Doctoral Thesis

Study of the mechanisms of transgenerational inheritance of behavioural alterations induced by early stress in mice

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STUDY OF THE MECHANISMS OF TRANSGENERATIONAL INHERITANCE OF BEHAVIOURAL ALTERATIONS INDUCED BY EARLY STRESS IN MICE

A dissertation submitted to

ETH ZURICH

for the degree of

Doctor of Sciences

presented by

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2009
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5HT1aR</td>
<td>serotonin receptor 1a</td>
</tr>
<tr>
<td>ABN</td>
<td>arch-back nursing</td>
</tr>
<tr>
<td>ABN-LG</td>
<td>arch-back nursing with licking/grooming</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Avy</td>
<td><em>Agouti viable yellow</em></td>
</tr>
<tr>
<td>Axin&lt;sup&gt;Fu&lt;/sup&gt;</td>
<td><em>axin-fused</em></td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bisulf.</td>
<td>bisulfite sequencing</td>
</tr>
<tr>
<td>BMA</td>
<td>basomedial amygdala</td>
</tr>
<tr>
<td>CeA</td>
<td>central amygdala</td>
</tr>
<tr>
<td>CB1</td>
<td>cannabinoid receptor</td>
</tr>
<tr>
<td>Cdk9</td>
<td>cyclin-dependent kinase 9</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyltransferase</td>
</tr>
<tr>
<td>CRFR</td>
<td>corticotropin releasing factor receptor</td>
</tr>
<tr>
<td>desip.</td>
<td>desipramine</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DPPA3</td>
<td>developmental pluripotency-associated 3</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>Ect</td>
<td>ectorhinal cortex</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAD67</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HPA axis</td>
<td>hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>ICR</td>
<td>imprinting control region</td>
</tr>
<tr>
<td>IAP</td>
<td>intracisternal A-particle</td>
</tr>
<tr>
<td>LH</td>
<td>lateral hypothalamus</td>
</tr>
<tr>
<td>LT-EE</td>
<td>long-term enriched environment</td>
</tr>
<tr>
<td>MAOA</td>
<td>monoamine oxidase A</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MeCP2</td>
<td>methyl CpG-binding protein 2</td>
</tr>
<tr>
<td>MePV/D</td>
<td>medial posteroverentral and medial posterodorsal amygdala</td>
</tr>
<tr>
<td>MBDP1</td>
<td>methyl binding protein domain 1</td>
</tr>
<tr>
<td>MSUS</td>
<td>maternal separation and unpredictable maternal stress</td>
</tr>
<tr>
<td>NSB</td>
<td>non-specific binding</td>
</tr>
<tr>
<td>PGC7</td>
<td>primordial germ cell 7</td>
</tr>
<tr>
<td>piRNA</td>
<td>Piwi-interacting RNA</td>
</tr>
<tr>
<td>PND</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PP1</td>
<td>protein phosphatase 1</td>
</tr>
<tr>
<td>PRh</td>
<td>perirhinal cortex</td>
</tr>
<tr>
<td>PVN</td>
<td>paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>Pyro.</td>
<td>Pyrosequencing</td>
</tr>
<tr>
<td>SAM</td>
<td>s-adenosylmethionine</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SOX 10</td>
<td>sex-determining region Y-box containing gene 10</td>
</tr>
<tr>
<td>SSRI</td>
<td>selective serotonin-reuptake inhibitor</td>
</tr>
<tr>
<td>ST-EE</td>
<td>short-term enriched environment</td>
</tr>
<tr>
<td>Svg</td>
<td>sauvagine</td>
</tr>
<tr>
<td>Xic</td>
<td>X-inactivation center</td>
</tr>
<tr>
<td>Xist</td>
<td>X-inactive specific transcript</td>
</tr>
<tr>
<td>ZEB</td>
<td>zebularine</td>
</tr>
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</table>
Abstract

Traumatic experiences in early life are risk factors for the development of behavioural and emotional disorders. Such disorders can persist through adulthood and have been reported to be transmitted across generations. Here, an epigenetic mechanism for the transgenerational inheritance of behavioural alterations caused by early stress is described. Chronic and unpredictable maternal separation, a mouse model of early stress, induces enhanced novelty-seeking and depressive-like behaviours in adulthood. Aspects of these behaviours can be reversed by environmental enrichment during adolescence only, or to a greater extent, when enrichment occurs throughout adolescence and adulthood. Interestingly, behavioural abnormalities induced by early stress are transmitted to the offspring of animals exposed to maternal separation, despite being reared under normal conditions. Transmission is associated with altered DNA methylation in the CpG island of several genes including corticotropin-releasing factor receptor 2 (CRFR2), methyl CpG-binding protein 2 (MeCP2) and cannabinoid receptor 1 (CB1), in both the germ line of separated animals and the brain of the offspring. Altered DNA methylation in the brain of the offspring is associated with changes in gene expression. Together, these findings highlight the importance of DNA methylation for the inheritance of behavioural disorders induced by early stress.
Résumé

Des traumatismes psychologiques et des conditions de stress subis pendant l'enfance sont des facteurs de risque pour le développement de troubles comportementaux et émotionnels chez l'homme. De tels troubles ont souvent la caractéristique de persister jusqu'à l'âge adulte et de plus, d'être transmis aux générations suivantes. Parce qu'ils sont provoqués pas des facteurs environnementaux, leur expression et leur transmission sont postulés être dépendants de mécanismes d'épigénèse. L'étude moléculaire de ces mécanismes est difficile et nécessite la mise au point de modèles animaux. Ce travail de thèse s'est attaché à développer un modèle de souris basée sur la séparation maternelle chronique et imprévisible reproduisant les effets de traumatismes et de stress précoces sur le comportement. Ce modèle manifeste de sèvres comportements impulsifs et dépressifs chez l'adulte, qui sont transmis aux générations suivantes. Certains aspects de ces comportements peuvent être reversés par l'enrichissement environnemental pendant l'adolescence et à l'âge adulte, et par des traitements pharmacologiques. L'étude de ces mes mécanismes de transmission démontre qu'ils sont associés à une altération de la méthylation de l'ADN dans des îlots CpG de plusieurs gènes candidats, tels que le gène codant pour le récepteur du facteur corticotropique 2 (CRFR2), et ceux codant pour le methyl-CpG binding protein 2 (MeCP2) et le récepteur au cannabinoïde 1 (CB1). Ces altérations affectent les cellules germinales mâles des animaux séparés, ainsi que le cerveau de leur progéniture, qui conduisent à des changements de l'expression des gènes dans le cerveau. Dans son ensemble, ces découvertes mettent en évidence l'importance de la méthylation de l'ADN comme mécanisme de transmission transgénérationnelle de troubles comportementaux induits par des traumatismes et stress précoces.
1.0 Epigenetics and Epigenetic Inheritance\textsuperscript{1}

\textsuperscript{1} Excerpts of the introduction included in Franklin and Mansuy (submitted) and Graeff, Franklin and Mansuy (submitted). See Appendix.
1.1 Introduction to Epigenetics and Heritable Epigenetic Variation

A core concept in biology is that it is the sequence of nucleotides in the genome which determines the traits of an organism. It is further accepted that heritable information is transmitted to offspring through these sequences of DNA and that any changes occurring on the chromosome during this process are random, and not due to environmental influences. However, recent work in the field of epigenetics has proposed that inheritance of the DNA sequence is not the only mechanism underlying the transgenerational transmission of physical, behavioural, and emotional traits.

The term “epigenetics” was coined by Conrad Waddington in the 1940s to describe the gene-environment interactions that ultimately lead to a particular phenotype (Waddington 1942). Waddington originally used this term in a developmental context to describe the permanent changes in gene activation and deactivation required for the differentiation of cell types. While the mechanism for these changes was unknown at the time, it is now suggested to be the result of post-translational chemical modifications to the DNA itself (Scarano 1971; Holliday and Pugh 1975; Riggs 1975). Thus, the current use of the term has shifted focus to emphasize the heritable changes (both mitotic and meiotic) in gene expression which are not due to the sequence of nucleotides, but rather to DNA and chromatin modifications. These modifications include biochemical modifications of the DNA or of the core histones, variant histones, DNA looping, and higher-order chromatin structure (Mager and Bartolomei 2005). Of these, the biochemical modification of nucleotides, more specifically DNA methylation, is thought to serve as a mark for the establishment and maintenance of the chromatin structure (Jaenisch and Bird 2003; Wang, Wysocka et al. 2004). The establishment or maintenance of DNA methylation patterns is regulated by DNA methyltransferases (DNMTs), methyl CpG-binding proteins, and most recently demonstrated, small RNA molecules (Cheng 1995a; Cheng 1995b; Morris, Chan et al. 2004; Fan and Hutnick 2005; Matzke and Birchler 2005; Wassenegger 2005; Zemach and Grafi 2007; Chahrour, Jung et al. 2008).
1.2 DNA methylation

DNA methylation has many well-known roles in the mammalian organism. During development, DNA methylation is necessary to establish tissue-specific gene expression patterns (Scarano 1971; Holliday and Pugh 1975). Additionally, X-chromosome inactivation, in which one X-chromosome in females is silenced (Riggs 1975; Delaval and Feil 2004) (see below section 1.2.1), and parental imprinting, in which one parental allele is inactivated by DNA methylation (see below section 1.3.1), have been particularly well studied.

In mammals, the most common epigenetic modification of the DNA strand involves the enzymatic transfer of a methyl group to the fifth position of cytosine residues in CpG dinucleotides (a cytosine immediately followed by a guanine) (Richards 2006). Across the genome, CpG dinucleotides are largely methylated, while the opposite is true in CpG-dense regions known as CpG islands (Bird 1986; Gardiner-Garden and Frommer 1987). The methylation level of CpG islands found within or near promoter regions are known to affect the transcription rate of the gene by altering local chromatin structure and thereby modifying the access that transcription factors have to the underlying DNA strand (Richards 2006).

1.2.1 The establishment and maintenance of DNA methylation

DNMTs are enzymes which catalyze the methylation reaction. Currently, there are four known DNMTs in mammals. DNMT1, known as a maintenance methylase, displays a preference for hemi-methylated CpGs occurring following DNA replication in the daughter strand, and acts to maintain the methylation patterns of the parent strand (Lyko, Ramsahoye et al. 1999). The biological role of DNMT2 is rather controversial, as it is currently unclear whether its main role is as a nuclear DNA methyltransferase, or as a cytoplasmic RNA methyltransferase. A recent paper has shown that the latter function is true in zebrafish development (Rai, Chidester et al. 2007). Moreover, human DNMT2 was shown to act as a RNA methyltransferase using a mechanism similar to DNA methyltransferases (Goll, Kirpekar et al. 2006; Jurkowski, Meusburger et al. 2006). Additionally, DNMT2 mutant mice and Drosophila lack phenotypes (Okano, Xie et al.
1998; Kunert, Marhold et al. 2003). DNMT3A and 3B, known as de novo methylases, play a critical role in determining methylation patterns during embryogenesis (Okano, Bell et al. 1999). Recently it has been demonstrated that DNMT3A/B may also have demethylating activity (Metivier, Gallais et al. 2008).

RNA-directed DNA methylation is well established in plants. However, despite the fact that some of the components required for RNA-directed DNA methylation to occur are present in mammals, evidence for its occurrence has been limited (Matzke and Birchler 2005; Wassenegger 2005). One study in mouse oocytes reports that siRNA knockdown of a gene does not involve de novo methylation (Svoboda, Stein et al. 2004). However, there is also one report in human cells of siRNA directed transcriptional silencing associated with increased DNA methylation (Morris, Chan et al. 2004). Obviously, further investigation is needed to better clarify what role, if any, RNA plays in the establishment of DNA methylation.

Demethylation is known to occur by both passive and active mechanisms. Passive demethylation occurs during DNA replication in the absence of DNMT1. Active demethylation is also known to occur although, in mammals, the mechanism for this has not yet been determined. However, two possible mechanisms for active demethylation have been suggested: (1) the direct removal of the methyl group from the fifth position of the cytosine residue (Bhattacharya, Ramchandani et al. 1999), and (2) the replacement of the methylated cytosine by base excision repair through the directed deamination of the methylated cytosine to uracil (Metivier, Gallais et al. 2008).

X-chromosome inactivation provides a clear example of the importance of DNA methylation in the regulation of gene transcription in mammals. In order to compensate for differences in gene dosage between males (XY) and females (XX), one X-chromosome is silenced in female mammals. In somatic cells, the only RNA to be transcribed from the inactive, but not active, X-chromosome is an untranslated RNA originating from the X-inactivation center (Xic), the X-inactive specific transcript (Xist) (Chang, Tucker et al. 2006). This gene is required for dosage equalization as it recruits repressive chromatin factors and silences one of the two X-chromosomes (Jaenisch and Bird 2003). Transcription of the antisense Tsix negatively regulates Xist by regulating
chromosome structure at the \textit{Xist} promoter, thereby protecting one X-chromosome from inactivation by \textit{Xist}. One of the features of the inactivated X-chromosome is increased DNA methylation at the 5’ end of genes on the inactive X-chromosome. Thus, while not playing a crucial role in the initiation of X-inactivation, the continuous activity of DNMT1 is suggested to be important in the maintenance of X-inactivation through mitotic cell divisions (Panning and Jaenisch 1996). Indeed, in the case of X-inactivation, the accuracy of DNA methylation patterns across mitotic divisions is extremely precise; the reversion events per cell division are in the $10^{-6}$ range (Kahan and DeMars 1980).

1.3 Transgenerational transmission of DNA methylation patterns

There are several examples in which DNA methylation is transmitted across generations. The following is a brief discussion of genomic imprinting, paramutation, and epigenetic inheritance occurring at two intracisternal A-particle (IAP) retrotransposons.

1.3.1 Genomic Imprinting

Perhaps the most obvious example of the transmission of DNA methylation patterns across generations is imprinting. Imprinted genes are genes which are expressed from only one of two parental alleles; paternally expressed genes are derived from the paternal allele and maternally expressed genes are derived from the maternal allele. Presently, it is believed that imprinted genes make up about 1% of all autosomal genes (for reviews see Reik and Walter 2001; Jirtle and Skinner 2007). Approximately 80% of imprinted genes occur in clusters in chromosomal domains, which also may contain imprinting control regions (ICRs). These ICRs are differentially methylated depending on the parental allele, and regulate gene silencing for all the genes in the cluster. A high proportion of imprinted genes contain CpG islands, and direct repeats near or within the CpG island. Further, a high number of imprinted genes are linked with fetal growth rates, leading to the theory that genetic conflict underlies the evolution of imprinted genes (Moore and Haig 1991). Thus, a number of maternally expressed genes minimize fetal growth, thereby conserving the mother’s resources and enhancing her overall reproductive success, while paternally expressed genes increase fetal growth, thereby
encouraging the success of each individual offspring.

Imprints are thought to be present during the development of the germ cell into sperm or oocytes, and are thought to be maintained after fertilization. This is despite the fact that there are two large-scale demethylation events occurring, one during gametogenesis and one following fertilization during preimplantation development (Reik 2007). In the mouse, the former is completed by embryonic day (E) 12-13 in both sexes and is followed by de novo methylation, which begins at the end of the embryonic period and continues into postnatal development (Kafri, Ariel et al. 1992; Brandeis, Ariel et al. 1993; Brandeis, Kafri et al. 1993; Tada, Tada et al. 1998).

Imprinted genes escape the massive demethylation and de novo methylation during gametogenesis. While the mechanism for this is not well understood, it has recently been suggested that the protein Stella (also called developmental pluripotency-associated 3 (DPAA3) or primordial germ cell 7 (PGC7)) can protect certain maternally methylated genes from demethylation (Nakamura, Arai et al. 2007). Increased levels of Stella are present during early primordial germ cell development, are expressed in high amounts in oocytes and are transferred to the zygote after fertilization (Nakamura, Arai et al. 2007).

Further, the binding of non-histone proteins to ICRs can prevent methylation during gametogenesis in one of two alleles, as is the case for the ICR which controls expression of the maternally imprinted gene insulin-like growth factor-2 in the mouse (Pant, Mariano et al. 2003; Schoenherr, Levorse et al. 2003). In this case, the ICR is methylated during late spermatogenesis, but in oogenesis the ICR is blocked from being methylated by the chromatin boundary element binding protein CTCF (Schoenherr, Levorse et al. 2003; Fedoriw, Stein et al. 2004). Additionally, a transgenic mouse model of Angelman’s syndrome, a disease characterized by prominent laughing and smiling, hypermotor behaviour, and stereotypies, has demonstrated that an ICR can both protect the Snrpn promoter in the maternal allele from being demethylated after fertilization, and the paternal allele from being de novo methylated in the embryo (Shemer, Hershko et al. 2000). It has been suggested that the sequence and allele-specific methylation present on imprinted genes may be established by miRNAs (Lin, Youngson et al. 2003; Seitz,
Chapter 1: Epigenetics and Epigenetic Inheritance

Youngson et al. 2003).

1.3.2 RNA-mediated paramutation in the mouse

Another well characterized example of the transmission of epigenetic information across generations is paramutation. Paramutation is when the silencing of one allele, by interaction in trans with the homologous allele, is meiotically heritable (Ashe and Whitelaw 2007). The large majority of examples of paramutation occur in plants (Ashe and Whitelaw 2007; Bond and Finnegan 2007; Chandler 2007). However, paramutation at the Kit locus has now also been demonstrated in the mouse (Rassoulzadegan, Grandjean et al. 2006; Wagner, Wagner et al. 2008). Mice heterozygous for Kit mutations display changes in pigment patterns, and reduced Kit mRNA. It was found that a proportion of the offspring of heterozygous Kit mice display this mutant phenotype while having a wild-type genotype (Rassoulzadegan, Grandjean et al. 2006). Abnormal RNA in the sperm of Kit mutant mice was suggested to be the cause of this phenomenon, as microinjection of sperm of Kit mutant mice or injection of miRNAs which target Kit mRNA produced the same effect (Rassoulzadegan, Grandjean et al. 2006). This suggests that functional information not encoded in the DNA sequence itself can be transmitted through the germ line and that this effect is RNA-mediated. This was further developed in a recent paper demonstrating that fertilized mouse eggs injected with miRNA targeting a regulator of cardiac growth, cyclin-dependent kinase 9 (Cdk9), induced cardiac hypertrophy in adulthood, and the presence of trace amounts of this same miRNA in sperm cells (Wagner, Wagner et al. 2008). Furthermore, this cardiac hypertrophy was inherited across at least three generations, mimicking human hypertrophic cardiomyopathy which is often familial (Wagner, Wagner et al. 2008).

1.3.3 Epigenetic Inheritance at Endogenous Alleles (Agouti viable yellow (A^vy) and axin-fused (Axin^{Fu}))

The agouti viable yellow (A^vy) and axin-fused (Axin^{Fu}) alleles, referred to as metastable epialleles, display inter-individual variability in expression state, as well as intra-individual variability in the case of A^vy. Thus, as a result of epigenetic modifications, there is a distribution of phenotypes which may occur despite being genetically identical. The transcriptional activity of these genes are regulated by an IAP retrotransposon.
These variations in expression state are not the result of genotypic variation, but rather variation in the level of DNA methylation of the IAP long terminal repeat upstream of the gene (in the case of $A^{\text{vy}}$) or the IAP within intron 6 of the gene (in the case of $Axin^{\text{Fu}}$) (Morgan, Sutherland et al. 1999; Rakyan, Chong et al. 2003). Ectopic expression of the $A^{\text{vy}}$ protein results in yellow fur, obesity, diabetes and increased tumour susceptibility and, in the case of $Axin^{\text{Fu}}$, a kinked tail (Reed 1937; Duhl, Vrieling et al. 1994).

Methylation levels of the $A^{\text{vy}}$ gene and $Axin^{\text{Fu}}$ genes can be affected by environmental input, specifically maternal diet. The offspring of dams fed a methyl-rich diet have increased levels of methylation of both the $A^{\text{vy}}$ gene and $Axin^{\text{Fu}}$ genes and therefore display the associated phenotypes (Waterland and Jirtle 2003; Waterland, Dolinoy et al. 2006). While naturally occurring variably expressed phenotypes resulting from differential methylation can be transmitted to the subsequent generation, paternally in the case of $A^{\text{vy}}$, and both maternally and paternally in the case of $Axin^{\text{Fu}}$ (Morgan, Sutherland et al. 1999; Rakyan, Chong et al. 2003; Blewitt, Vickaryous et al. 2006), the diet-induced change in $A^{\text{vy}}$ methylation can not be transmitted maternally to the next generation (Waterland, Travisano et al. 2007). Thus, while differential methylation of these two IAPs can induce variably expressed phenotypes in mice carrying the same genes in a transgenerational manner, the environmental impact of maternal diet on phenotype is reset between generations.

1.4 Aberrant DNA methylation and disease

1.4.1 Aging, cancer and DNA methylation

An emerging theory in the field of cancer research is that there is an increased risk of cancer as individuals age due to changes in DNA methylation which occur during the aging process. As cells age, overall methylation levels are decreased (Wilson and Jones 1983). This is thought to be largely the result of a progressive loss in efficiency of DNMT1 and thus an increase in passive demethylation (Lopatina, Haskell et al. 2002). Global DNA hypomethylation is linked to carcinogenesis because it is thought to induce aberrant gene expression, chromosomal instability, reactivation of retrotransposons, and
the loss of imprinting (Wilson, Power et al. 2007). In addition to the general hypomethylation, there are also localized increases in methylation in the CpG islands surrounding gene promoters (Richardson 2002; Fraga, Agrelo et al. 2007). It has been suggested that this increase in methylation of the CpG island is due to overexpression of DNMT3b that may occur as a compensatory mechanism for the general loss of methylation in the cell (Fraga, Agrelo et al. 2007). This hypermethylation in the CpG islands is thought to lead to the deactivation of tumour suppressor genes which should normally be active (Ahuja and Issa 2000; Fraga, Agrelo et al. 2007).

This provides an interesting treatment strategy in preventative and therapeutic medicine. Indeed, it has been shown that in mice predisposed to developing cancer, reduced DNA methylation induced by treatment with a DNMT inhibitor, 5-aza-2'‐deoxycytidine, decreases the number of intestinal polyps which develop (Laird, Jackson-Grusby et al. 1995). 5-aza-2'deoxycytidine treatment also reduced cell proliferation via an apoptotic mechanism in three different prostate cancer cell lines (Walton, Li et al. 2008). Interestingly, when these cell lines were treated with a combined treatment of 5-aza-2'deoxycytidine and an histone deacetylase inhibitor, trichostatin A, this effect was amplified (Walton, Li et al. 2008). Combined treatment with 5-aza-2'deoxycytidine and the histone deacetylase (HDAC) inhibitor valproic acid also prevented medulloblastoma and rhabdomyosarcoma in mice heterozygous for a tumour-suppressor gene (Ecke, Petry et al. 2009). Studies such as these have now led to the use of DNMT inhibitors, like Decitabine 6 and 5-Azacytidine, in the treatment of cancer, and additionally point to the synergistic role that promoter CpG island hypermethylation and histone hypoacetylation may play in tumour formation.

1.4.2 Cognitive function and DNA methylation

DNA methylation has been shown to play a role in memory formation. This relationship has been confirmed experimentally using contextual fear conditioning, a hippocampal-dependent learning model commonly used in rodents (Phillips and LeDoux 1992). Here, an animal learns to associate a novel context with an aversive stimulus, usually an electric shock (Phillips and LeDoux 1992). DNMT inhibition in the CA1 subregion of the hippocampus blocks memory formation when administered following contextual fear
conditioning (Miller and Sweatt 2007). This is consistent with findings that DNMT inhibition of acute hippocampal slices blocks the induction of long-term potentiation at the Schaeffer collateral synapse (Levenson, Roth et al. 2006). Additionally, fear conditioning itself is associated with methylation of the memory suppressor gene protein phosphatase 1 (PP1) and demethylation of the synaptic plasticity genes, reelin and brain derived neurotrophic factor (BDNF) (Miller and Sweatt 2007; Lubin, Roth et al. 2008). In turn, these methylation alterations are associated with a decrease in PP1 gene expression, an increase in reelin expression, and exon-specific alterations in BDNF gene expression (Miller and Sweatt 2007; Lubin, Roth et al. 2008).

Fittingly, many of the genetic diseases in humans linked to aberrant methylation are associated with intellectual disabilities. Germline inheritance of epimutations underlie a variety of diseases characterized by mental retardation and associated with abnormal genomic imprinting, including Angelman’s syndrome, Prader-Willi syndrome, and Beckwith-Wiedemann syndrome (Egger, Liang et al. 2004). It is also the germline mutation of methyl CpG-binding protein 2 (MeCP2), an X-linked methyl-CpG-binding domain protein, which results in the most common form of mental retardation in young girls, Rett syndrome.

1.4.3 Psychiatric disorders and DNA methylation

Depression

Depression is a common multifaceted mental disease characterized by pessimistic thoughts, reduced vitality and anhedonia. This chronic illness affects roughly five per cent of the US population and is difficult to treat; only half of depressed patients show complete remission (Berton and Nestler 2006; Tsankova, Renthal et al. 2007). One of the major questions in regard to therapies used for the treatment of depression is the delayed response between the onset of treatment and amelioration of the symptoms. This suggests the involvement of epigenetic modifications in the therapeutic actions of antidepressant treatments, and as a consequence, epigenetic dysregulation as an underlying mechanism in the etiology of depression.

There is evidence for the influence of antidepressant treatments on DNA methylation.
Chronic antidepressant treatment has been demonstrated to increase methyl MeCP2 and methyl binding domain protein 1 (MBD1), two proteins which bind methylated CpG dinucleotides and thereby act as either a transcriptional activator or repressor in the rodent brain (Cassel, Carouge et al. 2006). The antidepressant-dependent increase in MeCP2 is specific to gamma aminobutyric acid (GABA)-ergic interneurons (Cassel, Carouge et al. 2006). This finding is of particular interest, since abnormal GABAergic transmission and abnormalities in GABA-related gene methylation has been linked with suicide and major depression. Depressed patients who had died from suicide display higher methylation levels in the GABA-A α1 receptor subunit promoter and increased DNA methyltransferase 3b (DNMT3b) mRNA expression and protein levels in the prefrontal cortex when compared to control individuals who died of other causes (Poulter, Du et al. 2008). This suggests the intriguing possibility that antidepressant treatments specifically target the epigenetic machinery in cell types affected by depression.

