Journal of Neurochemistry JNC

JOURNAL OF NEUROCHEMISTRY | 2008 | 106 | 1378–1387 doi: 10.1111/j.1471-4159.2008.05484.x

# Long-lasting depression-like behavior and epigenetic changes of BDNF gene expression induced by perinatal exposure to methylmercury

Natalia Onishchenko,*[,1] Nina Karpova,†[,1] Farideh Sabri,* Eero Castrén† and Sandra Ceccatelli*

*Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden

†Neuroscience Center, University of Helsinki, Helsinki, Finland

**Abstract**

Substantial evidence indicates that predisposition to diseases can be acquired during early stages of development and interactions between environmental and genetic factors may be implicated in the onset of many pathological conditions. Data collected over several decades have shown that chemicals are among the relevant factors that can endanger CNS. We previously showed that perinatal exposure to methylmercury (MeHg) causes persistent changes in learning and motivational behavior in mice. In this study, we report that the depression-like behavior in MeHg-exposed male mice is reversed by chronic treatment with the antidepressant fluoxetine. Behavioral alterations are associated with a decrease in brain-derived neurotrophic factor (BDNF) mRNA in the hippocampal dentate gyrus and fluoxetine treatment restores BDNF mRNA expression. We also show that MeHg-exposure induces long-lasting repressive state of the chromatin structure at the BDNF promoter region, in particular DNA hypermethylation, an increase in histone H3-K27 tri-methylation and a decrease in H3 acetylation at the promoter IV. While fluoxetine treatment does not alter hypermethylation of H3-K27, it significantly up-regulates H3 acetylation at the BDNF promoter IV in MeHg-exposed mice. Our study shows that developmental exposure to low levels of MeHg predisposes mice to depression and induces epigenetic suppression of BDNF gene expression in the hippocampus.

**Keywords:** depression, fluoxetine, BDNF, environment, epigenetic changes, mice.

*J. Neurochem.* (2008) **106**, 1378–1387.

Predisposition to diseases can be acquired during early stages of development and resolve into an actual disorder later in life. Recent epidemiological data indicate that negative effects related to environmental factors should be of a particular concern in this regard. A National Research Council experts' committee concluded that in the USA 3% of developmental disabilities are the direct consequence of environmental exposure to toxic substances, and that another 25% occur via interactions between environmental factors and individual genetic susceptibility (National Research Council 2000). Frequent neurodevelopmental disorders include learning disabilities, sensory deficits, developmental delays, and attention deficit and hyperactivity disorder. In addition, a recent study has reported that functionally impairing depression occurs in 2–10% of children and adolescents (Dopheide 2006). The possible importance of developmental factors in the etiology of mood disorders is unclear and often underemphasized.

The CNS is particularly sensitive to alterations of the microenvironment during early development, when there are windows of susceptibility to adverse interference that are not present in the mature brain. Alterations in environmental conditions during development produce long-lasting and often permanent changes in the structure and function of the brain that reflect altered expression of key genes involved in

Received February 22, 2008; revised manuscript received May 7, 2008; accepted May 8, 2008.

Address correspondence and reprint requests to Dr. S. Ceccatelli, Department of Neuroscience, Karolinska Institutet, Box 210, SE-17177 Stockholm, Sweden. E-mail: Sandra.Ceccatelli@ki.se

[1]These authors have equally contributed to the work.

*Abbreviations used*: BDNF, brain-derived neurotrophic factor; FST, forced swim test; MeHg, methylmercury; MS-SNuPE, methylation-sensitive single nucleotide primer extension assay; PLSD, protected least significant difference; Trk B receptor, tropomyosine-related kinase B receptor.

neuronal development and plasticity (Meaney 2001; Colvis *et al.* 2005). Such conditions include sensory or social stimuli, maternal care, stress but also drug treatments and toxic compounds (McEwen 1999; Meaney 2001; Mendola *et al.* 2002; Ansorge *et al.* 2007).

Among the agents with neurotoxic potential is methylmercury (MeHg), a well-known environmental contaminant present in certain sea food, which is the main source for human exposure (Björnberg *et al.* 2005; Mahaffey 2005). Recently, we have shown that in addition to alterations in learning capabilities, developmental exposure to low levels of MeHg causes depression-like behavior in young and adult mice (Onishchenko *et al.* 2007). Long-lasting behavioral changes can be associated with changes in the expression of genes involved in neuronal plasticity and connectivity, particularly neurotrophin brain-derived neurotrophic factor (BDNF) and its tropomyosine-related kinase B receptor (TrkB) (Colvis *et al.* 2005; Tsankova *et al.* 2007), which are believed to play a central role in depression and antidepressant treatments (Nestler *et al.* 2002; Castrén *et al.* 2007). Epigenetic changes in chromatin structure of the BDNF gene were demonstrated in the mouse model of depression induced by chronic social stress (Tsankova *et al.* 2006). We now show that the depression-like behavioral changes induced by perinatal MeHg exposure are reversed by antidepressant treatment in adulthood. In addition, we show that depression-like behavior induced by perinatal exposure to low levels of MeHg is associated with long-lasting epigenetic changes of the BDNF gene in the hippocampus of exposed mice. Our data provide novel evidence for possible interactions between environmental and genetic factors, which can alter CNS system functions and increase the risk of disorders, including depression.

