly prone to epigenetic adaptation at the GR locus. These results were reproduced recently for the exon 1_F by another group, who also reported increases in DNA methylation of exons 1_B and 1_D in the cord blood mononuclear cells of newborns exposed to anxious mothers (Hompes et al., 2013). Similar to brain data, epigenetic regulation of the GR gene in the periphery likely implicate several genomic sites. Another study focused on 10–19 year-old children and on their mothers assessed retrospectively for a history of intimate partner violence (Radtke et al., 2011). Results indicated that such violence, when experienced by the mother during, but not before or after pregnancy, associates in the children with increased DNA methylation in the promoter region of the exon 1_F, as measured in whole blood cells. Concordant findings were recently reported (Mulligan et al., 2012) in newborns from women exposed to extreme events (war, rape), further suggesting that unfavorable prenatal stressors also regulate the methylation status of the GR gene. Finally, GR DNA methylation was recently assessed in the placenta, a key regulator of intra-uterine development (Bromer et al., 2012). Significant correlations were found between DNA methylation and neurobehavioural measures assessed at birth in healthy infants. While this study did not focus on any specific stress exposure or psychopathological antecedent, the results nevertheless suggests that GR may also function

in the placenta to regulate early neurodevelopment, with potential relevance for mental health outcomes. Altogether, these series of studies performed in very different populations, suggest that the methylation state of the GR promoter may serve as a robust marker of the regulation of the HPA axis by the early-life environment. It remains unclear how early-life adversity impacts the GR methylation state in some (hippocampus, blood leukocytes (McGowan et al., 2009, Perroud et al., 2011, Labonte et al., 2012b, Tyrka et al., 2012)), but not all (cingulate cortex (Labonte et al., 2012b)), tissues.

The activity of the HPA axis is also controlled by mechanisms other than hypothalamic peptides (AVP and CRH) and negative feedbacks (GR). At the intra-cellular level, FKBP5 is a chaperone protein that inhibits GR ligand binding and translocation of the GR-ligand complex to the nucleus. Several glucocorticoid response elements are present at the FKBP5 gene, so that expression of FKBP5 is stimulated by glucocorticoids, and represents an intracellular short negative feedback loop. Independent groups have reported that genetic polymorphisms in FKBP5 (including haplotypes rs1360780, rs9296158, rs3800373 and rs9470080) interact with a history of CM to predict post-traumatic stress disorder (PTSD), suicide attempts and depression in adulthood (Binder et al., 2008, Roy et al., 2010, Appel et al., 2011). Recently, an epigenetic mechanism was described at a glucocorticoid response element in the FKBP5 intron 2 (rs1360780), which may account for such an interaction (Klengel et al., 2012). Accordingly, *in vitro* experiments suggested that both baseline and corticosteroid-induced expression of FKBP5 are increased in carriers of the risk allele (rs1360780 A) compared to carriers of the protective allele (rs1360780 G). Using an elegant chromatin conformation capture approach, the authors were able to demonstrate that the risk allele, which creates a new TATAA box distant from the FKBP5 transcription start site (TSS), potentiates and favors the recruitment of the RNA polymerase II through a tridimensional interaction between intron 2, intron 7 and the TSS. Noteworthy, decreased DNA methylation levels were measured in the intron 7 of the gene as a function of CM (in peripheral blood), and likely resulted from this transcriptionally active conformation. Therefore, in risk carriers, FKBP5 negative feedback onto GR activity is potentiated, impairing the ability of glucocorticoids to retro-control the HPA axis, and ultimately potentiating the effects of CM-associated stress. These interesting data emphasize the need to combine analysis of DNA methylation and other measures of chromatin structure across large genomic regions to decipher complex, long-range epigenetic interactions.