**Schizophrenia**

Schizophrenia is a debilitating mental disorder, affecting approximately one percent of the US population, 18 years old or more (Regier, Narrow et al. 1993). Schizophrenia is characterized by two main psychotic symptoms: positive symptoms such as delusions and hallucinations, and negative symptoms such as social withdrawal, lack of motivation, and overall apathy. While the causes of schizophrenia are not well understood, they are likely to involve both a multigenic predisposition and environmental factors occurring during pre- and postnatal development. Accordingly, the concordance rate for schizophrenia in monozygotic twins is approximately 50 percent (Singh, Murphy et al. 2002). The question of how the environment can influence the development of schizophrenia is still open. However, there is increasing evidence for the role of aberrant epigenetic profiles in the pathogenesis of this disease, with the majority of the evidence pointing to abnormal GABAergic neurotransmission in cortical areas (Costa, Chen et al. 2009).

The first line of evidence concerns reelin, a glycoprotein expressed both during development and in adult GABAergic neurons that is important for appropriate neuronal
migration during brain development, and which has been linked with schizophrenia pathology (Fatemi 2005; Costa, Chen et al. 2009). Post-mortem samples taken from brains of schizophrenic patients indicate significantly reduced reelin mRNA expression and protein levels in several different brain regions, despite normal overall neuronal numbers (Impagnatiello, Guidotti et al. 1998; Guidotti, Auta et al. 2000). Since the reelin promoter contains a large CpG island, this suggests that DNA methylation in this promoter region may be particularly important in establishing its expression levels (Chen, Sharma et al. 2002; Tsankova, Renthal et al. 2007). Reelin expression is indeed sensitive to manipulations of DNA methylation, suggesting that lower reelin expression in schizophrenic patients may be the result of hypermethylation in the promoter region of the reelin gene. Thus, in vivo, repeated methionine administration increases methylation of the reelin promoter, induces binding of MeCP2 to the reelin promoter, and downregulates reelin expression (Tremolizzo, Carboni et al. 2002; Dong, Agis-Balboa et al. 2005), and in vitro administration of a methylation inhibitor, 5-aza-2'-deoxycytidine, increases its expression (Chen, Sharma et al. 2002).

Further evidence for the involvement of increased DNA methylation in the GABAergic dysfunction observed in schizophrenic patients involves glutamate decarboxylase (GAD67), an enzyme that catalyzes the production of GABA. GAD67 mRNA expression and protein levels are downregulated in cortical structures of schizophrenic patients (Impagnatiello, Guidotti et al. 1998; Guidotti, Auta et al. 2000) and this is correlated with increased methylation of the GAD67 promoter in prefrontal cortical samples taken from post-mortem brains of schizophrenic patients (Huang and Akbarian 2007).

In addition to the GABAergic system, increased methylation of the CpG island of an oligodendrocyte-specific transcription factor, sex-determining region Y-box containing gene 10 (SOX10), and decreased expression of SOX10 (Iwamoto, Bundo et al. 2005), was observed in brains of schizophrenic patients, providing a possible mechanism for the oligodendrocyte abnormalities observed in schizophrenic patients.

The involvement of abnormal hypermethylation of promoters such as reelin, GAD67, and SOX10, is suggested to be caused by elevated levels of s-adenosylmethioninne (SAM), and increased expression of DNMT1 mRNA observed in the brain of schizophrenic
patients (Veldic, Guidotti et al. 2005; Guidotti, Ruzicka et al. 2007). Accordingly, SAM administration per se can induce psychotic episodes in some schizophrenic patients (Antun, Burnett et al. 1971). Due to the involvement of aberrant hypermethylation in schizophrenic patients, DNMT inhibitors have been suggested as potential therapeutic agents for this disease (Chen, Sharma et al. 2002; Costa, Chen et al. 2002; Levenson and Sweatt 2005).

While in the GABAergic system and in oligodendrocytes several instances of hypermethylation have been demonstrated in schizophrenic patients, in the dopaminergic system there is also evidence of hypomethylation. Increased activation of catechol-O-methyltransferase (COMT), an enzyme involved in the degradation of neurotransmitters like dopamine, epinephrine, and norepinephrine, is associated with impairments in attention, executive cognition, and working memory, as well as an increased risk of several psychiatric disorders including schizophrenia (van Vliet, Oates et al. 2007). Reduced methylation of the COMT promoter is present in the frontal lobe of schizophrenia patients, and this is associated with increased activation of the gene (Abdolmaleky, Cheng et al. 2006). Moreover, aberrant methylation of genes within the dopaminergic system has also been observed in monozygotic twins studies. In one study, methylation profiles upstream of the dopamine D2 receptor was investigated in two sets of twins, one concordant and one discordant for schizophrenia. Of the discordant twins, the affected twin was epigenetically more similar to the twins concordant for schizophrenia than to his unaffected brother, suggesting that DNA methylation is playing an important role in the development of this disorder (Petronis, Gottesman et al. 2003).

1.4.4 Transgenerational transmission of disease states

Many of the diseases associated with aberrant DNA methylation have been shown to be transmitted across generations suggesting that there is not only mitotic but also meiotic transmission of DNA methylation patterns. Tumour susceptibility is one trait which has been found to be both associated with aberrant DNA methylation and transmissible. In Drosophila it has been demonstrated that an over-activated form of Janus kinase (JAK), an oncoprotein, blocks the epigenetic reprogramming which normally occurs in the early
embryo (Xing, Shi et al. 2007). Thus, an overexpression of JAK allows the inheritance of aberrant parental DNA methylation, thereby increasing tumor susceptibility in future generations (Xing, Shi et al. 2007). Furthermore, hypermethylation of two tumour-suppressor mismatch repair genes, *MLH1* and *MSH2*, have been associated with hereditary nonpolyposis colorectal cancer, and have been documented in families with inheritance of the disease (Herman, Umar et al. 1998; Chan, Yuen et al. 2006; Hitchins, Wong et al. 2007). Additionally, germline hypermethylation of both *MLH1* and *MSH2* was found, albeit in a low proportion of cells (Suter, Martin et al. 2004; Chan, Yuen et al. 2006).

There is also accumulating evidence that chemical toxins have detrimental effects not only on those exposed directly to the toxin, but on their offspring. Diethylstilbestrol is a synthetic nonsteroidal estrogen which was originally prescribed to prevent miscarriage in those with prior history. Mothers exposed to diethylstilbestrol during the first trimester of pregnancy had daughters with developmental abnormalities and an increased risk of developing both breast cancer and a rare form of adenocarcinoma (Palmer, Wise et al. 2006). Although the granddaughters of exposed mothers are not yet old enough for a full study of the transgenerational effects of diethylstilbestrol exposure to take place, there is one reported diagnosis of a 15 year-old girl, whose maternal grandmother was exposed to diethylstilbestrol during pregnancy, with a very rare case of small cell carcinoma in the ovary (Blatt, Van Le et al. 2003). The possibility of a transgenerational effect of diethylstilbestrol prompted researchers to reproduce this in the mouse. Similar to that seen in humans, perinatal exposure to diethylstilbestrol induced both abnormalities in uterine development and uterine cancer in both the F1 and F2 generation and this was suggested to be the result of abnormal DNA methylation in uterine cancer genes (Walker and Haven 1997; Li, Hansman et al. 2003; Newbold, Padilla-Banks et al. 2006).

Transgenerational transmission of detrimental effects of endocrine disruptors present in our environment has also been demonstrated in rats, this time through both the male and female line. Rats exposed to an endocrine disruptor (vinclozolin, a commonly used fungicide in agricultural fruit crops, or methoxychlor, a commonly used pesticide) during the period of gonadal sex determination (E8-E15) (F1) exhibit reduced epididymal sperm
counts and sperm motility, and increased spermatogenic cell apoptosis (Cupp, Uzumcu et al. 2003; Uzumcu, Suzuki et al. 2004). These traits were transmitted through the male germline to male offspring up to three generations downstream (F2-F4) from exposure to the toxin, and were associated with aberrant methylation in sperm (Anway, Cupp et al. 2005). Exposure to vinclozolin from E8-E14 was also found to induce pregnancy abnormalities, including uterine hemorrhage and/or anemia, up to two generations downstream from the original exposure in females (Nilsson, Anway et al. 2008). Besides affecting fertility, vinclozolin was also found to increase the incidence rate of tumor formation in aging males exposed to vinclozolin prenatally (F1) and their offspring (F2-F4) (Anway, Leathers et al. 2006).

It is also now becoming clear that poor nutrition or reduced food availability can exert its effects across several generations. For example, women subjected to severe food restriction during the last trimester of their pregnancies (the effects of a Nazi embargo on food supplies to Western Holland) delivered babies with lower birth weights. This was also true for the subsequent generation which was conceived and reared under no dietary restrictions suggesting a transgenerational effect of diet on birth weight (Susser and Stein 1994). Further, a recent study found that paternal grandfather’s and grandmother’s food supply were linked to mortality risk for grandsons and granddaughters, respectively, when reduced food supply was present during the slow growth period during mid-childhood in both grandparents or during early pre- and postnatal life of the grandmother (Pembrey, Bygren et al. 2006).

These findings in humans have also been demonstrated in controlled experimental environments in rats malnourished either before or during gestation, but not following delivery (Cowley and Griesel 1966; Zamenhof, van Marthens et al. 1971). Similar to that demonstrated by the Dutch famine in humans, female rats fed a low protein diet prior to and during gestation delivered pups with lower body and brain weights, and reduced levels of brain DNA and protein than controls. When these pups themselves had offspring, they also had abnormally low brain and body weights (Zamenhof, van Marthens et al. 1971). Additionally, a poor diet before, but not following conception also induced low birth weight, slower maturation rate, and poor performance in the Hebb-Williams maze in offspring two generations downstream from the treatment (Cowley and
Griesel 1966).  

Clearly, evidence from both animal experiments and epidemiological studies in the human population suggest that there are several incidences of transgenerational transmission of a disease state induced by the environment. Recent technological advances have identified aberrant DNA methylation resulting from differential environments as a possible mechanism underlying this phenomenon. Furthermore, it has been recently demonstrated that a poor postnatal environment, can not only induce changes in methylation patterns in the rat brain, but can also result in the transgenerational transmission of methylation abnormalities. Here, pups received abusive maternal care, including stepping, dropping, dragging, active avoidance, and rough handling, in general, from non-biological dams for a short period daily from postnatal day (PND)1-PND7. This treatment results in reduced BDNF expression and increased BDNF methylation in the prefrontal cortex in adulthood (Roth, Lubin et al. 2009). Moreover, the offspring of females exposed to maltreatment during early life also showed increased BDNF methylation in the prefrontal cortex (Roth, Lubin et al. 2009). This is especially interesting because, in humans, increased methylation of the BDNF gene in the frontal cortex has been previously associated with major psychoses such as schizophrenia and bipolar disorder (Mill, Tang et al. 2008). Thus, this study in rat suggests that maltreatment during early development may not only predispose individuals to major psychoses, but also their offspring, via transmission of abnormal methylation patterns across generations.
2.0 Persistent Effects of Stress during Early Development
2.1 Early trauma is a risk factor for psychiatric disorders in adulthood

The extent to which early environment predicts the adult life of an individual is controversial, although it is clear that early abuse or trauma continues to affect individuals into and throughout their adult life. Long-term studies have found that infant attachment predicts their ability to form appropriate peer relationships, sociability, risk-taking behaviours, school success and consequently school dropout rate. This last point could be predicted with 77% accuracy based solely on the quality of early care (Sroufe 2002; Carlson, Sroufe et al. 2004; Harper 2005). Maltreatment and childhood trauma are known to increase the risk of the occurrence of depression and anxiety disorders in adulthood (Jaffee, Moffitt et al. 2002; Iversen, Fear et al. 2007; Moffitt, Caspi et al. 2007; Heim, Mletzko et al. 2008; Rikhye, Tyrka et al. 2008). Further, while there is a high level of transmission of anxiety disorders and a strong link between parent and child anxiety disorders, this can not be explained by parenting alone, but instead can be predicted by constitutional factors, like temperament (Weissman, Leckman et al. 1984; Manasssis, Bradley et al. 1995; Merikangas, Swendsen et al. 1998; Dierker, Merikangas et al. 1999; Shamir-Essakow, Ungerer et al. 2005).

The persistence of disorders which may have arisen due to maltreatment in childhood drove researchers to investigate whether DNA methylation may play a role. In the rat, the level of maternal care provided has been shown to influence DNA methylation patterns in the brain of the pup when adult. Studies carried out by Meaney and colleagues have utilized the naturally occurring individual fluctuation in maternal care present in the rat to create two treatment groups, a high licking/grooming and arch-back nursing group (ABN-LG) and a low ABN-LG group. They have shown that pups reared with a high ABN-LG dam compared with a low ABN-LG dam have increased glucocorticoid receptor (GR) expression in the hippocampus and enhanced glucocorticoid feedback sensitivity as adults (Weaver, Cervoni et al. 2004). This was associated with lower DNA methylation in the promoter region of GR and, consequently, increased binding of the nerve growth factor-inducible protein-A transcription factor to the promoter region of GR (Weaver, Cervoni et al. 2004). They further showed that all effects of high ABN-LG rearing could be reversed in the adult offspring by intracerebroventricular infusion of L-methionine, which acts as a methyl-group donor.
This demonstrates that changes in the epigenome established by environment in early development may be reversed by environmental stimuli even in adults, emphasizing the plasticity of DNA methylation in the adult brain.

Findings from rodent experiments have led to a recent study in humans which has provided evidence that childhood abuse is also associated with abnormal methylation in the adult brain (McGowan, Sasaki et al., 2009). Methylation in the neuron-specific glucocorticoid receptor promoter in brains of suicide victims who had undergone childhood abuse was significantly higher than both suicide victims who were not abused and controls. This increase in methylation was associated with decreased glucocorticoid receptor expression. Thus, the findings in rodent studies implicating early environment in the establishment of methylation patterns has been confirmed in the human population.

2.2 Maternal separation is a model of early trauma in the rodent

Maternal separation is used as a rodent model of perturbed mother-infant interaction, early life deprivation, and/or neglect. Maternal separation procedures may vary both in length (1 to 24 hr), number of separations during the first two weeks following delivery, as well as rearing of the control group. While there are some inconsistencies based on the variety of paradigms used, many of these paradigms induce persistent increases in anxiety and depressive-like behaviours, as well as changes in the hypothalamic-pituitary-adrenal axis (HPA axis) response to stressful environments (Huot, Thrivikraman et al. 2001; Lehmann, Russig et al. 2002; Romeo, Mueller et al. 2003; Parfitt, Levin et al. 2004). Maternal separation has been contrasted with brief periods of separation termed handling (10-15 min), which has been found to have the opposite effects of maternal separation (i.e., reduced anxiety and HPA axis response) (Boccia and Pedersen 2001; Parfitt, Levin et al. 2004). This has been suggested to be the result of compensatory care, including additional licking and grooming, provided to the pups following this brief separation.

The effects of maternal separation itself can come from several causes. Firstly, it may
be due to the effect that the separation has directly on the dam. Evidence for this comes from a study demonstrating that the effects of maternal separation on rat pups (in this case, HPA axis hypersensitivity) are not present if dams were provided a foster litter during the time of the separation (Huot, Gonzalez et al. 2004). Thus, the stress of the separation on the dam may have important implications for her behaviour towards the pups upon their reunion, and on stress hormone levels in the milk provided to the pups. Secondly, the effects of maternal separation may be due to the effect of maternal separation on the pups themselves. Evidence for this comes from studies showing that the effects of maternal separation on rat pups are not present if pups are artificially stroked and fed during the separation period (van Oers, de Kloet et al. 1999; Groer, Hill et al. 2002). However, in mice artificial stroking during the maternal separation period only partially reversed increased neuronal and glial cell death seen in separated animals (Zhang, Levine et al. 2002). Thus, it is not clear whether the long-lasting effects of maternal separation are due to the effect of the separation on the dam, on the pups, or perhaps the most likely scenario, some combination of the two.

2.3 Adverse Early Environment Induces Increased Risk-Taking Behaviours in Adult

While there are conflicting reports as to the effects of maternal separation, several studies have demonstrated that maternal separation can lead to increased risk-taking and novelty-seeking behaviours:

- Adult rats previously exposed to six hours of daily maternal separation (PND1 and PND21) spend more time in the open arm of the elevated plus maze and demonstrate lower risk-assessment and increased risk-taking in the concentric square field test compared to control rats (Roman, Gustafsson et al. 2006)
- Adult rats previously exposed to six hours of daily maternal separation (PND6-21) demonstrate reduced latency to enter into the aversive area of an open field compared to control rats (Mathieu, Denis et al. 2008)
- Adult rats previously exposed to six hours of daily maternal separation (10 days during PND1-14) spend more time in the aversive areas of the open field and light-dark box compared to control rats (Colorado, Shumake et al. 2006)
• Adult rats previously exposed to three hours of daily separation (PND3-15) have a reduced latency to leave the closed protected arm of the T-maze compared to control rats (Slotten, Kalinichev et al. 2006)

• Adolescent rats previously exposed to three hours of daily separation (PND1-21) spend more time in the open arm of the elevated plus maze than control rats (McIntosh, Anisman et al. 1999).

• Adult mice previously exposed to 24 hr separation on PND9 spend more time in the open arms of the elevated plus maze than control mice (Fabricius, Wortwein et al. 2008)

2.4 Adverse Early Environment Induces Depressive-Like Behaviours in Adult

There are many reports of adverse early environment precipitating depressive-like behaviours during adulthood. Rodents exposed to three hours of daily separation during either the first two or three weeks post-delivery spend more time floating in the forced swim test compared to controls, a depressive-like behaviour in rodents (Ruedi-Bettschen, Pedersen et al. 2005; Veenema, Blume et al. 2006; Aisa, Tordera et al. 2007; Bhansali, Dunning et al. 2007; Lee, Kim et al. 2007; Lambas-Senas, Mnie-Filali et al. 2009). Three hours of daily separation during the first two weeks postnatal also induces two additional depressive-like behaviours, anhedonia, measured as reduced responsivity to pleasurable stimuli (sucrose), and ethanol preference (Huot, Thrivikraman et al. 2001; Ruedi-Bettschen, Pedersen et al. 2005).
3.0 Rationale and Main Goals
3.1 Rationale

Previous work in our lab has shown that C57Bl6/J mice exposed to a model of early stress, chronic maternal separation occurring between postnatal day 1 (PND1) and PND14, have long-term behavioural impairments that can be transmitted to the following generation (unpublished data). These are expressed as reduced risk-assessment and heightened depressive-like behaviours suggestive of a disturbance in the regulation of the behavioural response to stressful environments. Although females in litters that were exposed to chronic maternal separation demonstrate abnormal responses to novel or aversive environments, they exhibit normal maternal behaviour, indicating that transmission of behavioural alterations to their progeny occurs irrespective of maternal care. These results point to transgenerational epigenetic mechanisms underlying the transmission of behavioural traits in female pups exposed to chronic maternal separation to their offspring.

3.2 Goals of this Study

- To better characterize the behavioural phenotype induced by our maternal separation procedure

- To rule out the influence of maternal care as a mechanism for transmission of abnormal behaviours to F2 MSUS by performing cross-fostering and preparing and testing F2 offspring from males exposed to maternal separation

- To determine the extent to which all aspects of this behavioural phenotype are transmitted across generations

- To determine the mechanism behind this transmission by focusing on changes in the epigenome in genes related to stress pathways
4.0 Methods
4.1 Animals

C57Bl/6J mice were maintained in standard conditions under a reverse light-dark cycle with food and water available *ad libitum*. Unless otherwise described, animals were housed 3-4 per cage in standard Nalgene cages containing wood chip bedding and a small carton house for enrichment. All experimental manipulations were performed during the animals’ active cycle and all experiments were performed in accordance with guidelines and regulations of the cantonal veterinary office, Zurich.

4.2 Maternal Separation Paradigm

Maternal separation with unpredictable stress (MSUS) dams and litters were subjected to daily 3-hr proximal separation from PND1 to PND14. During separation, mothers and pups were placed in separate clean cages containing food and water (dams only), and bedding. Litters and dams were placed such that they had visual and olfactory contact. The timing of separation was unpredictable, but always occurred during the dark cycle. Maternal stress consisted of either 20-min restraint in a Plexiglas tube or 5-min forced swim in cold water (18°C) applied unpredictably and randomly during separation. Both MSUS and control dams and litters had their cages changed on PND1, PND7, PND14, and PND21, during which time the pups were also weighed. Only dams giving birth within one week of each other were used. Litters with less than 4 pups were excluded from analysis. Maternal care was scored shortly before, shortly after and 2-3 hours after separation and scoring included ABN, LG, ABN-LG, nest building alone, ABN + nest building, blanket nursing, carrying pups, and self-grooming on- or off-nest.

4.3 Enriched environment

Control and MSUS male mice were placed in an enriched environment (Marlau®; Viewpoint) immediately after weaning for a period of either two weeks (short-term enriched environment, ST-EE) or until behavioural testing which occurred during adulthood (long-term enriched environment, LT-EE). In the case of ST-EE, mice were returned to standard group-housing following the period of enrichment.
Enrichment consisted of group-housing (n=12) in a large box containing two levels. The bottom level was separated into two compartments, one containing food pellets, and the other containing access to water, running wheels, and a covered/protected area. The top level consisted of a maze which was changed three times per week with a total of 12 configurations. The box was organized such that animals were required to go through the maze to get to the food compartment.

4.4 Antidepressant administration

Desipramine (Desip.) (Sigma) was dissolved in 0.9% sterile saline immediately prior to use and injected intraperitoneally (i.p.) or subcutaneously (s.c.) using a volume of 10 ml/kg.

4.5 Behavioural Tests of Novelty Seeking and Behavioural Control

All behavioural tests were scored blind to treatment group. To avoid possible litter effects, pups from a minimum of 7 litters in the case of non cross-fostered animals, and 5 litters in the case of cross-fostered animals were tested in each behavioural test. All data presented is representative data from three independent experiments in the case of F1, two independent experiments in the case of F2, and one experiment in the case of F3.

4.5.1 Free exploratory paradigm

The test consists of a box (31.5 x 21 x 20.5 cm) with two rows of three-square areas which are all connected. Mice were habituated to three connected areas for 24 hours with food and water ad libitum. Testing began when mice were allowed access to the row of unfamiliar areas for 10 min exploration. Manual scoring was done to assess the latency to enter the unfamiliar areas.

4.5.2 Open field

Mice were placed in the open field (novel, 71 x 71.5 x 31 cm; repeated, 64 x 51 x 46.5 cm) and allowed free exploration for 10 min or repeatedly across 2 days, for 5
min 3 times daily. Latency to enter the center of the open field, and total distance was quantified using an automated scoring system (Noldus).

4.5.3 Elevated plus maze

Mice were placed for 5 min on a 4-arm plus maze made of two open and two closed arms (dark grey Plexiglas, 30 x 5 cm) raised 60 cm above the ground. Manual scoring was done to assess the first entry into an open arm.

4.6 Behavioural tests of depressive-like behaviours

4.6.1 Forced swim test

Mice were placed in a small tank of water (24 cm high, 19 cm diameter, 18±1°C, filled up to 18 cm) for 5 min. In order to determine the acute effects of desipramine, mice were administered saline or desipramine in three injections over a 24-h period (10 mg/kg, i.p. 24 h and 5 h, and 20 mg/kg s.c. 1 hr prior to the behavioural testing). For chronic experiments, mice continued to receive either saline or desipramine (20 mg/kg, i.p.) daily for 14 days with the last injection occurring 30 min prior to testing. Manual scoring was done to determine when the animal first assumed a floating posture, and to determine total floating duration.

4.6.2 Tail suspension test

Mice were suspended by their tail from a hook (35 cm from floor) for 6 min (Steru, Chermat et al. 1985). A plastic cylinder was placed around the tail to prevent tail climbing. In order to determine the acute and chronic effects of desipramine, mice were administered saline or desipramine (20 mg/kg, i.p.) daily for 1 (acute) or 14 (chronic) days with the last injection occurring 30 min prior to testing. Time spent immobile was quantified using an automated scoring system (Noldus).
4.6.3 Sucrose consumption test

Mice were singly housed and allowed free consumption of a 4% sucrose solution for 4 consecutive days. Results were normalized against water intake across the same time period.

4.7 In vitro methylation

In vitro methylation assays were carried out as previously described (Levenson, Roth et al. 2006). Male C57BL/6J mice were sacrificed, brains were immersed in oxygenated ice-cold cutting solution (CS) (110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH$_2$PO$_4$, 28 mM NaHCO$_3$, 0.5 mM CaCl$_2$, 7 mM MgCl$_2$, 5 mM glucose, 0.6 mM ascorbate), and transverse slices (400 µM) were prepared with a vibratome. After isolation, slices were randomized and equilibrated in a mixture of 50% CS and 50% artificial cerebrospinal fluid (ACSF) (125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 25 mM NaHCO$_3$, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 25 mM glucose) at room temperature for 45 min. Slices were treated with either vehicle (0.025% DMSO; 3 hrs) or zebularine (50 or 100 µM; 3 hrs). Immediately after treatment, cortical structures were isolated in ice-cold CS, immediately frozen and stored at -80°C.

4.8 DNA methylation assays

4.8.1 Sample preparation and bisulfite treatment

Genomic DNA was prepared from sperm and brain regions (hypothalamus and cortex) collected from F1 males, as well as brain, sperm, and lung collected from their offspring, and cortex isolated from acute brain slices (DNeasy blood and tissue kit; Qiagen). Purified DNA was processed for bisulfite modification (EZ DNA

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2 Drug treatment of acute brain slices was performed by Natacha Linder.
3 Sperm collection and brain region dissection was performed by Johannes Gräff.
methylation-gold kit, Zymo research).