## Materials and methods

### Animals and MeHg-exposure

All experiments were approved by the local Animal Ethics Committee (Stockholm Northern Ethics Board of Animal Experimentation). C57BL/6/Bkl mice were purchased from Scanbur BK (Sollentuna, Sweden) and kept under standard laboratory conditions (21°C, 12 h light-dark cycle) with free access to food and water. Pregnant dams were exposed to MeHg (CH3HgOH) at the dose of 0.5 mg/kg/day via drinking water from gestational day 7 till day 7 after delivery as described previously (Onishchenko *et al.* 2007). Briefly, pregnant mice were housed individually starting from gestational day 6. Concentration of MeHg in the drinking water was adjusted based on body weight and water consumption which were checked daily. This exposure protocol does not affect litter size or body weight gain and results in brain mercury concentrations similar to those found in infants from fish eating populations (Lapham *et al.* 1995). Control females received tap water. Based on our previous results showing depression-like behavior only in MeHg-exposed males, we performed all experiments in male offspring. Experimental groups included not more than two mice from the same litter.

### Forced swim test

Animals were individually placed in a glass cylinder (24 cm height, 12 cm diameter) filled up with water (25°C) to a height of 16 cm for 15 min (pretest) and for 6 min (test) 24 h after the previous session. Test sessions were videotaped and later analyzed for immobility duration. Immobility was defined as floating passively in the water at least 2 s or longer, without any movements or only small ones necessary to keep the head above the water surface. The animals were tested at the age of 9 weeks to verify the presence of depression-like behavior in MeHg-exposed mice. After that, control and MeHg-exposed animals were divided in groups receiving fluoxetine or tap water, and tested again at the age of 12 weeks. To find out whether depression-like behavior is a long-lasting effect of perinatal MeHg-exposure, we performed FST in additional groups of 14-month-old control and MeHg-exposed mice. For histological and molecular analyses animals were killed 24 h after the behavioral testing.

### Fluoxetine treatment

Control and MeHg-exposed male offspring received fluoxetine (SalutasPharma GmbH, Gerlingen, Germany) via drinking water at the concentration of 0.08 mg/mL, which did not reduce water intake and resulted in fluoxetine plasma levels within the therapeutic range in humans (Rantamäki *et al.* 2007). The treatment started when the animals were 9-week old and continued for 21 days.

### *In situ* hybridization

Mice were killed by cervical dislocation; brains were rapidly removed and frozen on dry ice. Sections at the level of the dorsal hippocampus were cut in a cryostat (Bright OTF, Huntingdon, UK) at 14 μm thickness and thaw-mounted on SuperFrost®Plus slides (Menzel GmbH & Co KG, Braunschweig, Germany).

Oligonucleotides complementary to mouse BDNF nucleotides 292–346 (Hofer *et al.* 1990) and TrkB nucleotides 2781–2829 (Wong *et al.* 1997) were labeled with $^{33}$P-γ-dATP (NEN) at the 3′-end using terminal deoxynucleotidyltransferase (Amersham, Amersham, UK) and purified using ProbeQuant™ G-50 Micro Columns (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The probes were added to hybridization solutions containing 50% of deionized formamide (Ambion, Inc., Austin, TX, USA), 4 × standard saline citrate (SSC), 1 × Denhardt's solution, 50 mg/L denaturated salmon testis DNA (Sigma, St Louis, MO, USA) and 200 mM dithiotreitol (Sigma). Sections were air-dried overnight and hybridized with the oligonucleotide probes (0.5 ng) in humidified chambers at 42°C for 16–18 h. After hybridization, sections were rinsed for 2 h (4 × 30 min) in 1 × SSC at 55°C followed by 1 h at 25°C. Sections were then rinsed in distilled water, rapidly dehydrated with alcohol, and air-dried. For control, the antisense probe was replaced with a sense probe, or an excess (100×) of the unlabeled antisense probe was added to the hybridization solution. No signal was detected with either procedure.