In another attempt to translate animal model findings to humans, Perroud et al explored the methylation state of the BDNF gene in peripheral blood leukocytes from borderline personality patients (Perroud et al., 2013). Results indicated that, reminiscent of rat findings from the model of maternal abusive behaviours (Roth et al., 2009), peripheral levels of DNA methylation in the BDNF promoter increased as a function of the number of childhood trauma in this population of patients. Of note, this study found no correlation between DNA methylation state and expression level of BDNF in the plasma, again. The serotonin system has also been investigated for early-life adversity-induced epigenetic regulation. Available studies, in a primate model and in humans, focused on the serotonin transporter (5-HTT), a gene previously implicated in the interaction between life stress events and the risk of depression (Caspi et al., 2003). In peripheral blood mononuclear cells from Rhesus macaques (Capitanio et al., 2005), an interaction was reported between early attachment

pattern and the methylation state of the 5-HTT promoter, whereby increased methylation in this genomic region associated with increased reactivity to stress in maternally deprived, but not in mother reared, infants. In the human study, EBV transformed lymphoblast cell lines derived from subjects recruited through the Iowa Adoption Study were used (Beach et al., 2010). A significant association emerged in that sample between sex abuse alone and overall DNA methylation of the CpG island at the promoter region of the 5HTT gene, among females. Building on this work, the authors explored another, non-overlapping cohort of female subjects (Beach et al., 2011). In this larger cohort, CM still associated with increased DNA methylation in the promoter of 5-HTT. Further, epigenetic patterns in this gene also associated with the emergence of antisocial personality disorder in adulthood. While methylation studies in DNA obtained from EBV transformed lymphoblast cell lines should be interpreted with caution, in subsequent studies the same group of investigators reported that parental history of psychopathology interacts with CM to predict the intensity of 5-HTT DNA methylation changes, and the risk of antisocial personality disorder (Beach et al., 2013). Finally, they also explored DNA methylation across other, non-promoter regions of the 5-HTT gene, and reported 2 additional sites of differential methylation as a result of CM (Vijayendran et al., 2012), although expression of the 5-HTT was unaffected. Results from these series of peripheral studies focusing on candidate gene are encouraging, and clearly suggest that epigenetic marks may serve as bio-markers of early stressful life experiences, and the risk of psychopathology. In the future, more research will be needed to assess the specificity and predictivity of epigenetic changes associated with CM in peripheral tissues.

Finally, the first genome-wide studies of DNA methylation changes in peripheral tissues following CM and related adverse experiences were recently reported. A first study tested correlations between DNA methylation in blood with histories of CM, PTSD and total life stress exposure (Smith et al., 2011). While differential methylation in 5 and 1 genes associated with PTSD and total life stress, respectively, no significant finding was reported for CM. In children exposed to institutional placement, compared to children raised by their biological parents (Naumova et al., 2012), more than 800 differentially methylated genes (among which 90% are hypermethylated) were detected, including genes implicated in cellular signaling, immune responses and brain function. In saliva samples from children having experienced severe enough CM to warrant out-of-home placement (Yang et al., 2013), more than 2600 CpG sites with differential methylation levels were reported in abused children compared to a control group of children that had no experience of maltreatment, and no psychiatric illness. In line with previous results in the brain, these studies suggest that the experience of CM, and related early stressful experiences, have profound, genome-wide epigenetic consequences in peripheral tissues. In the future, assessing epigenetic plasticity in both central and peripheral tissues of the same individuals, through similar technologies, will be required to explore their potential relationships. A recent study addressed this daunting task in relation to CM, and was able to provide evidence for some small overlap between epigenetic changes in the prefrontal cortex and circulating lymphocytes of rhesus monkeys (Provencal et al., 2012).

Specific challenges for human studies

Although recent technological breakthroughs now enable the characterization of epigenetic marks at genome-wide levels with single base-pair resolution, there are many challenges to the investigation of human epigenetic processes associated with CM. Chief among them is the necessary retrospective nature of these studies (although validated and robust instruments have been developed, such as the Childhood Experience of Care & Abuse, CECA questionnaire (Brewin et al., 1993, Roy and Perry, 2004)), and the impossibility of directly investigating CNS tissue in living subjects. Another difficulty arises from the plasticity of the brain epigenome. Animal studies revealed that experimental manipulations trigger complex, dynamic and sometimes rapid changes in the relation between DNA methylation levels and transcriptional activity (Sweatt, 2009, Guo et al., 2011a). As such, some authors have proposed the concept of epigenetic metaplasticity (Baker-Andresen et al., 2013), whereby “*reprogramming of gene expression may be encoded in the genome (i.e., through DNA methylation changes) and reflected in changes in gene expression only when required, such as during neuronal reactivation*”. Such dynamics remain out of reach of human studies using postmortem brain tissues, which capture a snapshot of epigenetic and transcriptomic states. New tools are being developed to visualize epigenetic processes in vivo, such as PET ligands for histone demethylase and deacetylase (see (Hooker et al., 2010, Neelamegam et al., 2012) and the review by Hooker and colleagues in this special issue).