4.8.2 Pyrosequencing

In F1 and F2 sperm and brain the percentage of methylated alleles at each CG site was quantified in bisulfite-converted DNA by pyrosequencing, a method which provides quantitative data for the relative proportion of the methylated versus unmethylated nucleotides, and known to have an inter-assay variability of approximately 5% (Tost and Gut 2007). To quantify methylation in the CpG island of corticotropin releasing factor receptor 2 (CRFR2), methyl CpG-binding protein 2 (MeCP2), cannabinoid receptor 1 (CB1), monoamine oxidase A (MAOA), and serotonin receptor 1a (5HT1aR), pyrosequencing appropriate methylation-unbiased primer sets (Table 1) were used for PCR amplification of bisulfite-converted DNA, with the reverse primer biotinylated to reduce the possibility of self-priming. Relative position of the region of interest is summarized for CRFR2 in Fig. 20a, MeCP2 in Fig. 25a, CB1 in Fig. 26a, MAOA in Fig. 28a, and 5HT1aR in Fig. 29a (represented in blue). Pyrosequencing was performed in PSQ™ 96MA system and data was analyzed using Pyro Q-CpG™ Software (Pyrosequencing AB) using an internal sequencing primer (not shown) and the appropriate dispensation order (Table 1). The dispensation order is designed to sequence the complementary strand of the bisulfite-converted DNA such that the percentage of G/A corresponds to the percentage of the methylated nucleotide. To confirm completeness of bisulfite conversion, a C nucleotide located in an internal non-CpG site was assessed.

4.8.3 Bisulfite sequencing

PCR amplification of the target region of CRFR2 (as described above; CRFR2124 Table 1), was performed on bisulfite-converted DNA extracted from F1 sperm. PCR products were cloned into a TOPO TA vector pCRII-TOPO (Invitrogen) and these constructs were used to transform TOP10 (Invitrogen) Escherichia coli competent cells. Positive clones were verified by PCR and products were sequenced using

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4 Pyrosequencing was performed by EpiGenDx.
standard methods. Sequenced clones were selected for inclusion in the analysis only if there was a conversion efficiency of at least 95% (no more than three non-CpG located cytosines per sequenced clone that remained unconverted by the bisulfite reaction).

Table 1. Methylation unbiased primer sets used for PCR amplification of bisulfite treated DNA

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Dispensation Order</th>
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<tr>
<td>CRFR2124FP</td>
<td>5’-TGGGTAGGAGGTTTACAGAT-3’</td>
<td>GTCGAGATGTCAGTCTGTTCGTATCGGTCGAGTGTGTTAATCGTGCAATGTGTTATCGTGTA</td>
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<tr>
<td>CRFR2124RPB</td>
<td>5’-GTGCATGTCTGTGTCAGTCTGCAGTCTGCAGTCTGCAGT</td>
<td>GTCGAGATGTCAGTCTGTTCGTATCGGTCGAGTGTGTTAATCGTGCAATGTGTTATCGTGTA</td>
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<tr>
<td>MeCP2129FP</td>
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<td></td>
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<tr>
<td>MeCP2129RPB</td>
<td>5’-GTCGAGATGTCAGTCTGTTCGTATCGGTCGAGTGTGTTAATCGTGCAATGTGTTATCGTGTA</td>
<td></td>
</tr>
<tr>
<td>CB1120FP</td>
<td>5’-GTCGAGATGTCAGTCTGTTCGTATCGGTCGAGTGTGTTAATCGTGCAATGTGTTATCGTGTA</td>
<td></td>
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<tr>
<td>CB1120RPB</td>
<td>5’-GTCGAGATGTCAGTCTGTTCGTATCGGTCGAGTGTGTTAATCGTGCAATGTGTTATCGTGTA</td>
<td></td>
</tr>
<tr>
<td>MAOA151FP</td>
<td>5’-GTCGAGATGTCAGTCTGTTCGTATCGGTCGAGTGTGTTAATCGTGCAATGTGTTATCGTGTA</td>
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<tr>
<td>MAOA151RPB</td>
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<tr>
<td>MAOA152FP</td>
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<tr>
<td>MAOA152RPB</td>
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<tr>
<td>MAOA153FP</td>
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<tr>
<td>MAOA153RPB</td>
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<td>MAOA154FP</td>
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<tr>
<td>MAOA154RPB</td>
<td>5’-GTCGAGATGTCAGTCTGTTCGTATCGGTCGAGTGTGTTAATCGTGCAATGTGTTATCGTGTA</td>
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</tbody>
</table>

4.8.4 Methylation-specific qRT-PCR

To quantify methylation changes induced by zebularine, methylation-specific primers were designed as follows: forward methylated (5’- TGGGTAGGAGGTTTACAGAT-3’), reverse methylated (5’-TTTTTGGGTATTTTAGAAG-3’), forward unmethylated (5’- AACTCTATCTCAACCACTACGT-3’), reverse unmethylated (5’- TAGTTTAGGTTGGTTTGAATTTTGA-3’).

5 Sequencing was performed by MicroSynth.
AAACTCTATCTCCAACCCCATC-3'). PCRs were performed in a total volume of 25 µl, consisting of 2 µl of bisulfite-modified DNA, 12.5 µl of SYBR Green Mix (Qiagen), 0.5 µl of primer (0.2 µM), and 9.5 µl of DEPC-treated double-distilled water. Reactions were performed in an ABI 7500 thermal cycler using the following cycling conditions: 95°C for 15 min, 45 cycles of 94°C for 15 sec, 52°C for 30 sec, and 72°C for 1 min. Detection of fluorescent products occurred at the end of the 1 min 72°C step. To verify product specificity, a melting curve analysis was performed by increasing the temperature in 1°C increments beginning at 60°C. For further verification, the amplified products were analysed by electrophoresis on an agarose gel.

4.9 Gene expression analysis with quantitative RT-PCR

For detection of CRFR2, MeCP2, and CB1 mRNAs, DNaseI-treated RNA isolated from hypothalamus, amygdala or cortex (RNeasy Mini Kit; Qiagen) was used for reverse transcription (RT). RT-reactions were carried out using the SuperScript First-Strand Synthesis System II for RT-PCR (Invitrogen) and quantitative RT-PCR was performed in an ABI 7500 thermal cycler using Taqman probes for β-actin, CRFR2, MeCP2, and CB1 in a total volume of 20 µL (Applied Biosystems). All probes except for CB1, which is a single-exon gene, were designed to span exon boundaries ensuring amplification of only mRNA. For each probe, samples were run in triplicate and Ct values were normalized to β-actin. Ct values were chosen in the linear range of amplification and the comparative Ct method was used to calculate differences in gene expression between samples (Livak and Schmittgen 2001; Pfaffl 2001).

4.10 CRFR binding

CRF receptor (CRFR) binding was carried out as previously described (Tezval, Jahn et al.). Briefly, brains of control and MSUS adult mice were extracted, embedded in Cryomatrix (Shandon) and frozen at -80°C. Serial coronal sections (20 µm) were cut.

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6 CRF receptor binding, quantification, and write-up in the Methods section was done by Dr. Sandor Vizi.
at Bregma -0.58 to -0.94 and -1.22 to -1.46. To confirm Bregma position during cutting, occasional sections were Nissl stained, magnified, and examined. Appropriate coronal sections were mounted onto APES-coated slides and stored at -80°C. Sections were preincubated in incubation buffer (PBS pH 7.2 with 10 mM MgCl₂, 2 mM EGTA, 0.1% BSA) for 1 min at room temperature. For CRFR1 binding, sections were treated in incubation buffer containing 200 pM [¹²⁵I-Tyr³]Sauvagine (a nonspecific CRFR ligand; PerkinElmer Life Sciences) and, for selective displacement, 1μM mouse UrocrortinII (a CRFR2 specific agonist, Phoenix Pharmaceuticals). For CRFR2 binding, the incubation buffer contained 100 pM [¹²⁵I-His³]Anti-sauvagine-30 (a CRFR2 specific ligand; GE Healthcare/Amersham). Nonspecific binding (NSB) was determined in the presence of 1 μM Sauvagine (Phoenix). Sections were washed in ice-cold PBS pH 7.2 0.01% Triton X-100 and in ice-cold water then air-dried. As control, CRFR binding was also performed with 200 pM [¹²⁵I-Tyr³]Svg (and with 1 μM Svg for nonspecific binding) on sections from CRFR1 and CRFR2 knock-out mice (kindly provided by Dr. Jan Deussing) (data not shown). Radioactively labeled sections were exposed to SR phosphor screens (Packard) for 24 hours at room temperature. After exposure, sections were Nissl stained with 2% cresyl violet and brain areas of interest were identified and outlined using a mouse brain atlas (Paxinos and Franklin 2001). Photographs of Nissl stained sections with outlined regions were then overlapped with corresponding phosphoimages in Photoshop CS2 (Adobe) and the phosphorimages were quantified using OptiQuant 4.0 (Packard). Labeling values of brain regions were corrected for screen background and nonspecific binding and expressed in net dlu/mm² units. Typically, for each animal and each brain region, four measurements were performed on two sections. Means of net labeling values were calculated for each brain region in each animal.

4.11 Statistical Analyses

One-way analysis of variance (ANOVA) followed by Fisher’s PLSD post-hoc tests were used to analyse maternal care and the effect of desipramine, unpaired t-tests with Welch’s correction in the case of unequal variance were used to compare F1 control and F1 MSUS, F2 control and F2 MSUS, and F3 control and F3 MSUS behavioural data, gene expression, and receptor binding data, and repeated
measures ANOVA were used to compare maternal care behaviours and weight across the postnatal period and to identify region of differential methylation. All correlations were performed using a correlation z-test. All data analysed matched the requirements for parametric statistical tests. Significance was set at $p<0.05$ for all tests. Error bars represent standard error of the mean (SEM).
5.0 Results
5.1 Chronic and unpredictable maternal separation alters behaviour in the mouse

A model of chronic postnatal stress in the mouse was used to evaluate the extent to which early trauma constitutes a risk factor for persistent and transmissible behavioural defects. Primiparous C57Bl/6J females (F0) and males were bred, and their litters (F1) were subjected to daily unpredictable maternal separation (3 hours) from birth to postnatal day fourteen (PND14) (MSUS). During the time of the separation, dams were also treated with maternal stress, unpredictably (Fig. 1a, b). The immediate effect of the manipulation was investigated by daily monitoring of maternal care supplied by F0 dams to F1 pups for two weeks after birth. Among the multiple parameters examined, arched-back nursing (ABN) and ABN associated with licking-grooming (ABN+ABN-LG) were used as an index of active maternal care (Liu, Diorio et al. 1997; Caldji, Diorio et al. 2000), and time off-nest as an index of absence of care. The overall resulting deficit in maternal care was apparent primarily during the first postnatal week, when the pups are the most dependent upon maternal attention (Fig. 2a, b). However, despite deficient maternal care the separated F1
pups grew normally, and had normal weight at weaning (PND21) (Fig. 2c).

The long-term effect of maternal separation, combined with physical stress, on the behaviour of dams who raised F1 MSUS (F0 MSUS) versus dams who raised F1 control (F0 control) was investigated. Following weaning of F1 control and MSUS, F0 dams were replaced into standard group-housing for one month before being behaviourally tested. Both the open field, and elevated plus maze were used in order to observe their response to aversive areas of a novel environment. F0 MSUS travelled less overall distance in the open field and made less entries into the aversive center of an open field, indicative of reduced motivation to explore often associated with depressive-like behaviours induced by chronic stress in rodents (Fig. 3a, b) (Grippo, Beltz et al. 2003; Lin, Liu et al. 2005; Zhao, Wang et al. 2008). Despite this general reduction in activity, there was a tendency for F0 MSUS to enter the most aversive region of the open field, the center, sooner than F0 controls, which may be indicative of enhanced novelty-seeking (Fig. 3c). Moreover, in the elevated plus maze, there was a tendency for F0 dams to spend more time in the least safe area of the maze (the open arm), make a greater proportion of entries into the open arm, and travel a greater proportion of their total path length in the open arm than controls, again

Figure 2. Poor maternal care provided to F1 MSUS pups did not result in abnormal weights during postnatal development. Dams undergoing maternal separation spent (a) less time actively nursing (F(1, 13)=7.25, p<0.05) and (b) more time off-nest (F(1, 13)=7.69, p<0.05) than control dams during the first week post-delivery. Average of maternal care scoring across three 30-min sessions per day. c, F1 MSUS grew similar to control pups (F(1, 14)=0.055, ns). *, p<0.05, as indicated by Fisher’s PLSD post-hoc tests.
suggesting inappropriate response to dangerous or aversive environments and reduced behavioural control (Fig. 4a-c). However, the possibility that this behaviour may also be due to reduced anxiety as a result of exposure to the physical stressors during the MSUS treatment can not be ruled out. Be that as it may, in F0 control dams the level of maternal care provided to F1 pups was correlated with behavioural traits. This analysis demonstrated that, on the elevated plus maze, increased time off-nest was significantly associated with risk-seeking behaviours in response to the aversive area of the elevated plus maze in F0 control dams (Fig. 4d-f). This correlation was not present in F0 MSUS, further demonstrating the perturbed maternal care induced by maternal separation.

To evaluate the long-term and multigenerational effects of maternal separation on behaviour, adult F1 mice and two subsequent generations were tested using several behavioural paradigms with gradually increasing levels of stress and aversion. In order to facilitate the reader, a summary of the main behavioural findings in F1, F2, and F3 control and MSUS mice is provided in Table 2. Table 2 clearly demonstrates transmission of abnormal behavioural traits from F1 MSUS male and female mice, to F2 MSUS mice derived from F1 MSUS females, and F2 and F3 MSUS mice derived from MSUS males. A further description of these results follows.
Figure 4. Increased exploration of aversive areas of the elevated plus maze in F0 MSUS dams. In the elevated plus maze, F0 MSUS dams (a) spent more time in the open arm (n=8; t(14)=1.94, p=0.073), (b) made more entries into the open arm (n=8; t(14)=1.78, p=0.097), and (c) travelled a greater proportion of total distance in the open arm (n=8; t(14)=2.01, p=0.064), than F0 control dams (n=8). 

(a) Exploration of the open arm is positively correlated with maternal care behaviours in F0 control dams, but not F0 MSUS. 

(d-f) Significant correlation between time spent in an open arm and time spent off-nest (p=0.03), entries into the an open arm and time spent off-nest (p=0.03), and distance travelled in an open arm and time spent off-nest (p=0.03) in F0 control, but not F0 MSUS (p=0.45, 0.79, 0.66 respectively).

+, 0.05<p<0.1, as indicated by unpaired t-tests.
<table>
<thead>
<tr>
<th></th>
<th>F1 male</th>
<th>F1 female</th>
<th>Male line</th>
<th>Female line</th>
<th>Male line</th>
<th>Male line</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>F2 male</td>
<td>F2 female</td>
<td>F2 male</td>
<td>F2 female</td>
</tr>
<tr>
<td>FEP latency to enter unfamiliar area</td>
<td>control: 33.4 ± 5.2 (25)</td>
<td>control: 13.3 ± 1.3 (25)</td>
<td>control: 28.4 ± 4.6 (29) MSUS: 25.4 ± 3.3 (29)</td>
<td>control: 22.7 ± 2.6 (15) MSUS: 13.2 ± 2.2* (15)</td>
<td>control pup-control dam: 39.17 ± 9.6 (18) MSUS pup-control dam: 48.8 ± 13.8 (13) MSUS pup-control dam: 24.3 ± 8.4* (10) MSUS pup-control dam: 22.3 ± 3.9* (16)</td>
<td>control pup-control dam: 16.3 ± 3.7 (15) MSUS pup-control dam: 21.6 ± 8.8 (14) MSUS pup-control dam: 35.1 ± 2.8 (15) MSUS pup-MSUS dam: 24.9 ± 3.7 (14)</td>
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<td></td>
<td>control: 17.4 ± 3.0 (19) MSUS: 33.4 ± 7.8 (18)*</td>
<td>control: 23.7 ± 4.3 (18) MSUS: 13.9 ± 2.0* (16)</td>
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<td>OF latency to enter center</td>
<td>control: 107.9 ± 18.5 (25) MSUS: 71.7 ± 11.8* (38)</td>
<td>control: 52.19 ± 7.7 (24) MSUS: 36.0 ± 4.9* (36)</td>
<td>control: 53.5 ± 7.8 (30) MSUS: 68.6 ± 9.5 (31)</td>
<td>control: 93.8 ± 18.3 (15) MSUS: 50.0 ± 8.3* (15)</td>
<td>control pup-control dam: 42.5 ± 7.2 (18) control pup-MSUS dam: 56.3 ± 11.1 (13) MSUS pup-MSUS dam: 83.8 ± 16.6* (11) MSUS pup-MSUS dam: 49.0 ± 5.6 (16)</td>
<td>control pup-control dam: 46.9 ± 6.9 (16) control pup-MSUS dam: 51.5 ± 10.4 (14) MSUS pup-MSUS dam: 49.9 ± 7.1 (15) MSUS pup-MSUS dam: 62.3 ± 10.3 (15)</td>
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<td></td>
<td>control: 46.2 ± 10.6 (17) MSUS: 52.1 ± 8.7(17)</td>
<td>control: 53.4 ± 7.2 (19) MSUS: 31.2 ± 3.9* (17)</td>
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<td>EPM latency to enter open arm</td>
<td>control: 91.2 ± 20.4 (25) MSUS: 105.3 ± 15.8 (42)</td>
<td>control: 193.3 ± 24.1 (26) MSUS: 125.1 ± 17.4* (39)</td>
<td>control: 129.7 ± 17.3 (30) MSUS: 57.2 ± 8.6* (25)</td>
<td>control: 231.0 ± 28.1 (15) MSUS: 142.4 ± 29.8* (16)</td>
<td>control pup-control dam: 117.5 ± 23.7 (20) control pup-MSUS dam: 134.6 ± 32.8 (14) MSUS pup-MSUS dam: 78.5 ± 10.8* (10) MSUS pup-MSUS dam: 50.4 ± 15.7* (16)</td>
<td>control pup-control dam: 160.9 ± 30.4 (16) control pup-MSUS dam: 175.9 ± 33.3 (13) MSUS pup-MSUS dam: 150.9 ± 31.2* (16) MSUS pup-MSUS dam: 51.6 ± 10.0* (14)</td>
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<td>control pup-control dam: 143.1 ± 22.7 (16) control pup-MSUS dam: 172.8 ± 26.0 (15) MSUS pup-control dam: 164.9 ± 25.0 (16) MSUS pup-MSUS dam: 147.9 ± 21.0 (16)</td>
<td>control pup-control dam: 162.0 ± 22.3 (20) MSUS: 155.2 ± 19.3 (19) MSUS: 141.1 ± 21.7 (20) MSUS: 49.5 ± 10.8* (13)</td>
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<td>FST latency to float</td>
<td>control: 90.4 ± 7.1 (14) MSUS: 73.5 ± 4.7* (29)</td>
<td>control: 86.7 ± 6.4 (23) MSUS: 105.1 ± 7.1± (37)</td>
<td>control: 103.7 ± 9.0 (31) MSUS: 112.7 ± 10.8 (31)</td>
<td>control: 162.4 ± 22.3 (16) MSUS:102.5 ± 9.5* (15)</td>
<td>control pup-control dam: 80.4 ± 6.1 (19) control pup-MSUS dam: 115.3 ± 10.2 (13) MSUS pup-MSUS dam: 144.0 ± 26.9 (11) MSUS pup-MSUS dam: 81.6 ± 6.5 (16)</td>
<td>control pup-control dam: 143.1 ± 22.7 (16) control pup-MSUS dam: 172.8 ± 26.0 (15) MSUS pup-control dam: 164.9 ± 25.0 (16) MSUS pup-MSUS dam: 147.9 ± 21.0 (16)</td>
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<td>control pup-control dam: 143.1 ± 22.7 (16) control pup-MSUS dam: 172.8 ± 26.0 (15) MSUS pup-control dam: 164.9 ± 25.0 (16) MSUS pup-MSUS dam: 147.9 ± 21.0 (16)</td>
<td>control pup-control dam: 162.0 ± 22.3 (20) MSUS: 155.2 ± 19.3 (19) MSUS: 141.1 ± 21.7 (20) MSUS: 49.5 ± 10.8* (13)</td>
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<td>control pup-control dam: 66.5 ± 5.0 (20) MSUS: 73.1 ± 8.2 (15)</td>
<td>control pup-control dam: 66.5 ± 5.0 (20) MSUS: 73.1 ± 8.2 (15)</td>
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<tr>
<td>time spent floating</td>
<td>control: 20.7 ± 5.05 (14) MSUS: 42.0 ± 4.0* (30)</td>
<td>control: 40.2 ± 5.4 (26) MSUS: 21.5 ± 3.9* (36)</td>
<td>control: 42.6 ± 6.7 (31) MSUS: 32.6 ± 5.4 (31)</td>
<td>control: 22.9 ± 5 (28) MSUS: 39.6 ± 6.2* (31)</td>
<td>control pup-control dam: 52.4 ± 8.3 (19) control pup-MSUS dam: 25.9 ± 4.7* (13) MSUS pup control dam: 33.5 ± 8.0 (10) MSUS pup-MSUS dam: 39.1 ± 6.9 (18)</td>
<td>control pup-control dam: 36.0 ± 5.3 (20) MSUS: 54.6 ± 6.4* (22) control pup-control dam: 41.3 ± 5.4 (19) MSUS: 30.8 ± 6.3 (18)</td>
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<td>Sucr. % sucrose intake</td>
<td>control: 285.4 ± 19.3 (18) MSUS: 234.5 ± 13.2* (20)</td>
<td>control: 291.0 ± 24.3 (18) MSUS: 275.7 ± 18.7 (20)</td>
<td>control: 286.1 ± 19.0 (14) MSUS: 264.4 ± 14.7 (16)</td>
<td>control: 277.8 ± 17.4 (16) MSUS: 257.3 ± 12.3 (16)</td>
<td>control pup-control dam: 273.5 ± 19.0 (19) control pup-MSUS dam: 277.2 ± 27.7 (14) MSUS pup-MSUS dam: 308.9 ± 21.5 (11) MSUS pup-MSUS dam: 335.2 ± 27.9 (18)</td>
<td>control pup-control dam: 274.3 ± 18.4 (15) control pup-MSUS dam: 337.8 ± 44.2 (14) MSUS pup-MSUS dam: 332.7 ± 29.4 (16) MSUS pup-MSUS dam: 311.2 ± 28.5 (16)</td>
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<td>control pup-control dam: 264.2 ± 20.5 (20) MSUS: 281.6 ± 24.9 (19)</td>
<td>control pup-control dam: 290.6 ± 18.2 (20) MSUS: 274.1 ± 15.6 (19)</td>
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</table>

FEP, free exploratory paradigm; OF, open field; EPM, elevated plus maze; FST, forced swim test; Sucr., sucrose consumption. *, significant interaction due to cross-fostering. **, p<0.05, +, 0.05<p<0.1 compared to control.
Animals were first tested in mildly stressful conditions using the free exploratory paradigm (Griebel, Belzung et al. 1993). This measures novelty-seeking behaviour in mice by providing access to an unfamiliar and familiar environment. Male F1 MSUS mice entered into unfamiliar areas of the testing arena sooner than control mice, indicating increased novelty-seeking behaviour and reduced assessment of the novel environment prior to entry compared to control mice (Fig. 5a). The effect of maternal separation on behaviour in the free exploratory paradigm was sex-specific, as female F1 MSUS mice demonstrated normal behaviour on this task (F1 control, 13.3 ± 1.3, n=23; F1 MSUS 14.5 ± 1.5, n=37; t(56)=.64, p=0.53). On a slightly more aversive task, the open field (Welker 1957; Dulawa, Grandy et al. 1999), a similar tendency to a reduction in latency to enter the aversive area of an arena was observed, in both male and female F1 MSUS animals (male: F1 control, 107.9 ±

Figure 5. Sex-specific increased novelty-seeking in F1 MSUS in the free exploratory paradigm, open field, and elevated plus maze. a, b, d, Images of the (a) free exploratory paradigm, (b) open field, and (d) elevated plus maze showing representative tracking of position and movements in F1 animals. Aversive areas are outlined by blue boxes. F1 MSUS mice (red trace) were more likely to enter aversive areas than female F1 control mice (black trace) in the (a) free exploratory paradigm, (b) open field, and (d) elevated plus maze. a, Shorter latency to enter unfamiliar areas following 24-hour habituation to familiar areas in the free exploratory paradigm in female F1 MSUS (n=41) versus F1 control (n=25) mice (t(64)=2.62, p<0.05). b, Shorter latency to enter the center of an open field in female F1 MSUS (n=36) versus F1 control (n=24) mice (t(58)=1.87, p=0.07). c, Similar total distance covered during 10-min free exploration of the open field in female F1 MSUS (n=36) and F1 control (n=24) mice (t(57)=1.30, p=0.20, ns). d, Reduced latency to first enter an open arm in female F1 MSUS (n=39) (F1, t(63)=2.35, p<0.05) than F1 control (n=26) *p<0.05, +=0.07, as indicated by unpaired t-tests.
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18.5, \(n=25\); F1 MSUS, 71.7 ± 11.8, \(n=24\); \(t(61)=1.74, p=0.087\); female: F1 control 52.2 ± 7.7, \(n=24\); F1 MSUS, 36.0 ± 4.9, \(n=36\); \(t(58)=2.6, p=0.070\). F1 MSUS had a shorter latency to enter the center area of an open field, but covered an overall similar distance confirming that the behavioural abnormalities demonstrated by F1 MSUS mice on initial exposure to a novel environment are not due to differences in overall arousal levels (Fig 5b, c). Finally on a highly aversive test, the four-arm elevated plus maze (Pellow, Chopin et al. 1985; Lister 1987), female F1 MSUS animals had significantly shorter latency to first enter the open arms than control animals (Fig. 5d), again indicating a reduction in behavioural control (Rodgers and Dalvi 1997). Here again the effect was sex-specific; male F1 MSUS mice demonstrated normal behaviour in the elevated plus maze (F1 control, 91.2 ± 20.4, \(n=25\); F1 MSUS, 105.3 ± 15.82, \(n=42\); \(t(65)=0.54\)).

We further tested the animals on paradigms classically used to examine depression. In a forced swim test (Porsolt, Le Pichon et al. 1977), male F1 MSUS animals adopted a floating posture significantly sooner than control mice and spent significantly more time floating (Fig. 6a, b), indicating altered stress-coping when faced with an inescapable stressor. Moreover, on a sucrose consumption test (Cryan and Mombereau 2004), they consumed less sucrose than control mice (Fig. 6c), reflecting anhedonia, a symptom commonly associated with depression in humans (Wong and Licinio 2001).