Sections were exposed to an autography film (Biomax MR, Kodak, Rochester, NY, USA) together with $^{14}$C standards (Amersham microscales) for 3–4 days at −20°C. After that, the films were developed in LX24 developer (Kodak) for 2 min, fixed in

AL4 X-Ray fixer (Kodak) for 15 min, rinsed in running tap water for 30 min, and air-dried.

Some sections were subsequently dipped into liquid photo emulsion NTB2 (Kodak) diluted 1 : 1 with distilled water, exposed for 3 weeks, developed in D19 (Kodak), fixed in Kodak Unifix and mounted in glycerol.

### Quantification and imaging

The autography films were scanned (Epson Perfection 1650, Seiko Epson Corp., Nagano, Japan) using Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA, USA) at the resolution of 2000 dpi. The measurements were performed using NIH ImageJ 1.37 software (http://rsb.info.nih.gov/ij/). Dentate gyrus, CA1 and CA3 regions of hippocampal formation were tracked on the digital images and amount of radioactivity was assessed in 3–5 sections of each mouse using [$^{14}$C]-standards as a reference.

Emulsion-dipped sections were analyzed using a Nikon Eclipse E600 microscope equipped with a dark field condenser and connected to a digital camera (Hamamatsu ORCA-ER C4742–80, Hamamatsu City, Japan). Digital images were imported into Adobe Photoshop and equally optimized for brightness and contrast.

### Chromatin immunoprecipitation assay

Chromatin extraction from hippocampal tissues was performed as previously described (Wells and Farnham 2002) with minor modifications. Hippocampi dissected from the mice brains were minced to approx. 1 mm sized pieces and frozen at −80C. Frozen minced hippocampus was cross-linked in 1% paraformaldehyde for 15 min at 25°C. The cross-linking reaction was stopped by adding freshly prepared glycine to a final concentration of 0.125 mol/L. The tissue was washed four times in cold phosphate-buffered saline and was homogenized by a Dounce homogenizer in a cell lysis buffer (1 × PBS, 0.4% NP-40, complete protease inhibitors cocktail, Roche Indianapolis, IN, USA). The homogenate was centrifuged at 200 *g* for 2 min to pellet extracellular debris, and the supernatant was centrifuged at 5500 *g* for 5 min to pellet the nuclei. Nuclei were resuspended in the nuclear lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate, protease inhibitors cocktail), in which the extracted chromatin was sheared to 150–300 bp using the Sonics VibraCell VCX 130 (Sonics & Materials, Inc., Newtown, CT, USA). One-fifth of the lysate was kept for the quantification of DNA present in different samples before immunoprecipitation ('input'). The chromatin was then subjected to immunoprecipitation using antibodies against trimethyl-Histone H3 (K27) and acetyl-Histone H3 (K9 + K14) (catalog numbers # 07–449 and # 06–599 respectively, Upstate, Temecula, CA, USA) overnight at 4°C. The specificity of these antibodies in ChIP assay has been established (Kumar *et al.* 2005; Bernstein *et al.* 2007). To control for the specificity of antibody binding in our conditions, non-immune rabbit IgG (catalog number # PP64, Chemicon, Temecula, CA, USA) and no-antibody immunoprecipitations were performed for each chromatin sample. Protein-DNA-antibody complexes were precipitated with protein A-sepharose beads (catalog number # 17–0780–01, GE Healthcare, Uppsala, Sweden) coated with sheared sperm DNA and bovine serum albumin for 2 h at 4°C, followed by two washes in each low salt, high salt, LiCl and 1 × TE buffers. The precipitated protein-DNA complexes were eluted from the antibody with 1% sodium dodecyl sulfate and 0.1 M $NaHCO_3$, then re-cross-linked in 0.3 M NaCl at 65°C overnight. Proteins were digested with proteinase K for 1 h at 45°C; the DNA was extracted with phenol/chloroform, precipitated with 100% ethanol, and finally resuspended in PCR-grade water and subjected to regular and real-time PCR. The following primers were used to selectively amplify the portion of BDNF promoter II: 5′-GGGCATATAATTGACATCCGCAA-3′ and 5′-TCCACCACTATCCTCACCTAAACTCT-3′, BDNF promoter IV: 5′-TGCGCGGAATTCTGATTCTGGTAAT-3′ and 5′-AGTCC-ACGAGAGGGCTCCACGCT-3′, and BDNF promoter VII: 5′-GTGTAGTCCGAGAATGGGTCTTGG-3′ and 5′-ACCTTGGA-CCTGTAAGTAAGCTTTG-3′ (nomenclature according to Aid *et al.* 2007). Control PCR with β-actin promoter specific primers (5′-AAAATGCTGCACTGTGCGGCGA-3′ and 5′-GGACGCGAC-TCGACAGTGGCTG-3′) was performed to ensure the validity of our ChIP assay. Quantitative real-time PCR was performed in an ABI Prism 7700 (Applied Biosystems, Foster City, CA, USA). 'Input' and immunoprecipitated DNA amplification reactions were run in triplicate at least two independent times in the presence of SYBR-Green (Applied Biosystems). Ct values from each sample were obtained using the Sequence Detector 1.1 software. Relative quantification of template was performed as described previously by Chakrabarti *et al.* (2002) and by the Applied Biosystems manual using ΔΔCt method, when ChIP data were normalized to 'input.'