In addition, studies of peripheral samples have the potential to start addressing these questions, while they simultaneously raise intriguing questions. Prospective studies of children who have been documented for a history of CM (using self-reported measures (Roy and Perry, 2004) and instruments to assess the risk of maltreatment (Begle et al., 2010)), combined with peripheral epigenetic measures, should prove instrumental in strengthening the relationships between early-life adversity, epigenetic changes, and mental health outcome. On the other hand, despite the extreme diversity of epigenetic organization across cell-types and genomic loci within the brain, available data suggest that cerebral CM-induced epigenetic adaptations may reflect, at least in part, into similar changes in peripheral tissues. Whether such changes may share some relationships, and through which molecular mechanisms, remains fully unknown.

Finally, another challenge will be to understand interactions between genetic variability, notably single nucleotide polymorphisms, and specific epigenetic marks. In particular, DNA methylation appears to be strongly determined by the DNA sequence (Kerkel et al., 2008, Schalkwyk et al., 2010, Gertz et al., 2011, Berlivet et al., 2012), and we presented above an example of this issue related to CM (see below and (Klengel et al., 2012)). Finally, the biggest challenge, in CM as well as for other phenotypes (Heyn and Esteller, 2012), will be to use DNA methylation in the diagnosis, prediction of treatment response (Lopez et al., 2012, Perroud et al., 2013), or for therapeutic innovation.

In conclusion, we summarized in the present review our current knowledge on epigenetic mechanisms mediating lifelong consequences of early life adversity. As the understanding of fundamental epigenetic mechanisms is rapidly progressing, future studies will likely provide deeper insight on the crucial role of DNA methylation, in combination with other epigenetic marks and genomic architecture, at the interface of genes and the environment.

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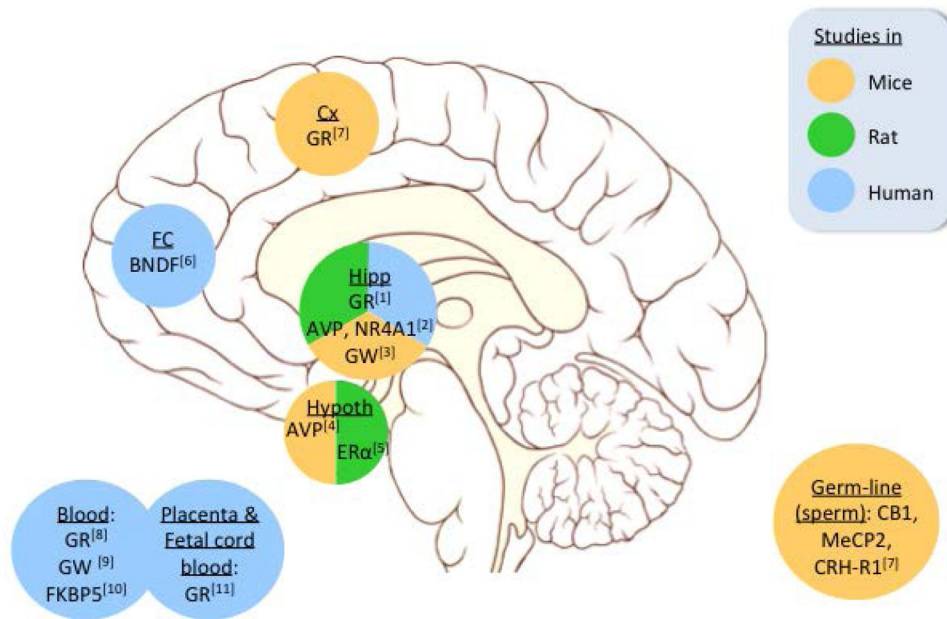


Figure 1. Early-life adversity triggers DNA methylation changes in several brain regions, as well as in peripheral tissues, across multiple genomic loci and mammalian species: rat, mouse and human