**Figure 6.** Depressive-like behaviours in male F1 mice. a, b, On the forced swim test, (a) shorter latency to float in male F1 MSUS (\(n=29\)) versus F1 control mice (\(n=14\)) (\(t(41)=2.03, p<0.05\)), and (b) increased time spent floating in male F1 MSUS (\(n=30\)) versus F1 control mice (\(n=14\)) (\(t(42)=2.25, p<0.05\)). c, Lower sucrose intake normalized to water intake over 4 days in male F1 MSUS (\(n=20\)) versus F1 control mice (\(n=18\)) (\(t(36)=2.22, p<0.05\)) due to (d) reduced overall sucrose consumption . *, \(p<0.05\) as indicated by unpaired t-test.
In contrast to the depressive-like behaviours present in male F1 MSUS mice, female F1 MSUS demonstrated a tendency for increased latency to float (F1 control, 86.7 ± 6.4, n=23; F1 MSUS, 105.1 ± 7.1, n=37; t(56)=0.06), as well as a significant decrease in time spent floating in the forced swim test (F1 control, 40.2 ± 5.4, n=26; F1 MSUS, 21.5 ± 3.3, n=36; t(63)=2.12, p=0.038), but no change in sucrose consumption (F1 control, 291.0 ± 24.34, n=18; F1 MSUS 275.7 ± 18.69, n=20; t(36)=0.51, p=0.62) compared to female F1 control mice. Altogether, these results indicate that chronic and unpredictable postnatal stress induces novelty-seeking behaviours in both male and female mice, and sex-specific depressive-like behaviours, with male MSUS mice demonstrating enhanced depressive-like behaviours, and female MSUS mice demonstrating decreased depressive-like behaviours in a forced swim test.

In order to see if depressive-like behaviours induced by maternal separation could be reversed in adulthood, male MSUS and control mice were placed in enriched environment immediately after weaning for a period of either two weeks (ST-EE) or until behavioural testing (LT-EE). Mice were tested on both the forced swim test and the tail suspension test, two behavioural tests which use time spent immobile when faced with an inescapable situation as a measure of depressive-like behaviours. Mice exposed to maternal separation living in standard housing exhibited depressive-like behaviours (more time immobile) in both the forced swim test and tail suspension test, confirming previous results (Fig. 7). Additionally, in the forced swim test, control mice exposed to 2-weeks of enrichment post-weaning spent more time floating than standard-housed control mice (Fig. 7a). This increase in depressive-like behaviour following short-term enrichment was unexpected, but is most likely the result of reinstatement into standard housing, a comparatively impoverished environment. Interestingly, this induction of depressive-like behaviours was not observed in ST-EE MSUS mice, suggesting that MSUS mice may be resistant to certain types of environmental stressors (Fig. 7a). In the forced swim test, either short-term or long-term enrichment had no effect on MSUS mice (Fig. 7a). In contrast, in the tail suspension test, both short-term and long-term enrichment had no effect on control mice but reversed the effect of maternal separation in MSUS mice (Fig. 7b). This suggests that while the behavioural response to the stressful condition (either immobility in the tail suspension, or floating in the forced swim) may appear outwardly similar, different neurochemical pathways underlie these behaviours. This
has been previously suggested by pharmacological studies demonstrating that, in control animals, selective serotonin-reuptake inhibitors (SSRIs) can reduce immobility in the tail suspension test, but not in the forced swim test. In contrast, atypical antidepressants tend to reduce immobility in the forced swim test, and not the tail suspension test (Bai, Clay et al. 2001; Cryan, Mombereau et al., 2005).

5.2 Behavioural alterations are transmitted to the subsequent two generations

To examine whether the behavioural traits induced by maternal separation are heritable, both adult F1 MSUS females and males were bred with wild-type animals to generate F2 MSUS offspring. These animals were reared under normal conditions (no maternal separation or stress involved) (Fig. 8). To ensure that rearing conditions were normal in these animals, maternal care in F1 MSUS females bred with wild-type males, and in wild-type females bred with F1 MSUS males, was monitored. In addition, cross-fostering was performed on PND0 such that the F2 offspring of F1 females include four treatment groups: F2 MSUS raised by F1 MSUS dams, F2 MSUS raised by F1 CON dams, F2 CON raised by F1 CON, and F2 CON raised by F1 MSUS. Maternal care was monitored during three 30-min periods within
their active cycle each day for one week after delivery (PND0-PND6 in cross-fostered female line, PND1-PND7 in male line). F1 MSUS females had normal fertility and provided adequate maternal care, not only to their own biological offspring (Fig. 9a) but also to the offspring of control dams after cross-fostering (Fig. 9b). Wild-type dams bred to F1 MSUS males also provided normal maternal care as expected (Fig. 10). Increased nursing observed in the female versus the male line is most likely

Figure 8. Generation of F2 MSUS and control mice. Experimental design to study transmission of the effect of MSUS on adult behaviour. Following behavioural testing, adult F1 control (blue, left) and F1 MSUS (orange, right) females and males were bred to C57BL6/J animals (black) and F2 offspring was raised in normal conditions (no maternal separation or maternal stress).

Figure 9. Similar levels of maternal care provided to F2 MSUS and control mice derived from experimental F1 females. a, b, Normal maternal care provided by F1 MSUS dams. Daily scoring of maternal care from birth to PND7 (three 30-min sessions/day) showing (a) similar ABN+ABN-LG, and (b) similar time off-nest in F1 control and MSUS dams raising their own offspring (F2 control and MSUS pups respectively) (control, n=9; MSUS, n=12) or cross-fostered offspring (F2 MSUS and control pups respectively) (n=3) (a, F(1, 23)=0.22, ns; b, F(3, 21)=0.26, ns).
due to the earlier developmental period (PND0-PND6 versus PND1-PND7) during which scoring took place. Consistently, F2 MSUS offspring from both groups of females grew normally and had body weight comparable to control animals at weaning (PND21) (Fig. 11).

Behaviour in F2 offspring was then tested using the same paradigms and conditions as in F1 animals. Strikingly, despite being adequately reared, F2 MSUS offspring from F1 MSUS males exhibited similar behavioural alterations as F1 MSUS animals. Interestingly, female mice seem more susceptible to transmission, as female F2 MSUS mice demonstrate similar alterations in all behavioural tests described above, while male F2 MSUS mice show similar alterations only in the elevated plus maze. On the free exploratory paradigm, F2 MSUS female mice derived from F1 MSUS males, had a
shorter latency to enter unfamiliar areas compared to F2 control mice (Fig. 12a). Similarly, they had increased propensity to enter into the aversive areas of the open field and elevated plus maze (Fig. 12b, d). Again, these behaviours were not due to increased activity or arousal since F2 MSUS mice covered a comparable total distance in the open field (Fig. 12c). In the free exploratory behaviour and open field, this altered behaviour was specific to females and was not observed in male F2 MSUS mice (free exploratory paradigm: F2 control, 28.4 ± 4.6, n=29; F2 MSUS, 25.4 ± 3.3, n=29; t(56)=0.54; open field: F2 control, 53.5 ± 7.82, n=30; F2 MSUS, 68.6 ± 9.52, n=31; t(59)=1.22, p=0.23). However, male F2 MSUS mice demonstrated similar behavioural alterations as female F2 MSUS mice in the elevated plus maze (elevated plus maze; control, 129.67 ± 17.34, n=30; MSUS, 57.2 ± 8.6, n=25; t(41)=3.74, p=0.0006).

**Figure 12. Increased novelty-seeking in the free exploratory paradigm, open field, and elevated plus maze in female F2 MSUS mice.** a, b, d, Images of the (a) free exploratory paradigm, (b) open field, and (d) elevated plus maze showing representative tracking of position and movements in F2 animals. Aversive areas are outlined by blue boxes. F2 MSUS mice (red trace) were more likely to enter aversive areas than F2 control mice (black trace) in the (a) free exploratory paradigm, (b) open field, and (d) elevated plus maze. a, Shorter latency to enter unfamiliar areas following 24-hour habituation to familiar areas in the free exploratory paradigm in female F2 MSUS (n=15) versus F2 control (n=15) mice (t(28)=7.75, p<0.01). b, Shorter latency to enter the center of an open field in female F2 MSUS (n=15) than F2 control (n=15) mice (t(28)=2.19, p<0.05). c, Similar total distance covered during 10-min free exploration of the open field in female F2 MSUS (n=15) and F2 control (n=16) mice (t(29)=0.45, ns). d, Female F2 MSUS mice had reduced latency to first enter an open arm (n=16) (t(29)=4.66, p<0.05) compared to F2 control (n=15). *p<0.05, **p<0.01 as indicated by unpaired t-tests.

Remarkably, whether derived from F1 MSUS males (Fig. 12) or F1 MSUS females (Fig. 13), either male or female MSUS offspring exhibited similar behavioural abnormalities when exposed to aversive environments, indicating a comparable transmission of this trait in both sexes. The transmission was sex-specific, as
transmission of abnormal behaviour in the free exploratory paradigm was seen in the male offspring of female MSUS mice, even though behaviour was normal in F1 MSUS females in this particular behavioural test. Abnormal behavioural traits in the offspring of F1 MSUS females was observed in both the free exploratory paradigm and the elevated plus maze, but not the open field, in male F2 MSUS mice (Fig. 13). Moreover, cross-fostering had no effect on the offspring of F1 MSUS dams in the free exploratory paradigm or elevated plus maze confirming that early environment provided by F1 MSUS dams does not contribute to the abnormal behavioural traits exhibited by male F2 MSUS (Fig. 13). While male F2 MSUS derived and raised by F1 MSUS females behaved normally in the open field, cross-fostering itself had a significant effect on these mice; male F2 MSUS derived from F1 MSUS females but raised by F1 control females demonstrated increased latency to enter the center of the open field (F2 control pup raised by F1 control dam, 42.5 ± 7.2, n=18; F2 control pup raised by F1 MSUS dam, 58.3 ± 11.1, n=13; F2 MSUS pup raised by F1 control dam, 83.8 ± 16.6, n=11; F2 MSUS pup raised by F1 MSUS dam, 49 ± 5.6, n=16; F(1, 54)=2.65, p=0.013). This effect of cross-fostering was not seen in male F2 control mice, suggesting that male F2 MSUS mice may be more susceptible to certain aspects of differential early environments. Female F2 MSUS mice derived from female F1 MSUS mice displayed normal behaviour in the free exploratory paradigm and open field, (free exploratory paradigm: F2 control pup raised by F1

**Figure 13.** Similar behavioural abnormalities in F2 MSUS derived from F1 MSUS females, whether raised with F1 control or F1 MSUS dams. a, Decreased latency to enter the unfamiliar areas in the free exploratory paradigm in male F2 MSUS offspring derived from F1 MSUS females, regardless of whether they were raised by F1 control (n=10 from 5 litters) or F1 MSUS dams (n=16 from 7 litters), compared to F2 controls (n=18 from 11 litters, 13 from 5 litters respectively) (F(1,53)=4.52, p<0.05). b, In the elevated plus maze, decreased latency to enter the open arms in male F2 MSUS offspring derived from F1 MSUS females, regardless of whether they were raised by F1 control (n=10 from 5 litters) or F1 MSUS dams (n=16 from 9 litters), compared to F2 controls, regardless of whether they were raised by F1 control (n=20 from 12 litters) or F1 MSUS dams (n=14 from 5 litters) (F(1,56)=6.24, p<0.05). * p<0.05, +=0.05 F2 control versus F2 MSUS within MSUS dams according to Fisher’s PLSD post-hoc tests.
control dam, 16.3 ± 3.7, n=15; F2 control pup raised by F1 MSUS dam, 21.6 ± 8.8, n=14; F2 MSUS pup raised by F1 control dam, 35.1 ± 9.8, n=15; F2 MSUS pup raised by F1 MSUS dam, 24.9 ± 3.7, n=14; F(1, 54)=0.12, p=0.73; open field: F2 control pup raised by F1 control dam, 46.9 ± 6.9, n=16; F2 control pup raised by F1 MSUS dam, 51.5 ± 10.4, n=14; F2 MSUS pup raised by F1 control dam, 49.9 ± 7.1, n=15; F2 MSUS pup raised by F1 MSUS dam, 62.3 ± 10.3, n=15; F(1, 56)=0.62, p=0.43) but similar to male F2 MSUS mice, female F2 MSUS mice had reduced latency to enter an open arm in the elevated plus maze when raised by MSUS dams. This abnormal behaviour was not present in F2 control mice cross-fostered to F1 MSUS dams, but was also not observed in F2 MSUS pups raised by control dams, demonstrating that while the environment provided by F1 MSUS dams can not induce the abnormal behaviour observed in the EPM, the environment provided by F1 control dams may be able to reverse some of the transmissible effects in a sex-specific manner (F2 control pup raised by F1 control dam, 160.9 ± 30.4, n=16; F2 control pup raised by F1 MSUS dam, 175.9 ± 33.3, n=13; F2 MSUS pup raised by F1 control dam, 150.9 ± 31.2, n=16; F2 MSUS pup raised by F1 MSUS dam, 51.6 ± 10.0, n=14; F(1, 55)=4.06, p=0.049).

In order to confirm that increased propensity to enter the aversive areas observed in female F2 MSUS mice are not due to an overall reduction in the level of anxiety, but instead an enhancement in novelty-seeking, F2 MSUS and control were placed repeatedly in an open field for two days. In this experiment, the center of the open field continues to be an aversive area within the arena, but the aspect of novelty is eliminated across time. While F2 MSUS initially covered a larger proportion of their total distance in the aversive center of the arena, on repeated exposure to the same arena, this proportion decreased to a similar level as in control mice (Fig. 14). Thus, F2 MSUS demonstrate a propensity to explore the aversive environment only in novel environments, demonstrating that it is not a general reduction in anxiety, but rather enhanced novelty-seeking associated with increased risk-taking.

We next tested whether depressive-like behaviours present in male F1 MSUS animals can also be transmitted to F2 animals. On the forced swim test, female F2 mice derived from F1 MSUS males adopted a floating posture significantly earlier than F2 control animals, and spent more time floating (Fig. 15 and saline in Fig. 16a, b, d, e), indicating depressive-like behaviours comparable to their F1 MSUS sires.
Female F2 MSUS animals also spent more time immobile in the tail suspension test (Steru, Chermat et al. 1985), further confirming the transmission of depressive-like behaviours from F1 males to their female offspring (saline in Fig. 16c, f). Notably, the behavioural alterations observed in the forced swim test and the tail suspension test in F2 MSUS animals could be fully reversed by both acute and chronic injection of the tricyclic antidepressant desipramine, consistent with their depressive-like nature (desip. in Fig. 16).

The transmission of depressive-like behaviours is specific to F2 female mice, and is not observed in the male offspring of F1 MSUS sires (latency to float: F2 control, 103.7 ± 9.0, n=31; F2 MSUS, 112.7 ± 10.8, n=31; t(60)=0.64; time spent floating: F2 control, 42.6 ± 6.7, n=31; F2 MSUS, 32.6 ± 5.41, n=31; t(60)=1.16, p=0.25). While immobility was clearly transmitted to female F2 animals, anhedonia was not present in F2 MSUS mice, as demonstrated by a non-significant decrease in sucrose consumption in male (F2 control, 277.8 ± 17.4, n=16; F2 MSUS, 257.3 ± 12.3, n=16; t(30)=0.97) and female F2 MSUS compared to F1 MSUS animals (Fig. 17). This suggests a lack of penetrance of this
behavioural trait. Moreover, unlike the abnormalities observed in F2 MSUS when exploring aversive environments, transmission of changes in depressive-like behaviours was not demonstrated in the female line, as F2 MSUS derived from F1 females demonstrated normal behaviours in the forced swim test and sucrose consumption (not shown). This difference in transmission may result from the fact that distinct mechanisms operate in female and male gametogenesis.

Figure 16. Reversal of depressive-like behaviours in F2 MSUS mice with acute and chronic antidepressant treatment. In the forced swim test (a, d) lower latency to float and (b, e) increased time spent floating in saline-treated female F2 MSUS (n=17) versus F2 control (n=14) was reversed by both (a, b) acute (1 day) and (d, e) chronic (14 day) treatment with desipramine (F2 MSUS n=14, F2 control, n=11) (a, F(3,52)=3.01, p<0.05; b, F(3,52)=2.93, p<0.05; d, F(3,48)=3.51, p<0.05; e, F(3,47)=3.22, p<0.05). In the tail suspension test, (c, f) increased time spent immobile in salinetreated female F2 MSUS (n=16) versus F2 control (n=14) was reversed by both (c) acute and (f) chronic treatment with desipramine (F2 MSUS, n=14, F2 control, n=11) (c, F(3,50)=5.93, p<0.001; f, F(3, 48)=7.29, p<0.001). *, p<0.05, MSUS versus control within generation/drug treatment, #, p<0.05, saline versus desipramine within MSUS or control as indicated by Fisher’s PLSD post-hoc tests.

Cross-fostering, itself, induced alterations in depressive-like behaviours demonstrated in the forced swim test in males derived from F1 MSUS dams. In the forced swim test, both male control and MSUS cross-fostered pups demonstrated increased latency to float and decreased time spent floating (latency to float: F2 control pup raised by F1 control dam, 80.4 ± 8.1, n=19; F2 control pup with F1 MSUS dam, 115.3 ± 10.2, n=13; F2 MSUS pup raised with F1 control dam, 144.0 ± 26.5, n=11; F2 MSUS pup raised by F1 MSUS dam, 81.6 ± 6.5, n=16; F(1, 55)=14.52, p=0.004; time spent floating: F2 control pup

Figure 17. Anhedonia in F2 mice. Lower sucrose consumption normalized to water intake over 4 days in female F2 MSUS (n=16) versus F2 control mice (n=13) t(27)=1.50, ns.)
raised by F1 control dam, 52.4 ± 8.3, n=19; F2 control pup raised by F1 MSUS dam, 25.9 ± 4.7, n=13; F2 MSUS pup raised by F1 control dam, 33.5 ± 8.0, n=10; F2 MSUS pup raised by F1 MSUS dam, 39.1 ± 6.9, n=18; F(1, 56)=4.28, p=0.043). The effect of cross-fostering was specific to males and was not observed in cross-fostered female F2 mice (latency to float: F2 control pup raised by F1 control dam, 143.1 ± 22.7, n=16; F2 control pup raised by F1 MSUS dam, 172.8 ± 26.0, n=15; F2 MSUS pup raised by F1 control dam, 164.9 ± 25.0; F2 MSUS pup raised by F1 MSUS dam, 147.9 ± 21.0, n=16; F(1, 59)=0.97, p=0.33); time spent floating: F2 control pup raised by F1 control dam, 27.4 ± 7.2, n=16; F2 control pup raised by F1 MSUS dam, 25.5 ± 7.3, n=15; F2 MSUS pup raised by F1 MSUS dam, 31.5 ± 6.6, n=16; F(1,58)=2.16, p=0.15). Both males and females derived from F1 MSUS dams consumed normal levels of sucrose, whether raised by control or MSUS dams (male: F2 control pup raised by F1 control dam, 275.3 ± 19, n=19; F2 control pup raised by F1 MSUS dam, 277.2 ± 27.7, n=14; F2 MSUS pup raised by F1 control dam, 308.8 ± 21.5, n=11; F2 MSUS pup raised by F1 MSUS dam, 335.2 ± 27.9, n=18; F(1,57)=3.305, ns; female: F2 control pup raised by F1 control dam, 274.3 ± 18.4, n=16; F2 control pup raised by F1 MSUS dam, 337.8 ± 44.2, n=14; F2 MSUS pup raised by F1 control dam, 332.7 ± 29.4, n=16; F2 MSUS pup raised by F1 MSUS dam, 311.2 ± 28.5, n=16; F(1,57)=1.89, ns).

Due to the increase in novelty-seeking behaviours present in F1 MSUS male and female mice, and the sex-specific transmission of these behaviours to their male and female offspring, as well as the increased depressive-like behaviours seen in F1 MSUS males and their female offspring, we wanted to determine the persistence of the transmission and confirm that it is germline-dependent, by further testing whether the behavioural phenotype could be transmitted to F3 generation. We bred F2 MSUS males with wild-type females and generated F3 MSUS offspring under normal rearing conditions (Fig. 18). Remarkably, adult F3 MSUS demonstrated similar behavioural alterations when exposed to aversive environments as F1 and F2 MSUS mice. Female F3 MSUS mice had increased novelty-seeking behaviour in the free exploratory paradigm (Fig. 19a), and increased propensity to enter into the aversive areas of the open field and elevated plus maze (Fig. 19b, c). Again, this effect is sex-specific; F3 MSUS males demonstrate normal behaviour in the open field and elevated plus maze, and a tendency towards increased latency to enter the
unfamiliar area in the free exploratory paradigm (latency to enter unfamiliar area in the free exploratory paradigm: F3 control, 17.4 ± 3.0, n=19; F3 MSUS, 33.4 ± 7.8, n=18; t(21)=1.91, p=0.07; latency to enter the center area of an open field: F3 control, 46.2 ± 10.6, n=17; F3 MSUS, 52.1 ± 8.7, n=17; t(32)=0.43; latency to enter open arm in the elevated plus maze: F3 control, 162.0 ± 22.3, n=20; F3 MSUS, 155.2 ± 19.3, n=19; t(37)=0.23). Despite the fact that female, but not male F2 MSUS demonstrated increased depressive-like behaviours in the forced swim test, male F2 MSUS transmitted aspects of this enhanced depressive-like behaviour to their male offspring. Male F3 MSUS animals spent overall more time floating in the forced swim test than F3 controls, although their latency to float was normal, indicating transmission of only some aspects of the immobility response to inescapable stress (Fig. 19d, e). Female F3 MSUS mice demonstrated normal behaviour in the forced swim test (latency to float; F3 control, 66.5 ± 5.0, n=20; F3 MSUS, 73.1 ± 8.2, n=15; t(33)=0.72, p=0.55; time spent floating; F3 control, 41.2 ± 5.4, n=18, F3 MSUS, 30.5 ± 6.3, n=18; t(35)=1.29, p=0.20). Both male and female F3 MSUS consumed normal levels of sucrose solution, confirming the lack of transmission of this behavioural trait (Fig. 19f) (male: F3 control, 264.2 ± 20.5; F3 MSUS, 281.6 ± 24.9, n=15; t(37)=0.54, p=0.60; female: F3 control, 290.6 ± 18.2; F3 MSUS, 274.1 ± 15.6, n=19; t(37)=0.69, p=0.47).

5.3 Aberrant DNA methylation in F1 germ cells and F2 brain

We next investigated the potential mechanisms underlying the inheritance of these behavioural traits. Since transmission occurs independently of maternal care, it is
likely to be mediated by epigenetic mechanisms in the germline. DNA methylation is a process that can persistently alter chromatin remodeling and gene expression and, importantly, can be inherited (Roemer, Reik et al. 1997; Morgan, Sutherland et al. 1999; Rakyan, Chong et al. 2003; Anway, Cupp et al. 2005; Klose and Bird 2006; Nilsson, Anway et al. 2008). To determine whether DNA methylation is involved in the observed transmission, we examined the level of methylation in F1 MSUS germline of five candidate genes: corticotropin-releasing factor receptor 2 (CRFR2),

Figure 19. Increased novelty-seeking, and depressive-like behaviours in F3 MSUS mice. a, b, d, Images of the (a) free exploratory paradigm, (b) open field, and (d) elevated plus maze showing representative tracking of position and movements in F3 animals. Aversive areas are outlined by blue boxes. F3 MSUS mice (red trace) were more likely to enter aversive areas than F3 control mice (black trace) in the (a) free exploratory paradigm, (b) open field, and (d) elevated plus maze. a, Shorter latency to enter unfamiliar areas following 24-hour habituation to familiar areas in the free exploratory paradigm in female F3 MSUS (n=16) versus F3 control (n=18) (t(24)=2.07, p<0.05) mice. b, Shorter latency to enter the center of an open field in female F3 MSUS (n=17) than F3 control (n=19) mice (t(27)=2.71, p<0.05). c, Shorter latency to first enter into an open arm in the elevated plus maze in female F3 MSUS mice (n=13) compared to F3 control mice (n=20) (t(27)=3.77, p<0.01). d, e, On the forced swim test, (d) Similar latency to float in male F3 MSUS (n=18) versus F3 control mice (n=18) (t(34)=0.75, ns), and (e) increased time spent floating in male F3 MSUS (n=22) versus F3 control mice (n=20) (t(40)=2.1, p<0.05). c, Similar sucrose consumption normalized to water intake over 4 days in male F3 MSUS (n=19) versus F3 control mice (n=19) (t(36)=0.32, ns). *, p<0.05, **p<0.01 as indicated by unpaired t-test.
methyl CpG-binding protein 2 (MeCP2), cannabinoid receptor-1 (CB1), monoamine oxidase A (MAOA), and serotonin receptor 1a (5HT1aR). These five genes were selected for their known involvement in emotional processing and have all been previously demonstrated to play a regulatory role in behavioural responses to stressful situations and in depressive-like behaviours (see discussion 6.2.3 and 6.3). To quantify the level of methylation of these genes, MSUS and control mice were randomly chosen after behavioural testing, and tissue samples were collected. Due to the prevalence of behavioural abnormalities demonstrated across behavioural tests, males were used for molecular analysis in the brain in the case of F1 and females in the case of F2. Genomic DNA was extracted from F1 MSUS male sperm cells and subjected to bisulfite conversion followed by pyrosequencing analyses. These analyses revealed that methylation in the CpG island surrounding the transcription initiation site of CRFR2 was significantly decreased in F1 MSUS sperm (Fig. 20a, b). Hypomethylation was prominent in a stretch of the CpG island (representing about 25% of CpGs) located 5’ of the transcription initiation site. The results were verified with bisulfite sequencing of individual sperm cells, which demonstrated an increase in the proportion of germ cells with lower methylation profiles (Fig. 20c). Next, to examine whether the aberrant methylation was transmitted to the offspring, similar analyses were performed in the brain of adult female F2 animals derived from F1 males. A comparable DNA hypomethylation in the same stretch of the CRFR2 CpG island was detected in female F2 MSUS brain (Fig. 20d). Notably, despite the fact that DNA hypomethylation is most commonly associated with increased gene expression (Jaenisch and Bird 2003; Caiafa and Zampieri 2005; Levenson, Roth et al. 2006; Lubin, Roth et al. 2008, but see also Polesskaya, Aston et al. 2006; Kangaspeska, Stride et al. 2008; Metivier, Gallais et al. 2008) CRFR2 CpG island hypomethylation was associated with decreased CRFR2 expression. Expression was decreased in brain areas involved in emotional processing (significantly in cortex and hypothalamus) in female F2 MSUS animals (Fig. 20e), and was associated with reduced CRFR2 binding in the lateral hypothalamus, a region involved in the regulation of emotional states activated by chronic stress (Matsuda, Peng et al. 1996; Winsky-Sommerer, Yamanaka et al. 2004; Chaki, Funakoshi et al. 2005) (Fig. 21). To test whether the relationship between hypomethylation and decreased CRFR2 mRNA expression is causal and not correlative, we conducted an in vitro methylation assay using the DNA methyltransferase (DNMT) inhibitor, zebularine. Zebularine decreased both DNA
methylation and CRFR2 gene expression in a dose-dependent manner (Fig. 22a), and the change in CRFR2 gene expression strongly correlated with the change in DNA methylation, both in vivo and in vitro ($r^2=0.979$) (Fig. 22b). This confirms the link between CRFR2 promoter hypomethylation and reduced CRFR2 expression. The in vitro assay was further validated by demonstrating the effect of zebularine on BDNF gene expression, a gene previously demonstrated to be increased following hypomethylation (Lubin, Roth et al. 2008; Roth, Lubin et al. 2009) (Fig. 22c).
Since CRFR2 is an important component of stress pathways and plays a key role in the integration of neuroendocrine and behavioural responses to stress (Takahashi 2001), it is likely to contribute to the observed behavioural phenotype. Thus, we examined whether its expression is also changed in the brain of F1 MSUS animals, and whether any change is associated with altered methylation. Quantitative RT-PCR revealed that CRFR2 mRNA expression was normal in cortex, amygdala and hypothalamus in male F1 animals (Fig 23b). Consistently, CRFR2 CpG island methylation was also not altered in the brain of male F1 animals (Fig. 23a). But despite normal CRFR2 mRNA expression and methylation levels, the behavioural abnormalities observed in F1 MSUS animals suggested that CRFR2 protein might be altered in these animals as well. Therefore, we examined the regional abundance of CRFR2 by quantifying receptor-binding. Despite the relatively mild phenotype observed in F1 MSUS females, CRFR2 binding was decreased in several brain areas including the hypothalamus and the amygdala in female F1 animals (Fig. 24). This decrease was specific to these structures and was not observed in hippocampus or cortical areas (data not shown). It was also specific for CRFR2, as the expression of CRFR1 was not altered in F1 MSUS brain (data not shown). The mechanisms involved in CRFR2 down-regulation in hypothalamus and amygdala are not known but most likely involve posttranscriptional regulatory processes. These results overall suggest that CRFR2 is likely to contribute to the behavioural traits observed in MSUS animals.
In addition to CRFR2, methylation of the CpG island surrounding the transcription initiation site of both MeCP2 and CB1 genes was altered in male F1 MSUS germline but, unlike CRFR2, methylation was increased (Fig. 25a, b, Fig. 26a, b). Importantly, hypermethylation of the same stretch of CpGs was observed in the brain of F2 MSUS animals, and hypermethylation was associated with reduced mRNA expression of both MeCP2 and CB1 genes (Fig. 25c, d, Fig. 26c, d).

To investigate whether the transmission of aberrant methylation profile is specific to brain tissue or is present across tissues, lung and sperm was taken from naive randomly chosen F2 MSUS and control mice. Decreased methylation of CRFR2 and increased methylation of MeCP2 and CB1 was also observed in sperm and lung cells of F2 MSUS animals, with some apparent correction in methylation level of CB1 in F2 MSUS sperm (Fig. 27).

This strongly suggests a broad transmission of the altered methylation profile, and is
consistent with behavioural data from F3 MSUS showing that most, but not all behavioural alterations were sex-specifically transmitted to the F3 generation (e.g., latency to float in the forced swim test, and anhedonia in sucrose consumption) (Fig. 19). It further demonstrates that DNA methylation changes in sperm are not the result of environmental factors occurring during behavioural testing, as F2 sperm was taken from naive mice. These data overall indicate that DNA methylation in the germline is
altered bidirectionally by early stress, and that these alterations are transmitted to several tissues in the offspring. Notably, the aberrant methylation was specific to particular genes, since the methylation profile of MAOA or 5HT1aR was not altered in F1 MSUS sperm (Figs. 28, 29).

Figure 25. Hypermethylation of MeCP2 CpG island in F1 MSUS germ line and F2 MSUS brain, and decreased mRNA expression in F2 MSUS brain.

a. Schematic representation of (a) MeCP2 showing the CpG island and transcription initiation site. Base-pair (bp) annotations are relative to the location of the initiation site. Target sequences used for pyrosequencing to quantify methylation are represented in blue. b, c. Increased methylation of MeCP2 CpG island in (b) sperm of F1 MSUS mice (F(1,7)=6.19, p<0.05) and (c) brain of female F2 MSUS mice (F(1,6)=13.31, p=0.01) (sperm, n=4-5; brain, n=4). d. Reduced MeCP2 mRNA expression in female F2 MSUS (n=7) compared to F2 control (n=5) brain (t(10)=2.80, p<0.05). For methylation data, horizontal bar highlights sequence significantly altered in F1 germ cell and F2 brain as indicated by repeated measures ANOVA. * p<0.05, ** p<0.01. For gene expression data (d), *, p<0.05, as indicated by unpaired t-test.
Figure 26. Hypermethylation of CB1 CpG island in F1 MSUS germ line and F2 MSUS brain, and decreased mRNA expression in F2 MSUS brain.  

a, Schematic representation of (a) CB1 showing the CpG island and transcription initiation site. Base-pair (bp) annotations are relative to the location of the initiation site. Target sequences used for pyrosequencing to quantify methylation are represented in blue.  

b, Increased methylation of CB1 CpG island in (b) sperm of F1 MSUS mice (F(1,9)=15.57, p<0.01) and (c) brain of female F2 MSUS mice (F(10, 40)=5.26, p<0.01) (sperm, n=5-6, brain, n=3).  

d, Reduced CB1 mRNA expression in female F2 MSUS (n=8) compared to F2 control (n=8) brain (t(14)=2.21, p<0.05). Horizontal bar highlights sequence significantly altered in F1 germ cell and F2 brain as indicated by repeated measures ANOVA, * p<0.05. For gene expression data (d), *, p<0.05, as indicated by unpaired t-test.
Figure 27. Aberrant methylation in the promoter-associated CpG island of CRFR2, MeCP2, and CB1 in F2 MSUS germ line and lung. a, b, Reduced methylation of CRFR2 CpG island in (a) sperm and (b) lung of male F2 MSUS mice compared to F2 control mice. c, d, Increased methylation of MeCP2 CpG island in (c) sperm and (d) lung of male F2 MSUS mice compared to F2 control mice. e, f, Increased methylation of CB1 CpG island in (e) sperm and (f) lung of male F2 MSUS mice. sperm, n=7 MSUS, n=7 control; lung, n=3 MSUS, n=3 control.
Figure 28. Normal methylation of MAOA CpG islands in F1 MSUS germ line. a, Schematic representation of MAOA gene showing two CpG islands and transcription initiation site. Base-pair (bp) annotations are relative to the location of the initiation site. b, c, Absolute level of methylation within identified regions of MAOA in sperm of F1 MSUS and F1 control mice in (b) CpG island 1 and (c) CpG island 2 (n=6).
Figure 29. Normal methylation of 5HT1aR CpG islands in F1 MSUS germ line. 

(a) Schematic representation of 5HT1aR gene showing two CpG islands and transcription initiation site. Base-pair (bp) annotations are relative to the location of the initiation site. 

(b) Absolute level of methylation within identified regions of MAOA in sperm of F1 MSUS and F1 control mice in (b) CpG island 1 and (c) CpG island 2 (n=6).
6.0 Discussion
The present results provide evidence that chronic and unpredictable stress experienced during early postnatal development in mice leads to persistent and transmissible stress-induced behaviours. Further, that the transgenerational transmission may result from epigenetic inheritance, more specifically the transmission of abnormal DNA methylation patterns through the germ line. Aberrant DNA methylation in the CpG islands of several genes have been demonstrated, both in the germline of animals exposed to stress, and the female brain and male germ cell of their progeny, and this is additionally associated with changes in gene expression in the female brain. The present findings are the first to demonstrate that postnatal stress in mice can persistently alter DNA methylation in the germline and affect behaviour across generations. This extends previous findings in plants, Drosophila, and mammals, that environmental manipulations or genetic mutations can induce epigenetic reprogramming and transmission of disease states (Roemer, Reik et al. 1997; Anway, Cupp et al. 2005; Molinier, Ries et al. 2006; Xing, Shi et al. 2007). These results demonstrate that unfavourable environmental factors during early development can negatively influence behaviour across generations in a sex-specific manner. Further, results presented here suggest a mechanism for a phenomenon that is well documented in humans and animals but, to this point, has been poorly understood (Chong and Whitelaw 2004; Harper 2005; Tsankova, Renthal et al. 2007).

6.1 Early stress induces transmission of abnormal behaviours across generations

Stress during postnatal development induced abnormal behavioural responses on initial exposure to aversive environments, as well as depressive-like behaviours in adulthood. The behavioural alterations induced by early stress were sex-specific, such that enhanced novelty-seeking was observed in both female and male mice, while increased depressive-like behaviours were only observed in male mice. The behavioural effects of the maternal separation itself were mild, in that a somewhat large number of animals were behaviourally tested in order to observe significant results. However, some of these behavioural deficits were clearly transmitted to the subsequent two generations without any additional environmental manipulation or changes in maternal care through both males and females exposed to the initial treatment. Transmission of behavioural alterations was again sex-specific; males
transmitted behavioural alterations primarily to female offspring. Moreover, while both males and females transmitted enhanced novelty-seeking behaviours similarly, depressive-like behaviours were only transmitted through the male line (for summary of behavioural data, see Table 2). Furthermore, depressive-like behaviours were only seen in F2 MSUS females and F3 MSUS males, demonstrating that F2 MSUS male mice can carry alterations responsible for enhanced depressive-like behaviours without, themselves, expressing the aberrant behaviour (Table 2). Taken together, behavioural data suggests the presence of an epigenetic mechanism underlying the robust transmission of behavioural abnormalities across generations.

6.1.1 Reduced anxiety or increased risk-taking?

The free exploratory paradigm, open field, and elevated plus maze, were used to challenge the animals’ response to novel and aversive environments. These tests take advantage of the approach-avoidance conflict naturally occurring in mice when exploring a novel area (Cryan and Holmes, 2005). On the one hand, mice are naturally curious and, thus, are motivated to explore a novel environment. On the other hand, they feel fear at the potential dangers associated with the novel arena. Additionally, open and unprotected areas are particularly aversive areas of a novel arena (Cryan and Holmes, 2005). Thus, there are two possible interpretations to reduced responsiveness to the dangers associated with an aversive area; either the animal feels less stress or anxiety when considering the challenge ahead, or the animal displays enhanced novelty-seeking and risk-taking which results in an inappropriate assessment of the dangers within the environment prior to exploration (Cryan and Holmes, 2005). The first interpretation is seen as beneficial, while the latter is a negative behavioural trait associated with a wide range of personality disorders in humans (Hinshaw, 2003). While perhaps the first explanation is most widely published, both interpretations are equally valid when considering the reduced latency to enter into the novel or aversive areas demonstrated by maternal separated animals and their offspring. Therefore, in order to make an important discrimination between these two interpretations, F2 mice were placed repeatedly in an open field to negate the factor of novelty within the environment. One would expect that if the abnormal behaviour in MSUS mice was the result of decreased anxiety they would continue to explore the aversive area of an open field more than control mice, despite multiple exposures. However, if the phenotype results from abnormal novelty-
seeking, MSUS would have normal levels of center exploration when the novelty of the environment is no longer a factor. Here, F2 MSUS mice explored the aversive area of the open field more than control mice on initial exposure, but this behaviour decreased to normal levels across repeated exposure, confirming that the transmissible abnormality in behaviour induced by early stress is indeed a deficit in behavioural control, and not reduced anxiety (Fig. 14). This additional experiment highlights the need for caution when interpreting animal behaviour.

6.1.2 Multiple neurochemical pathways involved in transmissible depressive-like behaviours

Aspects of depressive-like behaviours were also clearly induced by early stress and transmitted across generations. Animals exposed to early stress adopted an immobile posture more than control animals (Figure 6), and this was seen across two subsequent generations (Figs. 15, 19). However, traits such as anhedonia (as measured by sucrose consumption) and latency to enter into an immobile posture (as measured by the forced swim test) showed less penetrance (Figs. 6, 17, 19). This suggests that different molecular mechanisms with altered susceptibility to epigenetic inheritance across generations are responsible for the expression of these behavioural traits.

The observed depressive-like behaviour in both the forced swim test and tail suspension test is immobility, a behaviour which has been associated with an absence of escape-directed behaviours and, therefore, has been termed “behavioural despair” (Porsolt, Le Pichon et al. 1977). An alternative theory is that immobility represents a coping strategy, similar to the concept of “entrapment” often described in depressive patients (Thierry, Steru et al. 1984; Dixon, 1998; Gilbert and Allan, 1998; Cryan, Mombereau 2004). Consequently, immobility may be seen as a passive, rather than an active coping strategy, used to deal with stressful stimuli. In fact, animals with high immobility early in the forced swim test stay afloat, while animals which continue to struggle become unable to swim, and sink following extended exposure to the inescapable stress, providing evidence for the possible advantages of immobility as a coping strategy (Nishimura, Tsuda et al. 1988). Thus, here again caution must be used when interpreting this behavioural measure. However, whether the floating behaviour observed in MSUS mice is an indication of despair or coping, the clear effect of chronic antidepressant treatment (Fig. 16) does
suggest that similar neurochemical pathways are being affected by early stress in MSUS mice, as in depressive patients.

The tail suspension test has often been termed the “dry” version of the more popular forced swim test, as both tests measure time immobile induced by an inescapable situation. However, while the observed behaviour is similar, they are differentially affected by antidepressant administration, suggesting the involvement of different neurochemical pathways (Bai, Clay et al. 2001; see review Cryan, Mombereau et al., 2005). In contrast to the tail suspension test, the forced swim test is not generally seen as sensitive to selective serotonin reuptake inhibitors and is ineffective in a large proportion of mouse strains (Lucki, Dalvi et al. 2001). Furthermore, several atypical antidepressants, like rolipram (a type 4 cyclic adenosine monophosphate-specific phosphodiesterase inhibitor) and levoprotiline (a histamine H1 receptor antagonist), affect depressive-like behaviour in the forced swim test, but not in the tail suspension test (Maj, Rogóź et al., 1990; Noguchi, Fukuda et al., 1992). Additionally, there are also differences in sensitivity and in dose response curves to antidepressants between the two tests. While in the forced swim test, increasing doses of antidepressants tend to have a bi-phasic or U-shaped dose response, they have a linear response in the tail suspension test (Porsolt, Le Pichon et al. 1977; Bai, Clay et al. 2001; Li, Tizzano et al. 2001; Cryan, Mombereau et al. 2005). As well, decreased gamma-aminobutyric acid (GABA)-B signaling via administration of an antagonist or with genetic knockout reduces depressive-like behaviours in the forced swim test, but not in the tail suspension test (Mombereau, Kaupmann et al. 2004).

Due to the volume of evidence suggesting the involvement of different neurochemical pathways in the immobility response to the forced swim test and the tail suspension test, it may not be surprising that a differential effect of enriched environment on MSUS and control mice was observed in the forced swim test and tail suspension test. Thus, in the present study, both short-term and long-term enrichment completely reversed the depressive-like behaviours in maternal separated animals in the tail suspension test, but had no effect in the forced swim test (Fig. 7). This suggests that depressive-like behaviours in MSUS mice are the result of changes in several neurochemical pathways, of which enriched environment may only affect one. The clear reversal of immobility in the tail suspension task by enriched environment suggests that enriched environment may be altering the serotonergic
pathway, similar to the effect seen with selective serotonin reuptake inhibitors in the tail suspension test.

**6.1.3 Maternal care and environmental factors do not play a role in the transmission of behavioural traits**

While there was a clear transmission of an abnormal response to stressful situations, F2 MSUS dams did not provide abnormal maternal care to their offspring (Fig. 9). This, despite the fact that they themselves received poor maternal care during the maternal separation procedure (Fig. 2a, b). This is in contrast to studies done in rats that have shown behavioural transmission of differential maternal care (Francis, Diorio et al. 1999; Champagne, Francis et al. 2003; Weaver, Cervoni et al. 2004; Weaver, Chapagne et al. 2005). The difference between these previous studies and the one presented here may be due to the fact that previous studies observing a transmission of chronic (permanent) poor mothering results from a natural trait in female rats (high ABN-LG dams versus low ABN-LG dams). Such poor mothering, here characterized by low licking/grooming, is much more severe than the MSUS manipulation because it results in a persistent deficit in maternal behaviours experienced by the pups consistently throughout the day and throughout postnatal development until weaning. Our manipulation is restricted to only two weeks postnatal, with maternal care returning to normal levels during the second week of treatment (Fig. 2a, b). Consequently, the two-week treatment mainly perturbs the natural rhythm and regularity of care rather than its overall amount, as some compensation has previously been observed immediately after separation (data not shown). Additionally, care is reduced approximately five-fold, both in duration and number of licking/grooming bouts, in low licking/grooming rat dams compared to high licking/grooming dams (see for instance Champagne, Francis et al., 2003) in rat studies, while it was reduced by only 10-15% in MSUS mice compared to controls (Fig. 2a, b). The milder impact of MSUS on maternal behaviours most likely explains why maternal care is not a transmissible behaviour in the case of our maternal separation paradigm.

Observation of maternal care provided to F2 is necessary to ensure that postnatal environment is not a factor in the behavioural transmission demonstrated. Many measures of maternal care were observed, including several types of nursing behaviours and time spent off-nest, and F1 MSUS provided normal maternal care in
all measures. However, despite careful monitoring of maternal care it is clear that not all changes in postnatal environment can be observed externally. Nevertheless, several additional pieces of evidence suggest that behavioural transmission is not due to differential rearing environments. Cross-fostering studies demonstrated that F2 control animals raised by F1 MSUS did not display any of the behavioural abnormalities present in F2 MSUS (Fig. 13). Perhaps the strongest piece of additional evidence is that F1 MSUS males also transmit the environmentally-induced behavioural abnormalities, even to a greater extent than F1 MSUS females, despite being separated from females immediately after breeding.

Furthermore, it is important to note that chronic and unpredictable separation induces behavioural abnormalities two generations downstream of the original manipulation. This is important for reasons often overlooked in transgenerational epigenetic research (reviewed in Skinner, 2008). While transmission to F2 alone does suggest epigenetic, and not environmental factors, it does not completely rule out environmental influence, since the germ line which will ultimately generate F2 is present in F1 during the time of the postnatal treatment (Fig. 30). Although this may be unlikely, transmission to F3 is nonetheless important to demonstrate that the phenotype is indeed transgenerational, germ line dependent, and not the direct effect of the treatment itself (Skinner 2008). Here, early stress induces a phenotype which is transmitted through the paternal line at least two generations downstream, thereby ruling out any environmental basis for this transmission.
6.2 Early stress induces abnormal DNA methylation in the germline and in the brain of the subsequent generation

DNA methylation is an important mechanism for transcriptional regulation, and its establishment and maintenance are dynamically controlled during development (Klose and Bird 2006). In germ cells, it is acquired over successive prenatal and postnatal stages and is completed after birth (Chong and Whitelaw 2004; Trasler 2006). Aberrant DNA methylation is associated with multiple diseases including cancer, psychiatric disorders, immune and metabolic diseases (Egger, Liang et al. 2004; Anway, Cupp et al.; Jirtle and Skinner; Tsankova, Renthal et al. 2007; Wilson, Power et al. 2007). Further, environmental factors such as chemicals, nutritional
factors, hormonal manipulations and aging are known to contribute to abnormal DNA methylation (Rakyan, Chong et al. 2003; Anway, Cupp et al. 2005; Weaver, Champagne et al. 2005; Blewitt, Vickaryous et al. 2006; Nilsson, Anway et al. 2008). The present findings newly demonstrate that early life stress can also alter DNA methylation in sperm cells, and that the alterations persist and are transmitted to subsequent generations. These findings significantly extend previous data showing that DNA methylation in the brain is influenced by environmental factors such as maternal care, and recent evidence that early stress can result in transgenerational transmission of abnormal methylation in the brain (Weaver, Cervoni et al. 2004; Roth, Lubin et al. 2009). Additionally, these findings provide novel evidence that maternal care and inheritance of behavioural traits can be dissociated. Notably, the present findings indicate that the sensitivity to early stress is locus-dependent, since only three of the five genes investigated are affected, and suggest a differential sensitivity within the genome to stress-induced epigenetic regulation. Furthermore, the affected loci are perturbed differently, with some being hypomethylated (CRFR2) and others hypermethylated (MeCP2 and CB1) suggesting locus-specific regulation of methylation levels. Finally, it should be noted that even if only examined in the male germline, aberrant DNA methylation may also occur in oocytes since some of the MSUS-induced behavioural traits were similarly transmitted by F1 females and males.

6.2.1 Aberrant DNA methylation and associated gene expression changes

It is particularly interesting to note that the subtle change in DNA methylation (1-5% absolute value) is associated with a proportionally greater reduction of CRFR2 expression (12-45%) in female F2 MSUS brain. This amplifying effect on gene expression was true for both F2 MSUS mice (in vivo) and acute brain slices (in vitro) and was present to a similar extent in both conditions (Fig. 22b). This suggests that differential methylation at any (or a few) CpGs within the identified region is sufficient to affect gene expression. This extends previous evidence demonstrating that it is the density of DNA methylation rather than the pattern of DNA methylation itself which is most important for regulating gene transcription (Boyes and Bird, 1992; Lorincz, Schubeler et al, 2002). Evidence from MSUS mice suggest that a change in methylation at a subset of CpGs within the identified regions is sufficient to alter local chromatin structure and thereby modify the access that transcription factors have to
the underlying DNA strand (Jaenisch and Bird 2003; Wang, Wysocka et al. 2004; Richards 2006).

A single nucleosome consists of approximately 145 bp wrapped around an octamer of histones. Here, differential methylation was found across stretches of approximately 110-120 bp for CRFR2 and MeCP2, and 60 bp for CB1. Thus, it is possible that abnormal methylation at a subset of these CpGs may affect the wrapping within a particular nucleosome important for establishing the rate of gene transcription. Furthermore, the relatively large change in gene expression and the modest change in DNA methylation is associated with altered behaviour of the animal, however the extent to which each gene contributes to the behavioural phenotype is not known (discussed below in 6.3).

It is also important to realise that while the effect on DNA methylation is small in absolute terms, due to the low levels of overall methylation, when calculated in relative terms these changes are quite substantial and likely to have a considerable biological impact. Thus in F1, an overall 10% decrease in DNA methylation is observed for CRFR2, 40% increase for MeCP2, and 30% increase for CB1 in sperm. The finding that CpG islands are minimally methylated under normal conditions is consistent with previous data, and the biological impact of small changes in methylation has already been demonstrated for several genes in the context of cancer (Wilson, 2007).
The difference in CRFR2 methylation demonstrated by Pyrosequencing in F1 sperm was confirmed by repeating the methylation assays using classical bisulfite sequencing. These assays showed a comparable reduction in DNA methylation on the CRFR2 promoter of approximately 10% in MSUS germ cells compared to control (Fig. 31). Pyrosequencing data presented here also shows that the absolute value of DNA methylation is overall similar in brain, sperm, and lung although the profile of methylation differs slightly across tissues. This suggests the possibility that different mechanisms of establishment and/or maintenance of DNA methylation may be recruited in different tissues, as has been previously reported in the literature (Latham, 1995; Hanel and Wevrick, 2001; Raiche, Rodriguez-Juarez, et al., 2004; Ohgane, Hattori et al. 2005; Watanabe, Uchiyama, et al. 2006).

6.2.2 Aberrant germ cell methylation is associated with a high transmission rate of behavioural abnormalities

The subtle change in DNA methylation in the germline is also associated with a high degree of behavioural transmission of specific traits induced by maternal separation. Consequently, while the behavioural effects of the maternal separation itself are relatively mild, the transmission of these subtle behavioural abnormalities is robust across generations in a sex-specific manner. Thus, while there is correction of anhedonia across generations, there is no reduction in abnormal behaviours resulting from initial exposure to an aversive environment or in time spent immobile when faced with inescapable stress observed in the male line. Considering that altered DNA methylation at a subset of CpGs in the identified region is sufficient to induce changes in gene expression, this implies that MSUS alters DNA methylation not only in 1-5% of germ cells, which would result in 1-5% of inheritance, but rather at

![Figure 31. Comparison of Pyrosequencing and bisulfite sequencing of CRFR2 in F1 sperm. Similar reduction in DNA methylation at the CRFR2 promoter in germ cells between MSUS and control samples whether assessed by pyrosequencing (Pyro.) or bisulfite sequencing (Bisulf.) (Pyro. n=5-6; Bisulf., n=91-95 clones from 1 F1 control and 1 F1 MSUS).](image-url)
individual CpG sites in the identified region with a 1-5% probability (Fig. 32). This is further demonstrated by bisulfite sequencing of individual sperm cells that demonstrated an increase in the proportion of germ cells with DNA profiles characterized by lower levels of methylation (Fig. 20c). In addition, since F2 MSUS are only inheriting one affected allele, the high rate of transmission further suggests two possibilities for the underlying mechanism of transmission, of which one or both may be occurring. The first possibility is that maternal separation induces aberrant expression of factors which regulate DNA methylation inside the sperm head. These factors may act to alter methylation on the paternal allele within the sperm cell, and can be transferred on fertilization, to act on methylation on the maternal allele. The second possibility is that there is a mechanism by which methylation patterns from one allele is copied to the homologous allele after fertilization. Epigenetic mechanisms through which either situation can occur have been previously proposed. The first may occur by paramutation, in which the epigenetic state at one allele is conferred to the homologous allele in a meiotically heritable manner and the second, by transvection, in which the epigenetic state at one allele is conferred to the homologous allele in a mitotically heritable manner (Rassoulzadegan, Magliano, et al. 2002; Herman, Lu et al. 2003; Rassoulzadegan, Grandjean et al. 2006; Ashe and

Figure 32. Two possible explanations for transmission of behavioural traits due to aberrant DNA methylation in germ cell. Representation of two sperm samples with identical levels of overall methylation at each of the five CG sites (80% methylation; black circles). If only one unmethylated CG site (white circle) is necessary for transmission of a certain behavioural trait (purple mice), then example 1 results in a low transmission rate, and example 2 results in a high transmission rate to the next generation.
Whitelaw, 2007; Wagner, Wagner et al. 2008). Both phenomena have been demonstrated in mice and paramutation have been demonstrated to be RNA-mediated (see also 1.3.2).