Numbers indicate corresponding references. BDNF, Brain-derived neurotrophic factor; FC, frontal cortex; Hipp, hippocampus; Hypoth, hypothalamus; Cx, unspecified cortical region; GR, glucocorticoid receptor; GW, CM induced genome-wide adaptations. Numbers in brackets indicate the following references: [1], Weaver et al, 2004; McGowan et al, 2009; Kemner et al, 2012, LaBonte et al, 2012b; [2] Kemner et al, 2012; [3], LaBonte et al, 2012a; [4] Murgatroyd et al, 2009; [5], Champagne et al, 2006; [6] Roth et al, 2009; [7], Franklin et al, 2010; [8], Perroud et al, 2011, Radtke et al, 2011, Tyrka et al, 2012; [9], Smith et al, 2011, Naumova et al, 2012, Yang et al, 2013; [10], Klengel et al, 2013; [11], Oberlander et al, 2008, Bromer et al, 2012, Hompes et al, 2013.

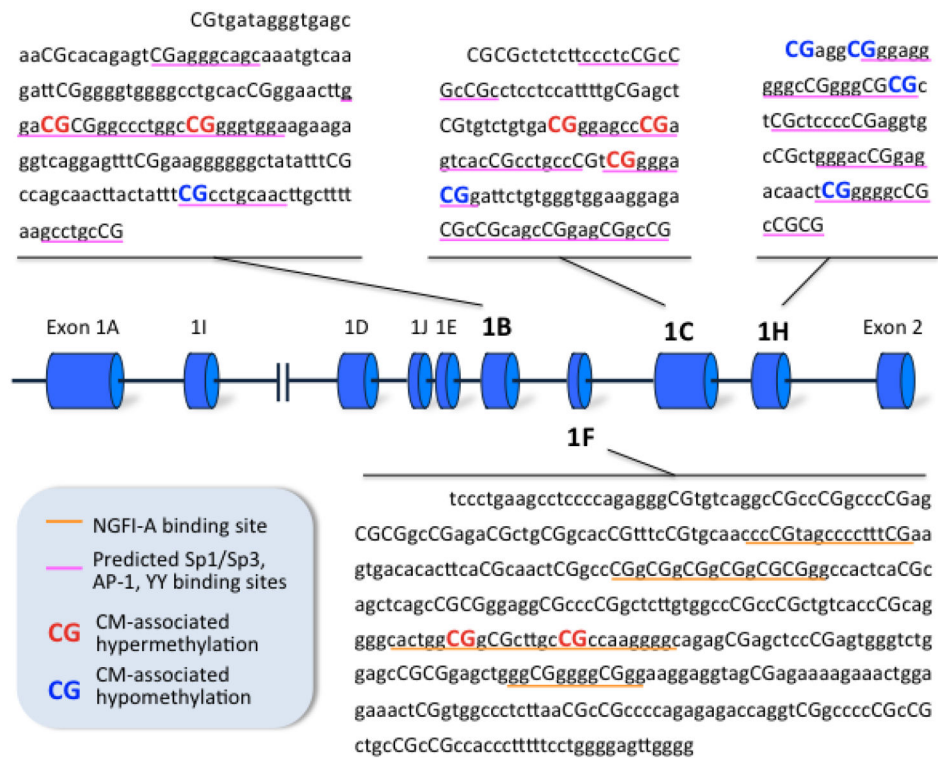


Figure 2. Childhood maltreatment modifies DNA methylation pattern at the human NR3C1 gene encoding for the glucocorticoid receptor, in post-mortem brain hippocampal tissues

Childhood maltreatment associates with both increases (red letters) and decreases (blue letters) in levels of DNA methylation across several CpG sites in the various 5'-untranslated exons of the human GR gene. DNA sequences from promoter regions of GR exons 1_B, 1_C, 1_F and 1_H are shown. Underlined sequences correspond to binding sites of several transcription factors, according to (i) *in silico* prediction for Sp1/Sp3, AP-1 and YY; and (ii) both *in silico* and *in vitro* studies for NGFI-A. Functional transcriptional relevance of DNA methylation levels has been assessed *in vitro* for exons 1_F (McGowan et al., 2009), but not yet for exons 1_B, 1_C and 1_H (Labonte et al., 2012b). Future studies may uncover structural and functional organization within the GR locus, and help understand why, where and how differential methylation occurs at specific CpGs as a function of childhood experiences.