While the precise nature of the mechanism underlying the high transmission rate of environmentally-induced transgenerational epigenetic effects is not known, this phenomenon has been observed previously in several rodent studies. Similar to the present findings, a model of early abuse induced DNA methylation changes to a similar degree in the brain of both male and female animals exposed to early trauma and the male and female offspring of females exposed to the early abuse (Roth, Lubin et al. 2009). Likewise, chemicals in the pre- and postnatal environment induce a wide range of adult-onset disease states and fertility abnormalities to a similar magnitude from F1 through F4 offspring, also as a result of the transmission of abnormal levels of DNA methylation (Anway, Cupp et al. 2005; Anway, Leathers et al. 2006; Anway, Memon et al., 2006; Anway, Rekow et al. 2008; Nilsson, Anway et al. 2008). Thus, it is likely that there are many circumstances in which one or both of these mechanisms occur and result in a high rate of transmission of environmentally induced abnormalities. However, further research is required to better understand the extent to which these mechanisms are occurring in mammals.

Female offspring of male F1 MSUS mice appear to be more susceptible to transmission of aberrant behavioural traits induced by early trauma than male offspring, except for behaviors on the elevated plus maze which were consistently transmitted to both F2 females and F2 males. The reason for the sex-biased expression of the behavioral traits induced by early trauma is not yet known. One potential mechanism may involve sex steroids, in particular, 17β-estradiol and testosterone. Both have been previously associated with altered level of methylation in a gene-specific manner in the mouse brain (Mani and Thakur, 2006; Singh and Prasad, 2008), and estradiol has also been suggested to decrease DNMT activity (Singh and Prasad, 2008). Thus, it is possible that the presence of male sex steroids may correct transmissible methylation in the brain of male offspring (only in the brain, not in the germline), and/or the presence of female sex steroids may act in concert with altered DNA methylation present in MSUS mice resulting in the observable sex-specific behavioural abnormalities. If the former is occurring, then the data also suggests that the presence of male sex steroids cannot correct abnormal methylation.
of DNA in the germ cell (Fig. 27a, c, e), thus resulting in the transmission of behavioral abnormalities to the F3 generation, despite normal behavior in the male F2 MSUS mice. Indeed, changes in DNA methylation were consistently observed in both F1 and F2 sperm despite the fact that aspects of the behavioral traits were not fully expressed by males. This strongly indicates transmission of the profile of DNA methylation in the germline, with potential correction in the brain. Parent-of-origin susceptibility to transmission of methylation patterns has been previously suggested by sex-dependent effects of imprinted genes (Hager, Cheverud et al. 2008). While such sex-linked epigenetic mechanism is not yet determined, it has been suggested that sex-dependent trans control of expression, i.e., differential expression at a sex-specific modifier locus during gametogenesis, may be responsible (Hager, Cheverud et al. 2008). If females are indeed more susceptible to epigenetic inheritance of stress-induced behavioural traits, this may be a contributing factor to the increased incidence rate of stress-induced affective disorders in the female population. Finally, a bias towards a transmission to females from fathers has been recently corroborated by a new study in mice showing an association of paternal open-field activity with the open-field activity of female but not of male offspring (Alter, Gilani et al. 2009). This data strongly suggests an epigenetic mode of transmission.

6.2.3 Differential methylation resulting from early stress is gene-specific

The high proportion of genes with methylation abnormalities (three of five candidates) results from the fact that these genes were carefully selected, and are not representative of the entire genome. New advances made towards high-throughput analyses across the epigenome will help better establish the extent of methylation changes induced by early stress, as well as the extent of transmission of aberrant methylation to the brain of the offspring. The two genes which did not demonstrate changes in DNA methylation in their CpG islands were both chosen as candidate genes because of the well-known link between the serotonergic system and depression-like behaviours. MAOA is an enzyme that catalyzes the degradation of the neurotransmitter serotonin. A deficiency in MAOA enhances emotional learning (Kim, Shih et al. 1997), and decreases time spent floating in the forced swim test in mice (Cases, Seif et al. 1995). It is also known that MAOA is increased following chronic stress (Filipenko, Beilina et al. 2002). 5HT1a receptors are present both presynaptically as autoreceptors in the dorsal and median raphe nuclei, and
postsynaptically throughout the forebrain including the hippocampus and amygdala. Mice deficient for 5HT1aR demonstrate enhanced fear responses (Kleimenhagen, Gordon et al. 2006; Tsetsenis, Ma et al. , 2007). Additionally, the 5HT1aR is a known target for antidepressants (for a review see Cryan, Valentino et al. 2005), and in vivo studies in humans have shown that patients with panic disorder or major depression have a reduction in 5HT1aR receptor binding in many areas of the brain (Sargent, Kjaer et al., 2000; Nash, Sargent et al. 2008). While it has been generally accepted that methylation levels in the promoter-associated CpG island of a gene is most likely to cause gene expression changes, it is now clear that methylation levels elsewhere within the gene may also have an effect (Flanagan and Wild, 2007; Song, Mahmood et al., 2009). Thus, the possibility that methylation levels may be altered elsewhere within these genes and that this may also affect gene expression of MAOA or 5HT1aR can not be ruled out at this time.

6.2.4 Alterations in CRFR2 protein levels in the brain of F1 MSUS are associated with changes in CRFR2 methylation in the germline

It is interesting that there was no difference in CRFR2 methylation in male F1 MSUS brain, but reduced CRFR2 binding in the hypothalamus and amygdala in the F1 MSUS female brain, and reduced CRFR2 methylation in F1 MSUS sperm. Since female F1 MSUS displayed a relatively milder behavioural phenotype than male F1 MSUS mice, it is likely that male F1 MSUS also demonstrate reduced CRFR2 binding. While this can not be confirmed, it is nonetheless important to consider a possible mechanism by which changes induced by maternal separation in F1 MSUS brain at the protein level may be associated with changes in sperm at the level of the epigenome. It is well known that the central nervous system can regulate gonadal function via the neuroendocrine system, and it has been recently established that there are certain brain areas directly connected with the gonads in a pituitary-independent manner (Gerendai, Toth et al. 2000). Importantly, there are descending connections from the amygdala, and regions of the hypothalamus important in emotional response, including the lateral hypothalamus and paraventricular nucleus of the hypothalamus, to the testis. These connections are known to exert a direct effect on local endocrine release, and have been suggested to be responsible for mediating subtle regulation of gonadal function (Gerendai, Banczerowski et al. 2005).
6.3 Early stress induces changes in gene expression in the subsequent generation

The present findings show that changes in DNA methylation affect several genes involved in stress coping in different brain areas, only a small number of which were identified in this study, and suggest that the observed behavioural phenotype results from the combination of alterations in these genes. The extent to which each individual gene contributes to the behavioural phenotype is not known and is difficult to evaluate at this point. However, all candidate genes were chosen based on their known involvement in stress and emotional pathways. Below is a brief description of these genes and evidence for their involvement in stress-related behavioural responses.

6.3.1 CRFR2

CRFR2 is a seven-transmembrane G-protein coupled receptor, which signals mainly by coupling to Gs, thereby activating adenylyl cyclase and protein kinase A. There are four known ligands for CRFR2: corticotropin releasing factor, urocortin I, urocortin II, also known as stresscopin-related gene, and urocortin III, also known as stresscopin. Corticotropin-releasing factor has a much higher affinity for CRFR1, urocortin I has equal affinities for CRFR1 and CRFR2, and urocortin II, and urocortin III appear to be selective for CRFR2 (for reviews, see Perrin and Vale 1999; Dautzenberg and Hauger 2002; Bale and Vale 2004). In rodents, CRFR2 has two splice-variants, CRFR2α and CRFR2β, which are produced by alternate 5’ exons (Chen, Perrin et al. 2005). CRFR2α is the predominant splice variant in rodent brain and CRFR2β is the predominant isoform in the periphery (Chen, Perrin et al. 2005). This differs from humans in which CRFR2α and not CRFR2β is the predominant isoform in the periphery (Kostich, Chen et al. 1998). In the present study, methylation levels were quantified in the CpG island 5’ to the start site of CRFR2α.

In contrast with CRFR1, CRFR2 is thought to reduce the stress response and has previously been associated with exploration in aversive environments and depression-like behaviours (Bale and Vale, 2004). Pharmacological antagonists to CRFR2 increase exploratory activity in aversive environments in mice (Pelleymounter, Joppa et al. 2002; Pelleymounter, Joppa et al. 2004), and CRFR2 deficient mice spend more time floating in the forced swim test (Pelleymounter,
Joppa et al. 2002; Bale and Vale 2003; Pellemounter, Joppa et al. 2004). These findings mimic the behaviours demonstrated by MSUS mice, which also display reduced CRFR2 gene expression and receptor binding.

6.3.2 MeCP2

MECP2 binds both methylated DNA and transcriptional repressor complexes, and thus was previously thought to function as a global transcriptional repressor (Lewis, Meehan et al. 1992; Nan, Campoy et al. 1997). However, recently it was demonstrated, with the use of MeCP2-null and overexpressing mice, that the majority of genes are activated by MeCP2 and thus, that MeCP2 acts as both an activator and a repressor (Chahrour, Jung et al. 2008). MeCP2 has also been suggested to interact with DNMT1, suggesting that it may guide DNMT1 to sites which require methylation following replication (Kimura and Shiota 2003). Furthermore, biophysical studies have suggested that MECP2 may act in a methylation- and HDAC-independent fashion to induce chromatin condensation via a chromatin-condensing domain (Georgel, Horowitz-Scherer et al. 2003). While the majority of studies involving the functional relevance of MeCP2 have focused on Rett syndrome, a neurodevelopmental disorder resulting from mutations in MeCP2, it has also been demonstrated that mice deficient for MeCP2, demonstrate increased aggression and have a heightened behavioural and physiological response to stress (Gemelli, Berton et al., 2006; McGill, Bundle et al. 2006; Fyffe, Neul et al. 2008). Additionally, MeCP2 has been demonstrated to bind the CRF promoter suggesting that MeCP2 can modulate CRF expression (McGill, Bundle et al. 2006). Furthermore, MeCP2 protein levels in the hippocampus are decreased by chronic stress (Dyuzhikova, Savenko et al. 2006).

6.3.3 CB1

CB1 is a presynaptic receptor which binds to endocannabinoids released into the synapse by the postsynaptic neuron. Once activated they inhibit the release of neurotransmitters. Pharmacological blockade of CB1 or elimination by gene knock-out also alters emotionality in mice (Haller, Bakos et al. 2002; Martin, Ledent et al. 2002; Griebel, Stemmelin et al. 2005; Rutkowska, Jamontt et al. 2006; Steiner, Wanisch et al. 2007). CB1 knockout mice have increased expression of CRF mRNA
in the hypothalamus, decreased expression of GR mRNA in the CA1 subregion of the hippocampus, and release higher levels of ACTH and glucocorticoids during a stressful event (Haller, Varga et al. 2004; Cota, Steiner et al. 2007). Thus, CB1 has been linked with basal HPA-axis function.

6.3.4 The effect of maternal separation is multigenic

Due to the multigenic effect of the maternal separation treatment, the extent to which these genes are mediating the behavioural effects observed can not be determined at this time. It is likely that it is the interaction of many genes that induces the behavioural phenotype seen in MSUS. This is clearly demonstrated by data from bisulfite sequencing of CRFR2 in the F1 sperm cell in which there is a range of different profiles of DNA methylation in both F1 control and F1 MSUS mice. While there is an overall shift towards unmethylated DNA in F1 sperm cells, each F2 MSUS comes from a single sperm cell. Thus, the high rate of behavioural transmission suggests that the behavioural phenotype is not the result of altered methylation in CRFR2 alone, but is likely to involve interactions between several genes in a sex-specific manner. Data from genome-wide methylation and gene expression assays will help to discern the extent of the transmissible changes induced by maternal separation. However, behavioural results from gene-targeting and pharmacological studies do suggest that alterations in the expression levels of CRFR2, MeCP2, and CB1 in MSUS mice are a contributing factor to the aberrant response to stressful environments observed in MSUS mice.

6.4 The use of environmental manipulations as animal models for human situations

6.4.1 Maternal separation involves multiple factors which could contribute to behavioural and methylation abnormalities

Unlike the pharmacological or gene manipulation studies described above, maternal separation induces widespread and global changes in mice exposed to the treatment. This points to both the benefits and disadvantages using differential environments, such as maternal separation, or enriched environment, in animal research. Thus, environmental manipulations like maternal separation offer the
benefit of applying ethologically valid environments to mice, with easy-to-draw parallels to human situations and disorders. For example, mice exposed to MSUS are reared in a stressful and unpredictable environment during the time when they are most dependent on dams for care, clearly mimicking aspects of detachment or poor parental relationships which may occur during childhood in humans. Unfortunately, due to the global nature of this treatment, it is difficult to evaluate the precise cause of the induction of abnormalities in animals exposed to maternal separation. For instance, from the data presented here, it is clear that maternal care provided to pups undergoing maternal separation is perturbed. However, maternal separation in combination with maternal stress has multiple long-term effects on the behavioural response of the dam, including reduced motivation to explore and inappropriate behaviours towards aversive environments, suggesting that a variety of neurochemical and hormonal pathways may be affected in F0 MSUS dams during the treatment (Figs. 3, 4). Interestingly, in F0 control dams, the natural fluctuation in caregiver traits which occurs across animals is correlated with their innate behavioural responses to the aversive areas of the elevated plus maze (Fig. 4d-f). However, this was not the case in F0 MSUS dams, further demonstrating that behaviour in F0 MSUS dams is greatly perturbed by the maternal separation paradigm used here. Therefore, considering the obvious stress which the dams undergo during maternal separation, and its long-term ramifications, it is especially important to consider that there are factors other than maternal care received, which may be the cause of the behavioural abnormalities and associated methylation changes observed in F1 MSUS mice. In particular, along with abnormal maternal care, the effect of the stress on the dam is likely to alter milk content, including stress hormone levels present in the milk that is provided to the pups.

Additionally, the effects of the maternal separation paradigm may be due to factors which occur during the period of separation itself. For instance, during the period of separation, the nest temperature of the pups is not regulated. Nest temperature has previously been shown to be important for healthy development in mice, most likely due to the additional energy expenditure, and brown fat thermogenesis required for survival at reduced temperatures (Haidmayer and Hagmüller 1981; Schneider, Hamilton et al. 1987; Bult and Lynch 2000). This additional energy expenditure may be especially difficult for the pups as milk is not provided during the three-hour separation. Furthermore, we have observed that licking/grooming activity occurs
quite frequently (10-15% of the time) during the first postnatal week under normal conditions (data not shown), and this important aspect of postnatal environment is absent during the three-hour separation. Previous findings have suggested licking/grooming to be a particularly important aspect of the effects of maternal separation as several studies have shown that the effects of maternal separation on rat pups are not present if pups are artificially stroked and fed during the separation period (van Oers, de Kloet et al. 1999; Groer, Hill et al. 2002). Due to the number of factors likely to contribute to the phenotype presented here, it is not possible to pinpoint at this time which are involved, and to what extent their involvement is necessary for the induction of the behavioural phenotype or for behavioural transmission to occur. However, it is likely that a combination of these factors induced by the severity of the treatment is required for such persistent transgenerational changes to occur.

**6.4.2 Extrapolating postnatal manipulations from mouse to human**

Another limitation to postnatal manipulations in mice occurs when drawing parallels between the animal model and human circumstances due to differences in developmental rate. This is because mice are born extremely immature; anatomical studies have suggested that humans are born at a stage of development similar to that of third postnatal week in the mouse (Clancy, Darlington et al. 2001). That being said, mice are able to live independent from the dam as early as two to three weeks post-delivery (soon after eye-opening), while humans are dependent on their parents for many years following birth. Thus, the maternal separation treatment occurs during the entire two week period, from a time of extreme immaturity which might parallel prenatal development in humans, to a time of near independence from the litter, making it difficult to extrapolate, from the mouse data, a critical period for the effects of differential environment in humans.

**6.4.3 Enriched environment as a means of reversing disease states**

Similarly, many factors are known to be involved in the effect of enriched environment. Mice living in enriched environment have greater somatosensory, olfactory, and visual input, as well as increased cognitive stimulation due to enhanced spatial complexity, object recognition, and novelty within the environment.
These enhancements to the environment are known to affect various cortical areas, the cerebellum, and the hippocampus (for reviews, see Nithianantharajah and Hannan 2006; Laviola, Hannan et al. 2008). In particular, enriched environment is thought to increase the expression of genes which promote cellular plasticity and reduce cell death, such as the neurotrophins (Faherty, Raviie Shepherd et al. 2005; Franklin, Murphy et al. 2006; Wolf, Kronenberg et al., 2006; Spires, Grote et al. 2004). Enriched environment has also been effective in rodent models of Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and traumatic brain injury (Passineau, Green et al. 2001; Spires, Grote et al. 2004; Faherty, Raviie Shepherd et al. 2005; Wolf, Kronenberg et al. 2006).

In addition to its effectiveness in the above-stated disease models, enrichment has also been shown to be beneficial in ameliorating the symptoms of stress-induced depression. In rats exposed to juvenile stress (PND27-29), living in enrichment during adulthood reversed depressive-like behaviours in two-way shuttle avoidance learning (Ilin and Richter-Levin, 2009). Further, several studies have shown that enriched environment post-weaning is effective in reversing depressive-like behaviours in the forced swim test induced by either prenatal or postnatal stress (Yang, Li et al. 2006; Bhansali, Dunning et al. 2007). However, similar to the present experiment, it has also been reported that post-weaning enrichment can not reverse the depressive-like behaviours in the forced swim test induced by postnatal stress (Cui, Yang et al. 2006). Such conflicting reports of the effect of enriched environment is frequent due to the lack of standardization of enrichment protocols across labs and experiments. In fact, Donald Hebb first used the term when comparing observations he had made about the behavioural differences in rats allowed to roam freely in his home versus those in the laboratory (Hebb 1947). While enriched environment experiments run currently are more controlled than this, there are still several factors which remain variable from lab to lab. For instance, many enriched environments involve the placement of objects which are rotated during the treatment. These objects, as well as the number of times these objects are changed, are not standard and the objects themselves are often not well described in published accounts. The use of running wheels in the enriched environment is also not standardized, neither in their usage or in the number of running wheels available. Enrichment can also involve housing one strain of mice with a strain of mouse less susceptible to stress (Holmes, le Guisquet et al. 2005; Bhansali, Dunning et al. 2007). The protocol itself
may vary in whether the animals are housed permanently in an enriched environment, or placed in an enriched environment for a period of time each day.

Due to the variety of enrichment protocols currently in use, a standardized version of enriched environment, called the Marlau cage (Viewpoint) was used in the present experiment. This cage was created to reduce variation and increase reproducibility across laboratories. The enriched environment provided by the Marlau cage provides increased social interaction (12 mice per cage, instead of 4), increased somatosensory and visual input, voluntary exercise and increased motor stimulation provided by the running wheels and larger cage size, as well as increased novelty and cognitive stimulation due to the rotation of maze configurations which need to be learned or recalled several times during the week. Thus, reversal of the depressive-like behaviour demonstrated in the tail suspension test may be the result of one, or a combination of these factors. Despite this, it is interesting to note that reversal due to housing in the Marlau cage is only present in the tail suspension test, and not in the forced swim test, suggesting some specificity in its effects.

6.5 Changing views on the impact of methylation on gene transcription

The findings presented here bring attention to a common perception of DNA methylation which has recently been called into question. Traditionally, DNA methylation has been viewed as a silencing mechanism for gene expression. Here, reduced methylation of CRFR2 and increased methylation of CB1 and MeCP2 have all been associated with decreased gene expression. This is consistent with several recent reports that provide evidence that DNA methylation in the promoter region is associated with, not only decreased gene expression, but also increased gene expression. For instance, in the human brain, DNA methylation in the promoter region of the 5HT2A gene was shown to correlate with higher gene expression (Polesskaya, Aston et al. 2006). In human somatic cells, many active genes were shown to have methylated promoters, while many inactive genes had unmethylated promoters (Weber, Hellmann et al. 2007). Additionally, recent evidence that a methyl Cpg-binding protein, MeCP2, acts as both an activator and a repressor of gene transcription, further suggests that decreased methylation can be mechanistically associated with decreased gene expression (Chahrour, Jung et al. 2008). Thus,
while there are several examples to support the traditional view that increased promoter methylation is associated with gene silencing, there is growing evidence that the opposite also does occur.

Further, DNA methylation has been recently shown to be highly dynamic and rapidly cycling. Cyclical methylation and demethylation of CpG dinucleotides was found in the promoter region of five different transcriptionally active genes, including estrogen receptor α, with a periodicity of approximately 100 minutes (Kangaspeska, Stride, et al. 2008). Importantly, increased methylation at the promoter region coincides with the initiation of transcription (Kangaspeska, Stride, et al. 2008), suggesting that DNA hypomethylation can be mechanistically associated with decreased transcription. Furthermore, DNMT3a/b was found to be recruited to the DNA strand when cyclical demethylation occurs demonstrating that it can have both methylating and demethylating activities (Metivier, Gallais et al. 2008). In vitro, DNMT3a/b was demonstrated to have deamination activity (Metivier, Gallais et al. 2008). Thus, the induction of deaminated cytosines by DNMT3a/b, and their repair via base excision repair, are a proposed mechanism for cyclical demethylation in promoter regions (Metivier, Gallais, et al. 2008). These very recent findings have greatly changed the prior view of DNA methylation as generally permanent, and demonstrates the need to better define the factors mediating the effects of DNA methylation on gene transcription.

6.6 Conclusions

Early stress induces behavioural deficits which are transmissible to two generations downstream. Male MSUS mice demonstrate a significant increase in propensity to enter into aversive areas in the free exploratory paradigm and elevated plus maze, a tendency to enter into the aversive center of the open field, as well as enhanced depressive-like behaviours in the forced swim test and sucrose consumption test. Female MSUS mice demonstrate a significant increase in propensity to enter into aversive areas in the elevated plus maze. The offspring of male MSUS mice also exhibit aspects of these behavioural abnormalities in a sex-specific manner: male offspring exhibit similar behavioural effects in the elevated plus maze, and female offspring exhibit similar behavioural effects in the free exploratory paradigm, open
field, elevated plus maze, and forced swim test. Comparably, the offspring of female MSUS mice exhibit similar behavioural abnormalities in a sex specific-manner: male offspring in the free exploratory paradigm and both male and female offspring in the elevated plus maze. F3 MSUS mice derived from F2 MSUS males also demonstrate similar behavioural abnormalities in a sex-specific manner: females in the free exploratory paradigm, open field, and elevated plus maze, and males in the forced swim test. This behavioural transmission is associated with aberrant DNA methylation in the germ line of male animals exposed to maternal separation and in the female brain and male germ line of their offspring. Aberrant methylation is associated with changes in gene expression which are likely to contribute to the behavioural abnormalities present in the offspring of animals exposed to maternal separation. This provides evidence for a rapid form of environmentally-driven evolution distinct from traditional views that the evolutionary process is driven solely by random mutations in the DNA sequence itself. Instead, it suggests the prospect of a mechanism for the environment to, not only directly influence the organism, but its offspring as well. The extent to which this occurs, as well as the persistence and plasticity of the mechanism, has yet to be determined. However, due to the multigenerational transmission demonstrated here, and suggested by previous epidemiological studies in humans, transgenerational epigenetic inheritance is likely to continue to be of interest to people across scientific spectrums in the coming years.

Lastly, the transmissible behavioral traits induced by our early stress model, such as inappropriate response to stressful environments and depressive-like behaviours, are reminiscent of symptoms of major neuropsychiatric disorders such as borderline personality disorder and depression. Thus, the present findings may be relevant when considering the underlying mechanisms and incidence rate of diseases within clinical psychiatry and may ultimately provide the basis for research into novel therapeutic strategies to prevent or treat these disorders.

6.7 Future directions

The results discussed here lead to several important experimental questions which may be developed in future studies. Evidence presented here suggests that abnormal DNA methylation patterns are responsible for the transmission of
behavioural abnormalities across generations. Although the mechanisms that alter DNA methylation in the present model are not known, they may involve changes in DNMTs, non-coding RNAs, and/or chromatin remodeling complexes (Kawasaki and Taira 2004; Siedlecki and Zielenkiewicz 2006; Aravin, Sachidanandam et al. 2007; Carmell, Girard et al. 2007), or a combination of these processes. One intriguing possibility for the mechanism underlying germ line abnormalities in DNA methylation has come from the recent discovery of sperm/oocyte specific non-coding small RNAs, termed Piwi-interacting RNAs (piRNAs). piRNAs are dynamically expressed during the development of germ cells, and are thought to be key to the establishment of methylation patterns in the germ cell (Aravin, Sachidanandam et al. 2008). This is particularly interesting in regards to the present study because aberrant RNA content has been shown to be transmitted through meiosis to the fertilized embryo (Rassoulzadegan, Grandjean, et al., 2006; Wagner, Wagner, et al., 2008). Further, abnormal piRNA expression would alter methylation in a locus-specific and gene-specific manner, similar to that observed in the present study. This possibility is currently under investigation.

Additionally, both short-term and long-term housing in enriched environment was able to reverse one aspect of depressive-like behaviours demonstrated in mice exposed to maternal separation. It is expected that this reversal in one depressive-like behaviour would also be associated with an absence in the observed transmission of increased immobility in the tail suspension test to F2 MSUS. It would also be expected that blockade of one aspect of the transmitted behavioural phenotype would be associated with partial correction in aberrant DNA methylation in germ cell from maternal-separated mice.

It is also interesting that short-term enriched environment induced increased time spent floating in the forced swim test in control mice, but not in MSUS mice. This suggests that MSUS mice may be resistant to certain forms of stress in adulthood. Resistance to chronic stress (chronic social defeat stress) has also been induced with chronic systemic treatment with histone deacetylase (HDAC) inhibitors (Tsankova, Berton et al. 2006). This raises the question of whether aberrant DNA methylation is also associated with changes in histone acetylation in adult mice subjected to maternal separation as pups.
Finally, due to the volume of human studies suggesting the presence of transgenerational epigenetic inheritance, and the number of disorders already linked with aberrant methylation of specific genes, it would be interesting to quantify methylation in germ cells of patients with and without stress-induced affective disorders across generations to determine if aberrant methylation in genes previously associated with these disorders may indeed be observed across generations. This type of evidence in the human population will draw these findings closer to clinical relevance and help shift the focus towards the epigenome as a target for novel pharmaceuticals in the future.
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Appendix
Epigenetic inheritance in mammals: evidence for the impact of adverse environmental effects

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Abstract

The epigenome is the overall epigenetic state of a cell, and represents the ensemble of chromatin modifications. It is an essential mechanism for the regulation of the genome that depends on modifications of DNA and histones, but does not involve any change of the DNA sequence. It was previously assumed that in order for appropriate cellular development and differentiation to occur in mammals, the epigenome was fully erased and reestablished between generations. However, several examples of incomplete erasure at specific genes have been reported, and this is suggested to be associated with the epigenetic inheritance of gene profiles. Although the existence of such a mode of inheritance has been controversial, there is increasing evidence that it does occur in rodents and humans. In this review, we discuss the evidence that adverse environmental factors can affect not only the individuals directly exposed to these factors, but also their offspring. Because the epigenome is sensitive to environmental influence and, in some cases can, in part, be transmitted across generations, it provides a mechanism for the transgenerational transmission of the impact of environmental factors. Environmental factors examined include exposure to toxicants, diet, and postnatal care. DNA methylation is the main mechanism discussed in this review.

Keywords: epigenetic, DNA methylation, transmission, nutrition, chemical toxicants, postnatal environment
It is commonly accepted that heritable information is transmitted to offspring through sequences of DNA, and that any change of the DNA sequence during transmission is random, and not influenced by environmental factors (except for mutagens). However, recent work in the field of epigenetics has proposed that inheritance of DNA sequences is not the only mechanism underlying the transgenerational transmission of physical, behavioural, and emotional traits in mammals. Further, it is now known that the epigenome can be modulated by a variety of environmental factors, including chemicals, nutrition and early environment, as well as by aging (Anway et al., 2005; Anway et al., 2006; Roth et al., 2009; Waterland et al., 2006; Weaver et al., 2004; Weaver et al., 2005; Wilson and Jones, 1983). The epigenome therefore provides an important interface between genes and the environment, and may provide a mechanism for a rapid form of environmentally-driven transgenerational adaptation.

Conrad Waddington coined the term “epigenetics” in the 1940s to describe gene-environment interactions that ultimately lead to a particular phenotype (Waddington, 1942). Waddington originally used this term in a developmental context to depict the permanent changes in gene activation and deactivation required for cellular differentiation. While the mechanisms for these changes were unknown at the time, they are now considered to be the result of altered DNA methylation and/or post-translational chemical modifications of the chromatin (Holliday and Pugh, 1975; Riggs, 1975; Scarano, 1971). Thus, the current use of the term has shifted to emphasize mitotically or meiotically heritable changes in gene expression, that are not due to any alteration in the nucleotide sequence, but rather to modifications of the DNA molecule itself and of the chromatin. These modifications on the DNA include biochemical alterations of specific base pairs on the DNA, in particular methylation of CpGs (cytosines immediately followed by a guanine), or of core or variant histone proteins, as well as DNA looping or
In mammals, the most common epigenetic modification of the DNA strand involves the enzymatic transfer of a methyl group to the fifth position of cytosine residues in CpG dinucleotides (Richards, 2006). DNA methylation has many well-known roles in the mammalian organism. During development, it is necessary to establish tissue-specific patterns of gene expression (Scarano, 1971). It is also crucial for X-chromosome inactivation, in which one X-chromosome is silenced in females, and parental imprinting, in which one parental allele is inactivated (Delaval and Feil, 2004; Riggs, 1975). The pattern of DNA methylation in CpG islands located within or near promoter regions is known to affect the transcription rate of the gene by altering local chromatin structure and thereby modifying the access of transcription factors to DNA (Richards, 2006; Wang et al., 2004). DNA methylation patterns are established and maintained by DNA methyltransferases (DNMTs), methyl CpG-binding proteins and, as recently demonstrated, small RNA molecules (Chahrour et al., 2008; Cheng, 1995a; Cheng, 1995b; Fan and Hutnick, 2005; Matzke and Birchler, 2005; Ooi et al., 2009; Wassenegger, 2005; Zemach and Grafi, 2007).

Transgenerational transmission of epigenetic information

RNA-mediated paramutation in the mouse

A well-characterized example of transmission of epigenetic information across generations is paramutation. Paramutation is a phenomenon by which the silencing of one allele is meiotically inherited by interaction in trans with the homologous allele (Ashe and Whitelaw, 2007). Most examples of paramutation reported to date are in plants (Ashe and Whitelaw, 2007; Bond and Finnegan, 2007; Chandler, 2007), but
paramutation has also now been demonstrated in the mouse, firstly at the Kit locus that codes for a tyrosine kinase receptor (Rassoulzadegan et al., 2006; Wagner et al., 2008). Mice heterozygous for Kit mutations have reduced Kit mRNA expression, and an altered pigment pattern (white spots). However, some of the offspring from heterozygous Kit mice have a similar altered pigmentation, despite carrying two wild-type alleles (Rassoulzadegan et al., 2006). Abnormal RNA in the sperm of Kit mutant mice was suggested to be responsible for this phenomenon because microinjection of sperm RNA from Kit mutant mice, or injection of miRNAs targeting Kit mRNA reproduce this effect (Rassoulzadegan et al., 2006). These results suggested that functional information not encoded in the DNA sequence itself, but likely to be carried by RNA, can be transmitted through the germ line. This idea was corroborated by a recent study demonstrating that injection of a miRNA targeting cyclin-dependent kinase 9 (Cdk9), a regulator of cardiac growth, in fertilized mouse eggs induces cardiac hypertrophy in adult animals (Wagner et al., 2008). This correlated with the presence of trace amounts of the miRNA in sperm cells. Importantly, the cardiac hypertrophy was inherited across at least three generations, and mimicked human hypertrophic cardiomyopathy, a disease that is often familial (Wagner et al., 2008). The injection of a $\text{miR}-124$ microRNA into fertilized eggs resulting in a giant phenotype in the progeny of transgenic males was also recently reported as a model of RNA-mediated heritable epigenetic modifications (Grandjean et al., 2009).

**Epigenetic inheritance at endogenous alleles Agouti viable yellow ($A^{vy}$) and axin-fused (Axin$^{Fu}$)**

The agouti viable yellow ($A^{vy}$) and axin-fused (Axin$^{Fu}$) alleles are metastable epialleles, characterized by an inter- or intra-individual variability in expression state. Here, variations in expression are not the result of any genotypic alteration, but result from
modifications in the level of DNA methylation of an intra-cisternal A particle (IAP) long terminal repeat. This IAP is a retrotransposon located upstream of the coding sequence in the case of $A^v$, and within intron 6 in the case of $Axin^{Fu}$ (Morgan et al., 1999; Rakyan et al., 2003). These variations in DNA methylation and gene expression induce a diversity in phenotypes that occur despite the genetic identity (isogenic $A^v$ or $Axin^{Fu}$ mice). The ectopic expression of the $A^v$ protein results in yellow fur, obesity, and diabetes and increases tumor susceptibility, while the ectopic expression of $Axin^{Fu}$ induces a kinked tail (Duhl et al., 1994; Reed, 1937). These naturally occurring variable phenotypes resulting from differential methylation can be transmitted to the subsequent generation, paternally for $A^v$, and both maternally and paternally for $Axin^{Fu}$ (Blewitt et al., 2006; Morgan et al., 1999; Rakyan et al., 2003).

Further, the level of methylation of $A^v$ and $Axin^{Fu}$ DNA methylation and gene expression can also be modulated by environmental factors, in particular maternal diet. Administration of a methyl-rich diet to pregnant mouse females was shown to increase the level of DNA methylation at the $A^v$ and $Axin^{Fu}$ genes in the offspring, and induce the associated variability in phenotype (Waterland et al., 2006; Waterland and Jirtle, 2003). This is most likely mediated by in utero effects occurring as a result of the methyl supplement. Somewhat unexpectedly, diet-induced changes in $A^v$ methylation are not transmitted maternally to the next generation (Waterland et al., 2007). Thus, while the differential methylation of IAPs in $A^v$ and $Axin^{Fu}$ genes can induce variable phenotypes across generations in isogenic mice, data from $A^v$ mice suggest that the impact of maternal diet on these phenotypes may be reset between generations.

The $A^v$ case provides an additional example of transgenerational mechanisms that are independent of epigenetic variation at the $A^v$ gene (Waterland et al., 2008). $A^v$ mice
have a tendency to become obese when adult, even when fed regular mouse diet ad libitum (Duhl et al., 1994; Waterland et al., 2008). Interestingly, the interindividual variation in maternal obesity demonstrates a transgenerational amplification of body weight across generations, which is independent of methylation at $A^{vy}$ (Waterland et al., 2008). Furthermore, diet-induced hypermethylation blocks the transgenerational effect of maternal adiposity on the body weight of their offspring (Waterland et al., 2008). Thus, while diet-induced hypermethylation at $A^{vy}$ is not transmitted, methyl supplementation may interact with epigenetic mechanisms at other genomic loci, in particular those involved in body weight regulation (Waterland et al., 2008).

**DNA methylation and familial cancer**

Aberrant genome-wide changes in DNA methylation have been consistently observed in cancer cells. Although a global hypomethylation is often detected in tumors, localized increases in DNA methylation in the promoter-associated CpG islands of genes also occur (Fleming et al., 2008; Wilson et al., 2007). The cause of such concomitant hypo- and hypermethylation is not known, but could result from local alterations in DNMTs, which are expressed at higher levels in tumor cells (Fleming et al., 2008; Jones and Baylin, 2002). Global hypomethylation is linked to carcinogenesis because it can induce aberrant gene expression, chromosomal instability, reactivation of retrotransposons, and/or loss of imprinting (Fleming et al., 2008; Wilson et al., 2007). In contrast, hypermethylation in CpG islands may lead to a deactivation of tumor suppressor genes that should normally be active (Ahuja and Issa, 2000; Fraga et al., 2007). These mechanisms provide a potential treatment strategy in preventive and therapeutic medicine. Indeed, hypomethylating agents like decitabine 6 and 5-azacytidine are currently used for the treatment of cancer (Kurkjian et al., 2008).
Hypermethylation at the promoter of two tumor suppressor mismatch repair genes, \textit{MLH1} and \textit{MSH2}, has been associated with hereditary non-polyposis colorectal cancer (Chan et al., 2006; Herman et al., 1998; Hitchins et al., 2007). Hypermethylation of both \textit{MLH1} and \textit{MSH2} was also observed in the germline, albeit in a low proportion of cells (Chan et al., 2006; Suter et al., 2004). Intriguingly, the \textit{MLH1} epimutation appears to be more easily transmitted through the maternal line, raising the possibility that epigenetic errors may be more likely to occur during oogenesis than spermatogenesis (Fleming et al., 2008). The heritable germline epimutation described here suggests transgenerational epigenetic inheritance. However, because the subjects are not genetically identical, it is possible that DNA variants are present within the family, and may predispose the individuals to an atypical epigenetic state at each generation (Chong et al., 2007). Indeed, it was recently demonstrated that a deletion in the last exons of a gene located directly upstream of \textit{MSH2} correlates with epigenetic inactivation of the \textit{MSH2} allele. This suggests that, at least in the case of \textit{MSH2}, DNA variants and not epigenetic inheritance, may lead to epigenetic alterations present in each generation (Ligtenberg et al., 2009).

**Transgenerational transmission of environmental effects**

**Epigenetic inheritance and environmental toxicants**

There is accumulating evidence that chemical toxicants have detrimental effects not only on individuals directly exposed to the toxicant, but also on their offspring. One of the most dramatic examples is with diethylstilbestrol, a synthetic nonsteroidal estrogen prescribed in the 1970s to prevent miscarriage in women with prior history. While the drug helped pregnancies to go to term, it induced severe developmental abnormalities
and increased the risk of developing breast cancer and a rare form of adenocarcinoma in girls whose mothers was exposed to diethylstilbestrol during the first trimester of pregnancy (Palmer et al., 2006). Further, the risk of cancer appeared to be transmitted to the following generation. A clinical study reported that a 15 year-old girl, whose maternal grandmother was exposed to diethylstilbestrol during pregnancy, was diagnosed with a very rare case of small cell carcinoma in the ovary (Blatt et al., 2003). A larger number of maternal granddaughters than expected also developed ovarian cancer (Titus-Ernstoff et al., 2008). Although these findings are among the first and need to be confirmed by further transgenerational studies, they suggest that the detrimental effect of a drug can be transmitted across generations. Such transgenerational effect of diethylstilbestrol was also observed in mice. Similar to humans, perinatal exposure to diethylstilbestrol induced abnormalities in uterine development and uterine cancer in both F1 and F2 generations. These abnormalities were suggested to result from aberrant DNA methylation in a gene that controls uterine development (homeobox gene HOXA10) and in uterine cancer genes (Bromer et al., 2009; Li et al., 2003; Newbold et al., 2006; Walker and Haven, 1997).

Transgenerational transmission of the detrimental effects of endocrine disruptors present in our environment was also demonstrated in rats, through both females and males. Rats exposed to the endocrine disruptor vinclozolin, a fungicide commonly used for agricultural fruit crops, or the pesticide methoxychlor, during the period of gonadal sex determination (embryonic stage E8-E15, F1), have reduced epididymal sperm counts and sperm motility, and increased spermatogenic cell apoptosis (Cupp et al., 2003; Uzumcu et al., 2004). These drug-induced traits are transmitted to the male offspring through the male germline down to three generations from exposure to the toxicant (F2-F4), and were associated with aberrant DNA methylation in sperm (Anway et al., 2005).
Exposure to vinclozolin from E8 to E14 was also found to induce pregnancy abnormalities, including uterine hemorrhage and/or anemia, down to two generations in females (Nilsson et al., 2008). Further to affecting fertility, vinclozolin was also found to increase the incidence rate of tumor formation in aging males exposed to vinclozolin prenatally (F1) and their offspring (F2-F4) (Anway et al., 2006). Thus, it is now apparent that exposure to chemical toxicants during key periods of development impacts fertility and tumor development across multiple generations via transmission through both females and males. These effects persist across the lifespan and are still present in aged animals. The mechanism of transmission itself is suggested to be the result of aberrant DNA methylation in the germline.

**Epigenetic inheritance and diet**

It is now becoming clear that poor nutrition or reduced food availability can have detrimental effects across several generations. A marked example of such effect is of women subjected to severe food restriction during the last trimester of pregnancy, due to Nazi embargo on food supplies in Western Holland during World War II. Babies born from these women were reported to have lower birth weight, and this was also observed in the subsequent generation despite no further dietary restriction during conception or rearing. These observations suggest a transgenerational effect of diet on birth weight (Susser and Stein, 1994). A recent study also reported that paternal grandmother’s and grandfather’s food supply is linked to the risk of mortality in granddaughters and grandsons respectively. This was observed when food supply was insufficient during the slow growth period in mid-childhood in both grandparents, or during early pre- and postnatal life of the grandmother (Pembrey et al., 2006).

A similar phenomenon was observed in experimental animals, in particular in rats
malnourished before or during gestation (Cowley and Griesel, 1966; Zamenhof et al., 1971). Like for the Dutch famine, female rats fed a low protein diet prior to and during gestation delivered pups with lower body and brain weight, an effect that correlated with reduced level of DNA and protein in the brain. This reduction was not observed when food restriction occurred after delivery. But the effect was inherited by the offspring of the malnourished pups which also had abnormally low brain and body weight (Zamenhof et al., 1971). Second generation offspring had comparable low birth weight, slower maturation rate, and displayed poor cognitive performance in the Hebb-Williams maze (Cowley and Griesel, 1966). Since these studies in rodents were carried out prior to molecular advances in epigenetics, the mechanisms underlying the transmission of the effects of a poor diet remain unknown, but would be interesting to investigate.

Due to the increase in obesity in Western countries, the transgenerational effect of a maternal high-fat diet on subsequent generations has also been studied. Maternal high-fat diet exposure increases body length and reduces insulin sensitivity two generations downstream from the initial exposure (Dunn and Bale, 2009). These abnormalities can be transmitted both maternally and paternally, and the effect is further amplified when female and male offspring of maternal high-fat diet exposure are bred (Dunn and Bale, 2009).

These findings in human and rodent suggest that epigenetic and environmental factors are involved in the transmission of the effect of insufficient or excessive diet, but these factors remain unknown. They are likely to be multiple, but a recent study showed that alterations in DNA methylation of the gonadotrophin hormone secretagogue receptor (GHSR) promoter in the brain of offspring of mice exposed to a high-fat diet (Dunn and Bale, 2009). More research is required to determine the extent of the contribution of
epigenetic mechanisms.

Epigenetic inheritance and poor early environment

The influence of the quality of the environment early in life has been under intense research. It is now largely recognized that early abuse or trauma strongly affects individuals throughout their adult life. Long-term studies revealed that infant attachment predicts the ability of the child to form appropriate peer relationships, and his/her sociability, risk-taking behaviors, school success or dropout rate. Success in school is indeed an important factor that can be predicted with 77% accuracy, based solely on the quality of early care (Harper, 2005; Sroufe, 2002). Further, maltreatment and childhood trauma are known to increase the risk of depression and anxiety disorders in adult individuals (Iversen et al., 2007). While there is a high level of transmission of anxiety disorders and a strong link between parent and child anxiety disorders, this can not be explained by parenting alone, but instead can be predicted by constitutional factors, like temperament (Dierker et al., 1999; Manassis et al., 1995; Merikangas et al., 1998; Shamir-Essakow et al., 2005; Weissman et al., 1984).

The persistence of disorders that may have arisen from maltreatment in childhood drove researchers to investigate whether DNA methylation plays a role. In rat, the amount of maternal care provided during early development has been shown to influence the pattern of DNA methylation in the pup’s brain when adult. Based on naturally occurring individual fluctuations in maternal care provided by female rats, Michael Meaney and colleagues explored the mechanisms that may underlie such fluctuations. Female rats were selected based on their nurturing ability and placed in two treatment groups, high licking/grooming and arch-back nursing (ABN-LG), and low ABN-LG. Studies of pups
reared by high ABN-LG dams showed that these pups have higher glucocorticoid receptor (GR) expression in the hippocampus and glucocorticoid feedback sensitivity than pups from low ABN-LG dams, when adult (Weaver et al., 2004). These effects were associated with lower DNA methylation in the promoter region of GR and an associated increase in binding of the nerve growth factor-inducible protein-A transcription factor to the promoter region of GR (Weaver et al., 2004). They further showed that all effects of high ABN-LG rearing could be reversed in the adult offspring by intracerebroventricular infusion of L-methionine, which acts as a methyl-group donor (Weaver et al., 2005). This demonstrates that changes in the epigenome established by the environment during early development may be reversed by environmental stimuli even in adults, emphasizing the plasticity of DNA methylation in the adult brain.

Findings from rodent experiments were recently corroborated by a study in humans, and implicated the early environment in the establishment of methylation patterns. This study provided evidence that childhood abuse is also associated with abnormal methylation in the adult human brain (McGowan et al., 2008). Methylation in the neuron-specific glucocorticoid receptor promoter in the brain of suicide victims who experienced childhood abuse was significantly higher than in suicide victims who were not abused, or in controls. The increase in methylation was associated with decreased glucocorticoid receptor expression (McGowan et al., 2008).

Further to altering methylation, a poor postnatal environment was also recently demonstrated to result in transgenerational transmission of methylation anomalies in the rat brain. Pups receiving daily abusive maternal care including stepping, dropping, dragging and active avoidance, from non-biological dams during postnatal day (PND) 1 to 7 have reduced BDNF expression and increased methylation of the BDNF gene in the
prefrontal cortex when adult (Roth et al., 2009). The offspring of females exposed to maltreatment during early life also showed increased BDNF methylation in the prefrontal cortex (Roth et al., 2009). The findings are particularly interesting because, in humans, increased methylation of the BDNF gene in the frontal cortex is associated with major psychoses such as schizophrenia and bipolar disorder (Mill et al., 2008). Thus, this study in rat suggests that maltreatment during early development may not only predispose individuals to major psychoses, but also their offspring via transmission of abnormal methylation patterns across generations. Importantly, maltreatment during early development induced poor maternal care in the female offspring when they themselves, had pups (Roth et al., 2009). Thus, the alterations in BDNF methylation in the brain of the offspring from abused dams may result from either transmission of methylation via the gametes, or changes in methylation elicited by the transmission of poor maternal care across generations. Interestingly, cross-fostering did not fully reverse alterations in methylation levels, suggesting that postnatal experience is not the only factor contributing to this transmission (Roth et al., 2009).

Conclusions

A growing number of reports describe transmission of complex behavioral traits and adult-onset disease states across multiple generations that are non-genomic. The extent to which transgenerational epigenetic inheritance occurs, as well as the persistence and plasticity of this mechanism, still need to be determined. A point sometimes overlooked in transgenerational epigenetic research is the need for transmission two generations downstream from the original manipulation (reviewed in Skinner, 2008). While transmission to the offspring of perturbed animals may suggest epigenetic and not environmental factors, it does not completely rule out environmental
influence, because the cells that ultimately generate the offspring are present at the time of treatment. Thus, transmission to a subsequent third generation is important to demonstrate that the phenotype is indeed transgenerational, germline dependent, and not a direct effect of the treatment itself (Skinner, 2008). Several studies have taken this point in consideration and provided evidence for transgenerational transmission across several generations. Further, growing interest in the field also provided new experimental evidence that alteration in DNA methylation in germ cells may underlie epigenetic inheritance. Because a wide range of environmental factors are known to be associated with changes in DNA methylation in both humans and rodents, it is now clear that epigenetic inheritance may be occurring on a much broader scale than previously thought. Finally, further to DNA methylation, other epigenetic mechanisms such as RNA interference, histone posttranslational modifications or DNA repair may also contribute to epigenetic inheritance (Godmann et al., 2009).
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Epigenetics of Brain Disorders

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The brain is one of the most complex and most complexly regulated organs in the human body and its proper functioning requires that regulatory networks are tightly controlled. Over the past decade, it has become increasingly apparent that epigenetic mechanisms are an essential part of these networks and largely contribute to higher-order brain processes such as synaptic plasticity, and learning and memory. Not surprisingly, therefore, can a dysfunction thereof lead to severe brain disorders including neuro-developmental diseases such as Rett syndrome, Fragile X syndrome and Rubinstein Taybi syndrome, neurodegenerative disorders such as Huntington’s and Alzheimer’s disease, as well as autism. Moreover, dysregulated epigenetic mechanisms also underlie psychiatric disorders such as depression and schizophrenia, and they can also determine, in part, how the brain deals with stressful situations in infancy and adulthood.

In this chapter, we will describe the evidence that suggests how a dysregulation of epigenetic mechanisms is a major factor in the etiology and occurrence of Rett syndrome and Alzheimer’s disease as examples of neurodevelopmental and neurodegenerative diseases, respectively. Further, we will also cover the involvement of epigenetic mechanisms in depression and schizophrenia to illustrate the role of the epigenome in psychiatric disorders. Where applicable, we will describe animal models that reproduce human pathophysiological features and that are currently explored to test potential therapeutic treatments targeting the epigenetic machinery. Finally, this chapter ends with a description of how early-life stress can have life-long influence on emotional behaviors.
Introduction

Epigenetics is most commonly defined as the study of alterations in gene function which are heritable through both mitosis and meiosis, but which cannot be explained by changes in the DNA sequence itself [1] (reviewed in [2, 3]). On the molecular level, epigenetic mechanisms encompass first and foremost chemical modifications of the DNA and of the histone proteins, the two constituents of chromatin. In extension, RNA interference and prion mechanisms can also constitute epigenetic mechanisms [4], however, in this chapter we will concentrate our focus on direct chemical modifications of the chromatin. Such modifications entail on the one hand methylation of the DNA at cytosine-guanine dinucleotides, which are often found in sequences called CpG islands being characterized by a high incidence of CG repeats. DNA methylation is most commonly associated with transcriptional silencing either by directly inhibiting the binding of transcription factors or by indirectly recruiting methyl-CpG binding proteins (MBPs) and their associated repressive chromatin-remodeling activities [5, 6]. However, since DNA methylation has also be found in the promoter and the coding region of actively transcribed genes, the relationship between this epigenetic mark and transcriptional activity is not clear-cut [7, 8].

On the other hand, epigenetic modifications at the chromatin refer to posttranslational modifications of histone proteins. Histones are basic proteins that function to condensate and to regulate the accessibility of the chromatin. They consist of a globular histone core and an N-terminal histone tail, a loosely structured sequence of amino acids protruding out of the core. Posttranslational histone modifications most prominently occur on this N-tail, and involve acetylation, methylation, phosphorylation, ubiquitination in mammals (reviewed in [4]), and
sumoylation in yeast [9]. Due to their chemical properties, these epigenetic modifications are capable of altering the condensation of the chromatin and, as a consequence, the accessibility of the DNA to the transcriptional machinery.

Both the acetylation and the phosphorylation of histones, occurring on lysine (K) as well as on serine (S), threonine (T) and tyrosine (Y) residues, respectively, have been linked to transcriptional activation. This is because the addition of the partially negatively charged acetyl- and phospho- groups results in a repulsive force between the histone tail and the DNA, itself being negatively charged, which relaxes the chromatin structure [10]. In contrast, histone methylation has been shown to associate with both actively transcribed but also with silenced genes [11, 12], depending on which residue they occur. What is more, these residues can be mono-, di-, and trimethylated, the combination of which can have differing roles in the regulation of gene expression [13]. Histone methylation can also occur on arginine (R) residues in both a mono- or dimethylated form, with its effect on the organization of chromatin not being clearly understood [13]. Next, protein ubiquitination (also called ubiquitylation), the attachment of the highly conserved 76 amino acid-long polypeptide group ubiquitin, is most commonly associated with marking proteins for degradation by the proteasome, but has also been found to occur on histone tails, where it serves a different, yet not well characterized purpose. The ubiquitination of histone tails has been identified to be a prerequisite for subsequent histone methylation and it has also been shown to be a correlate of both transcriptional activation and nucleosome loosening [13, 14]. Finally, histone sumoylation is the least known posttranslational histone modification. In yeast, it has been shown to occur on all four core histones and to negatively regulate transcription, possibly by interfering with histone acetylation and ubiquitination [9, 15].
The repertoire of DNA and histone modifications is insured by specific enzymes including DNA methyltransferases (DNMTs), histone acetyltransferases (HATs) and deacetylases (HDACs), histone methyltransferases (HMTs), protein kinases and phosphatases, and ubiquitin- and SUMO-associated enzymes [4, 13, 15]. These enzymes operate both independently and in synergy to establish a “histone code”, a highly dynamic and variable chromatin marking that in combination with chromatin-associated proteins determines the pattern of gene expression [16, 17].

1. Epigenetic dysregulation in neurodevelopmental disorders - Rett Syndrome

Neurodevelopmental disorders commonly define impairments of the central nervous system that occur during development. Such impairments can have their onset during the development as such, for instance in the case of fetal alcohol syndrome, whereas others are genetically predisposed, for instance in Down Syndrome. Several genetically predisposed neurodevelopmental disorders have been documented to involve at least in part an epigenetic dysregulation of physiological functions. These disorders include Fragile-X syndrome, Rett Syndrome and Rubinstein-Taybi Syndrome. In this chapter we will focus exclusively on Rett Syndrome, as it implicates both aberrant DNA methylation and posttranslational histone modifications. For a recent review of epigenetic dysregulation in other neurodevelopmental disorders the reader is referred to [18].

Rett Syndrome (RS) is a relatively common cognitive disorder characterized by an arrest of neurological development leading to decelerated head growth and cognitive impairment. RS is caused by a loss-of-function mutation in the X-linked methyl-CpG-binding protein 2 (MeCP2) gene [19] (Table 1), which is lethal when heterozygous. This disease thus develops exclusively in females with a worldwide prevalence of 1:10'000. Because MeCP2 is a transcriptional regulator that binds specifically to
methylated DNA, its deficiency alters chromatin remodeling and induces a general dysregulation of gene transcription. Although MeCP2 is a member of the transcriptional repressors methyl-binding proteins (MBP) (for a review see [5]), it was recently found to play also a role in the activation of transcription, and can therefore act both as a transcriptional silencer and activator [20].

In mice, both MeCP2-null mutation and neuron-specific deletion of MeCP2 recapitulate some of the symptoms of RS. MeCP2-deficient mice have reduced brain weight and impaired neuroanatomical structures (smaller neuronal cells). They exhibit an overall decrease in exploratory activity [21, 22], and have cognitive deficits and impaired synaptic plasticity [23]. The cognitive deficits can be reversed by overexpression of wild-type human MeCP2 protein (by two-fold) in young animals (10-week old) [23]. Intriguingly, however, in 20-week old mice, human MeCP2 overexpression induces seizures, suggesting that an overdose of MeCP2 can have deleterious effects, presumably by silencing genes that - under physiological conditions - are active and required for a proper brain functioning.

One identified target of MeCP2 is brain-derived neurotrophic factor (Bdnf). Calcium-dependent phosphorylation of MeCP2 reduces MeCP2 binding to the Bdnf promoter, and thereby decreases promoter methylation and increases Bdnf expression [24-26], an effect similar to that observed in RS-related mutations of MeCP2. This would suggest that RS is associated with increased Bdnf expression. However, this appears not to be the case [27] as MeCP2 mutant mice have reduced BDNF levels. This reduction and the neuronal atrophy characteristic of RS can be reversed by forebrain-specific overexpression of Bdnf in vivo [27]. This beneficial effect of Bdnf overexpression was recently confirmed in cultured hippocampal neurons [28]. It is therefore thought that the substantial lack of neuronal activity in MeCP2 mutant mice is more important as to determine the levels of Bdnf than what would be caused by
the lack of MeCP2 binding [29]. Alternatively, since MeCP2 is also expressed in astrocytes [30], it is conceivable that MeCP2 affects the levels of Bdnf in a cell-type specific manner, which might explain the apparent discrepancy between the two lines of evidence. More refined analyses are required to solve this issue.

In addition to Bdnf, a recent study identified seven other genes as direct binding targets of MeCP2 in the mouse brain [31], including myelin-associated proteins and dopamine decarboxylase. Yet more genes are likely to be discovered, as MeCP2 phosphorylation is associated with dendritic growth and spine maturation [26], which is thought to occur through the de-repression of target genes involved in development processes [20, 32].

Further to regulating DNA methylation, MeCP2 also influences posttranslational modifications of histone protein, in particular histone acetylation and methylation. At the promoter region of Bdnf, this is mediated by the formation of a complex between MeCP2 and HDAC1 [25], which reduces the acetylation of both histone H3 and H4. This is paralleled by increased dimethylation of H3K9 but decreased dimethylation of H3K4, two post-translational histone modifications that inhibit and promote gene transcription respectively [33]. This suggests a cooperative contribution of epigenetic modifications for gene silencing, and is consistent with the observation that mice with deficient MeCP2 have hyperacetylated H3 [34]. Taken together, these results strongly argue for an overall increase in gene transcription in RS, due to loss of MeCP2-mediated gene silencing. However, in light of the recent observation that MeCP2 also acts as a transcriptional activator, it is essential to determine whether the transcription of specific genes is also decreased. In animal models and in patients, the effect of MeCP2 deficiency may be pharmacologically attenuated by direct administration of exogenous MeCP2, or with drugs targeting transcriptional processes, DNA methylation, histone acetylation and/or histone methylation.
However, such drugs can have significant side effects and unspecific targets, and thus need to be further investigated for potential future use.

2. Epigenetic dysregulation in neurodegenerative disorders - Alzheimer's disease

Neurodegenerative diseases are conditions in which cells of the central nervous system are lost. Since cells in the central nervous system are only marginally regenerated, cell loss will have devastating consequences on cognition and locomotion. Alzheimer’s Disease (AD) and Huntington’s Disease are two neurodegenerative diseases for which there is ample evidence that they are caused - at least in part - by epigenetic dysregulation. For an extensive overview of the implication of epigenetic mechanisms in Huntington’s, the reader is referred to [35].

AD is one of the most common neurodegenerative diseases worldwide, and has an estimated prevalence of 1:100 in the population over 65 of age in Western countries. Despite years of intense research and multiple clinical trials, AD remains a non-curable brain disease [36]. It is primarily characterized by a progressive cognitive decline that gradually develops with age. Its pathophysiology in the brain is manifested by the presence of two major hallmarks: extracellular amyloid plaques and intracellular neurofibrillary tangles [37]. Amyloid plaques are deposits of the amyloid β (Aβ) peptide, produced through enzymatic cleavage of the amyloid precursor protein (APP) by β and γ secretase. Neurofibrillary tangles (NFTs) are intraneuronal aggregates of hyperphosphorylated tau protein, a microtubule-binding protein.

Several epigenetic modifications such as aberrant histone acetylation and DNA methylation are present in the APP-Aβ pathway and may contribute to AD. One line
of evidence suggests that histone acetylation is increased in AD. A potential mechanism for this increase is thought to involve the formation of a complex between the APP intracellular domain (AICD), produced from APP by γ secretase, and the nuclear adaptor protein Fe65 and the histone acetyltransferase (HAT) TIP60, a transcriptional activator [38]. At the same time, presenilin 1 (PS1), a gene coding for the γ secretase complex, was also shown to contribute to histone hyperacetylation in AD pathology. Loss-of-function mutations in PS1 or mutations associated with familial AD, an early onset form of AD affecting genetically predisposed individuals, inhibit the proteasomal degradation of the HAT cAMP-responsive element binding protein (CBP), and result in increased CREB-mediated gene expression in neurons in culture [39]. Consistently, in a mouse model of AD with severe neurodegeneration and memory loss due to cyclin-dependent kinase 5 (Cdk5) hyperactivation (CK-p25 mouse model), lentivirus-mediated overexpression of the HDAC SIRT1 (silent mating type information regulation 2 homolog) confers substantial protection against AD-related neurodegeneration and memory loss [40]. Although in this case it is not known whether SIRT1 acts via the epigenetic machinery or rather via cytoplasmic substrates, these findings suggest that pharmacological treatment activating SIRT1 such as the polyphenol resveratrol might be beneficial for treating AD.

In contrast, a parallel line of evidence suggested that a decrease in histone acetylation is causally linked to AD. In cultured cortical neurons, the overexpression of APP leading to cell death is accompanied by a decrease in the level of CBP, and in H3 and H4 acetylation [41]. Similarly, loss-of-function mutations in PS1 and PS2 genes in mice reduce the expression of CBP, and of CBP/CREB target genes such as c-fos and Bdnf. They also result in impaired synaptic plasticity, and defective spatial and contextual memory [42]. Moreover in CK-p25 mice, the intracerebroventricular injection of sodium butyrate, a potent HDAC inhibitor, rescues memory and synaptic connectivity [43], suggesting that HDAC inhibitors might be
used for potential treatment of AD-related pathologies. Notably, several inhibitors are currently being tested in pre-clinical or phase I/II trials for the treatment of AD. However, since histone acetylation appears to be dysregulated bidirectionally and possibly, in a gene-specific fashion, more research is required to fully evaluate the potential of HDAC inhibitors.

Interestingly, besides pharmacological treatment, natural manipulations such as environmental enrichment may also represent a promising means to treat AD. In wild-type mice, exposure to an enriched environment for four weeks was shown to improve synaptic connectivity and cognitive abilities in mice [43]. The improvement was comparable to that achieved with HDAC inhibitors [43], which highlights the potential of environmental stimulation for the reversal of cognitive deficits in AD.

Further to histone acetylation, DNA methylation is also involved in the etiology of AD. In cell culture, hypomethylation of the promoter region of PS1 increases presenilin expression, which enhances β-amyloid formation [44]. This effect can be reversed by application of the methyl donor S-adenosylmethionine (SAM) that rescues methylation, increases presenilin expression and reduces β-amyloid formation. These observations therefore suggest that methyl donors or drugs targeting the methyl metabolism may be potential therapeutic agents to treat AD [45]. The finding that DNA hypomethylation underlies some aspects of AD pathology was also recently confirmed in mouse and primate models of AD. In these models, exposure to lead (Pb) was found to reduce the enzymatic activity of DNMT1 in cortical neurons and is associated with increased APP expression [46]. Further, a recent post mortem study in human also reported hypomethylation of the presenilin promoter region in late-onset AD patients when compared to age-matched healthy subjects [47]. These findings therefore support the hypothesis that DNA hypomethylation, at least in the presenilin promoter, is causally associated with AD. However, other AD-related
susceptibility genes such as \( BACE1 \), which codes for \( \beta \)-secretase, or the gene coding for apolipoprotein E that facilitates amyloid plaque formation, are hypermethylated in late-onset AD [47]. This suggests that alteration in DNA methylation is presumably bidirectional and gene-specific altered in AD, similar to histone acetylation. Further studies are thus needed to identify genes with an altered DNA methylation profile, and the direction and extent of their expression changes.

3. Epigenetic dysregulation in psychiatric disorders - Depression

Psychiatry disorders range from personality and anxiety disorders to addiction and depression, which are often difficult to define and even more so to treat. One of the most prominent psychiatric disorders is depression, for which a substantial body of evidence now suggests that several depressive-like phenotypes are partly caused by epigenetic mechanisms.

Depression is a common mental disease characterized by pessimistic thoughts, lack of enthusiasm and vitality, feelings of sadness and anhedonia. This chronic illness affects roughly five per cent of the population worldwide, although this number is likely to underestimate the actual prevalence rate. Depression is difficult to treat. Indeed, only half of depressed patients show complete remission [48, 49]. One of the major issues with therapeutic treatment of depression is that the beneficial effect of anti-depressant drugs is delayed, and symptoms are usually ameliorated only after a couple of weeks. The reasons for such delay are not known but are thought to result from the fact that the mechanisms underlying the etiology of depression are of epigenetic nature. Initial evidence for this hypothesis came from studies examining the effect of electroconvulsive therapy, a treatment effective only after repeated administration. Chronic electroconvulsive seizures (ECS) were shown to increase \( Bdnf \) and \( CREB \) expression, and BDNF protein in the hippocampus [50-52]. This
increase is associated with H3 hyperacetylation at BDNF promoter 3 [53], suggesting that chromatin remodelling contributes to the beneficial effects of chronic ECS.

The importance of histone posttranslational modifications in depression was further demonstrated in a model of social defeat in rodent. This model of chronic stress induces symptoms of depression that can be reversed by chronic, but not acute, antidepressant treatment, mimicking what is observed in human patients [54]. In mice, chronic social defeat decreases the expression of two splice variants of Bdnf (Bdnf III and Bdnf IV) in the hippocampus, and is associated with increased dimethylation of H3K27 [54], a mark for transcriptional repression [33] (Figure 1). While behavioural abnormalities induced by chronic stress can be reversed by chronic antidepressant treatment, the increase of H3K27 dimethylation can not [54]. Instead, antidepressant treatment appears to reverse the downregulation of Bdnf expression by increasing H3 acetylation, and H3K4 methylation, both marks of transcriptional activation [33] at the same promoters [54, 55]. Additionally, chronic antidepressant treatment downregulates the expression of HDAC5, specifically in animals exposed to chronic stress [56]. Thus, chronic stress-induced histone modifications at the Bdnf gene in the hippocampus are likely to be an important mechanism for the development of depressive behaviours, and may also be a target for antidepressant treatments.

In addition, there is evidence for the influence of antidepressant treatments on DNA methylation. Chronic antidepressant treatment was demonstrated to increase methyl MeCP2 and MBD1, two proteins which bind methylated CpG dinucleotides and act as either a transcriptional activator or repressor in the rodent brain [57]. The antidepressant-dependent increase in MeCP2 was specific to gamma aminobutyric acid (GABA)-ergic interneurons [57]. This finding is of particular interest, since abnormal GABAergic transmission and abnormalities in GABA-related gene
methylation have been linked to major depression and suicide. Depressed patients who committed suicide have higher level of methylation in the GABA-A α1 receptor subunit promoter, and increased DNMT3b mRNA and protein in the prefrontal cortex when compared to control individuals who died of other causes [58]. This suggests the intriguing possibility that antidepressant treatments specifically target the epigenetic machinery in cell types affected by depression.

4. Epigenetic dysregulation in psychotic disorders - Schizophrenia

Psychotic disorders refer to mental illnesses that are characterized by distorted perceptions of reality. The most common form of psychosis is Schizophrenia, with an approximate prevalence of one percent worldwide among people 18 years old or more [59]. Schizophrenia is characterized by two main types of symptoms: positive symptoms such as delusions and hallucinations, and negative symptoms such as social withdrawal, lack of motivation, and overall apathy. While the causes of schizophrenia are not well understood, they are likely to involve a genetic predisposition and environmental factors during pre- and postnatal development. The importance of the environment is indeed illustrated by the fact that the concordance rate for schizophrenia in monozygotic twins is of only 50 percent [60]. But the question of how environmental factors influence the development of schizophrenia is still open.

There is now increasing evidence that schizophrenia is associated with aberrant epigenetic profiling, and abnormal GABAergic neurotransmission in cortical areas [61]. The first line of evidence involves reelin, a glycoprotein expressed in GABAergic neurons during development and adulthood, and which contributes to neuronal migration [61, 62]. Post-mortem analyses in schizophrenic patients revealed that reelin mRNA and protein expression is significantly reduced in several brain regions,
despite an overall normal number of neuronal cells [63, 64]. This reduction may be caused by an alteration of the methylation profile, possibly hypermethylation, at the reelin promoter since this promoter contains a large CpG island [49, 65]. Reelin expression is indeed sensitive to pharmacological manipulations of DNA methylation. In vivo, repeated methionine administration increases methylation of the reelin promoter, induces binding of MeCP2 to the reelin promoter, and downregulates reelin expression [66] [67]. Consistently, in vitro administration of the DNMT inhibitor, 5-aza-2'-deoxycytidine, increases reelin expression [65].

Further evidence for the involvement of DNA hypermethylation in GABAergic dysfunction observed in schizophrenic patients involves glutamate decarboxylase (GAD67), an enzyme that catalyzes the production of GABA. GAD67 mRNA and protein expression is downregulated in cortical structures of schizophrenic patients [63, 64], which correlates with increased methylation of the GAD67 promoter in prefrontal cortical samples from post-mortem brains of schizophrenic patients [68].

In addition to the GABAergic system, increased methylation of the CpG island of an oligodendrocyte-specific transcription factor, sex-determining region Y-box containing gene 10 (SOX10), and decreased expression of SOX10 [69], was observed in the brains schizophrenic patients. This may provide a possible mechanism for the abnormalities in oligodendrocytes observed in schizophrenic patients.

The mechanisms that underlie the abnormal hypermethylation of promoters such as reelin, GAD67, and SOX10 in the brain of schizophrenic patients are suggested to be caused by elevated levels of SAM, and increased expression of DNMT1 mRNA [70, 71]. Indeed, the administration of SAM can induce psychotic episodes in some schizophrenic patients [72]. Due to the involvement of aberrant hypermethylation in
schizophrenic patients, DNMT inhibitors have been suggested as potential therapeutic agents for this disease [4, 65, 73].

While in the GABAergic system and in oligodendrocytes, several instances of hypermethylation were observed in schizophrenic patients, hypomethylation was detected in the dopaminergic system. Increased activation of catechol-O-methyltransferase (COMT), an enzyme involved in the degradation of neurotransmitters such as dopamine, epinephrine, and norepinephrine, is associated with impaired attention, executive cognition, and working memory, and with an increased risk to develop diseases such as schizophrenia [74]. Reduced methylation of the COMT promoter is detected in the frontal lobe of schizophrenic patients, and is associated with increased activation of the gene [75]. Moreover, aberrant methylation of genes within the dopaminergic system has also been observed in monozygotic twins. In one study, methylation upstream of the locus for dopamine D2 receptor was investigated in two sets of twins, one concordant and one discordant for schizophrenia. Of the discordant twins, the epigenetic profile of the affected twin was more similar to that in twins concordant for schizophrenia than to his own unaffected brother [76], confirming the implication of DNA methylation in schizophrenia.

Finally, the brain of schizophrenic patients is also characterized by differential histone posttranslational modifications. Valproic acid (also known as valproate), a common mood stabilizer prescribed to treat schizophrenia, is a potent HDAC inhibitor [77]. Its administration not only decreases HDAC activity, but also increases reelin expression both in vitro and in vivo. Further, it decreases methylation of the reelin promoter [65, 66]. In vitro, other HDAC inhibitors such as trichostatin A or MS-275 (for a review see [78, 79]) have also been demonstrated to activate the expression of reelin and GAD67 [80]. While the mechanism for the link between HDAC inhibition and altered DNA methylation is currently unknown, it is thought to operate via
regulating the accessibility of DNMTs to promoter regions, or by direct induction of DNA demethylase activity [49, 80]. Valproate has further been demonstrated to reduce schizophrenia-like behaviours in a epigenetic mouse model of schizophrenia induced by methionine [81]. Additionally, two antipsychotic drugs acting as dopamine D2 receptor antagonists, haloperidol and raclopride, induce phospho-acetylation of H3 in mouse striatum [82]. Thus, it appears that decreased acetylation may be involved in the pathology of schizophrenia, which can be readily reversed by HDAC inhibitors. However, due to the non-specific effects of the antipsychotics described above, direct evidence for this hypothesis is still needed.

5. Epigenetic dysregulation in stress - how early life events influence behavior in adulthood

Increased stress vulnerability and other forms of pathological and inappropriate stress-coping behaviors are also common brain disorders. These disorders are complex and have intricate mechanisms, which are still poorly understood. It is recognized that they are strongly influenced by environmental factors, in particular by early life detrimental experiences (pre- and postnatal). These experiences are thought to induce lasting epigenetic modifications resulting in changes in gene expression in adult animals. In mammals, the quality of early life is primarily defined by maternal care and nutrition. In mice and rats, maternal care is provided by arched-back nursing (AB) and licking/grooming (LG), two behavioral traits exhibited by most rodent species but which vary greatly between strains [83]. These traits critically influence the offspring’s behavior and determine their responsiveness to stress and their level of anxiety [84]. At the molecular level, such responsiveness is in part regulated by glucocorticoid and glucocorticoid receptors (GR). High levels of circulating glucocorticoids raise the body alertness and increase the stress response, while lower levels result in a more “relaxed” behavior and attenuate the stress
response. Conversely, high levels of GR in forebrain areas such as the hippocampus provide a negative feedback that reduces the production of glucocorticoids and thereby dampens the stress response (reviewed in [85]). Offspring of high-LG-ABN mothers show increased GR expression and reduced reactivity to stress, whereas offspring of low-LG-ABN mothers have decreased GR expression and increased stress reactivity [86].

Stress reactivity and the GR system are subject to epigenetic modifications in early life. In the offspring of high LG-ABN females, DNA methylation is reduced and H3K9 acetylation is increased in the promoter of the GR gene, a promoter that is in part, controlled by binding of the transcription factor NGFI-A (also known as Egr-1 or Zif268) [87]. In contrast, in the offspring of low LG-ABN females, promoter methylation was increased (but acetylation was not changed), suggesting that differential epigenetic marking underlies changes in GR expression. Recent evidence further indicates that NGFI-A itself conveys these epigenetic changes, because its binding to the GR promoter region is required for these changes to occur [88]. Importantly, although stable, these epigenetic changes can be reversed by environmental or pharmacological manipulations. Cross-fostering of pups, or treatment with the HDAC inhibitor trichostatin (TSA) lead to GR promoter DNA hypomethylation and histone hyperacetylation in low LG-ABN offspring [87]. Likewise, methyl supplementation via the administration of L-methionine, a SAM precursor, can reverse maternal programming of stress responses via glucocorticoid receptors [89]. Both treatments have further been shown to modulate the transcriptome in the hippocampus in both high and low LG-ABN offspring [90], which suggests that the type of maternal care not only influences DNA methylation and histone acetylation at the GR promoter, but also on other genes.
Importantly, the implication of epigenetic regulation of GR was recently confirmed in a study involving post-mortem human brain samples [91]. In this study, the correlated decrease in hippocampal GR expression, and increase in DNA methylation in the GR promoter were found in suicide victims with a history of childhood abuse, but not in age-matched control subjects including suicide victims without such history and people having died from other causes. These findings strongly suggest that in rodents and humans alike, childhood neglect can cause lifelong epigenetic alterations of gene expression in the brain’s stress system. Remarkably, rat pups raised by mothers that display high nurturing levels become high nurturing mothers themselves, raising the intriguing possibility that this early-life induced behavioral trait can be transmitted to subsequent generations [92].

Conclusions and outlook

It is now clear that epigenetic mechanisms play a pivotal role in higher-order brain functions under both physiological and pathological conditions. A deeper understanding of these mechanisms is therefore of utmost importance for the development of potential treatments against brain disorders involving aberrant epigenetic modifications (for all brain disorders not described herein, the reader is referred to [4, 18, 35, 93]. Promising results have already been obtained, in particular with the use of HDAC inhibitors [94]. However, most of these inhibitors still act rather unspecifically, and further research is required before such treatments can be safely administered. It is therefore important to identify more specific HDAC inhibitors, but also to determine which HDACs are involved in different disease states. For instance, it was recently demonstrated that although HDAC1 and HDAC2, two structurally related members of the class I HDACs that often form functional
heterodimers, it is only HDAC2 that is causally implicated in negatively regulating memory formation and synaptic plasticity [95].

In addition to the best-understood instances of aberrant DNA methylation and posttranslational histone modifications in brain disorders, other forms of epigenetic regulation such as RNAi are likely to merit further attention in the future. Although so far, no example of naturally occurring RNAi-mediated epigenetic silencing of gene expression in the nervous system has been documented, this mechanism is quite common in yeast, where RNAi regulate the heterochromatin structure around centromeres [96], and in eukaryotic development, where RNAi is implicated in X-chromosome inactivation [97]. Moreover, RNAi has proven an efficient means to artificially silence genes of interest, which has recently also been achieved in the mouse brain [98]. As a result, RNAi-mediated gene silencing has the potential to emerge as a powerful tool against various brain disorders [99], including the neurodevelopmental and neurodegenerative examples mentioned herein.

In summary, although the importance of epigenetic programming in brain diseases is now fully appreciated, a more thorough understanding of epigenetic processes is still required until safe and efficient “epigenetic treatments” of these diseases can be envisaged.
References

Recovery of learning


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Figure 1. The importance of posttranslational histone modifications in a rodent model of depression. (a) Under physiological conditions, i.e. in the absence of stress, the promoter region of the Bdnf gene is characterized by moderate levels of histone H3 acetylation and H3K27 dimethylation, and is bound by the histone deacetylase HDAC5. (b) Upon chronic social defeat stress, a rodent model of depression, H3K27 dimethylation is increased, leading to increased condensation of the Bdnf promoter, which shuts down Bdnf gene expression. (c) Upon chronic antidepressent (imipramine) treatment, HDAC5 levels are reduced, which leads to increased H3 acetylation, whereas H3K27 dimethylation remains unaffected. Nonetheless, the increase in H3 acetylation is sufficient to reinstate Bdnf gene expression. A, acetyl; Bdnf, brain-derived neurotrophic factor; HDAC, histone deacetylase; K, lysine; M, methyl. Figure reproduced, with permission, from [49].
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