### Methylation-sensitive single nucleotide primer extension assay

Genomic DNA was converted by sodium bisulfite (Frommer *et al.* 1992; Clark *et al.* 1994). Then BDNF promoter IV region was amplified using the primers Methyl-Fwd 5′-GTGTGTGAATTTGT-TAGGATTGGAAGTG-3′ and Methyl-Rev 5′-CAACAAAAAACT-CCATTTAATCTAAACAA-3′ (Fig. 4a). MS-SNuPE reactions were performed essentially as described (Gonzalgo and Jones 1997). PCR product was purified from nucleotides and primers using the Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA). For each CpG site, two reactions were prepared: 20 ng of purified PCR template, 1 × PCR buffer, 1 pM of the primer, 1 U Taq polymerase and 1 μCi of [$^{32}$P]dCTP or [$^{32}$P]dTTP for forward primer or [$^{32}$P]dGTP or [$^{32}$P]dATP for reverse primer in 20 μL reaction volume. Following primers were used: CpG-109-reverse 5′-CACAAATTACCAAAATCAAAATTCC-3′; CpG-66-forward 5′-GTAATTTGTGTATTAGAGTGTTTATTT-3′; CpG-35-forward 5′-GA-GGAGGTATTATATGATAGTTTA-3′; CpG-24-forward 5′-TTATA-TGATAGTTTATGTTAAGGTAG-3′ (Fig. 4a). Conditions for primer extension were as follows: 95°C, 1 min; 56°C, 2 min; 72°C, 2 min. Extended primers were separated from non-incorporated nucleotides on 10% denaturing polyacrylamide gel (7 M Urea). Gel was dried on a vacuum drier and processed for autoradiography. The autoradiogram was aligned with the gel and pieces corresponding to the radioactive bands were excised and quantitated (Wallac 1414 WinSpectral Liquid Scintillation Counter). The signal of methylated C over the signal sum of both methylated and unmethylated C [$C^m/(C + C^m)$] equals the percentage methylation.

### Statistical analysis

Values reported in the text and figures are represented as mean ± SEM. For comparisons between two groups Student's *t*-test was used. ANOVA followed by Fisher's protected least significant difference (PLSD) *post hoc* test was employed if more than two

groups were compared. Two-way ANOVA was used to reveal interaction between effects of MeHg-exposure and fluoxetine treatment. The type of ANOVA statistical analysis is specified for each experiment in the results chapter. A $p$-value $< 0.05$ was considered as statistically significant.

## Results

### Depression-like behavior induced by developmental exposure to MeHg is reversed by fluoxetine

Forced swim test evaluates rodent's active response and immobility in an inescapable aversive situation (placement into the water). The inactive state (floating) is interpreted as a measure of depressive-like behavior, and is dose-dependently reduced by antidepressant treatments (Porsolt *et al.* 1977). In our experiment, 9-week-old animals were tested and the immobility time was longer in perinatally MeHg-exposed mice than in controls (264.4 ± 16.5 s vs. 189.3 ± 18.9 s, respectively; $p = 0.01$, Student's $t$-test). This difference was also present when the mice were tested at the age of 12 weeks [$F(3,37) = 5.18$, $p = 0.007$, ANOVA followed by Fisher's PLSD *post hoc* test] (Fig. 1a). Similarly, 14-month-old exposed mice had a significantly higher immobility time in the FST than controls [$F(1,10) = 5.26$, $p = 0.044$, one-way ANOVA] (Fig. 1b) pointing to long-lasting effect of MeHg. Treatment of the 9-week-old MeHg exposed mice with the selective serotonin reuptake inhibitor fluoxetine in drinking water for 21 days reduced the immobility time in MeHg-exposed animals as compared to age-matched exposed mice, which had not received fluoxetine [$F(3,37) = 5.18$, $p = 0.0005$, ANOVA followed by Fisher's PLSD *post hoc* test] (Fig. 1a). Antidepressant administration had no effect in control mice, which is in agreement with previous studies where either acute or chronic treatment with fluoxetine did not affect behavior of C57BL/6 mice (Dulawa *et al.* 2004; Crowley *et al.* 2005).

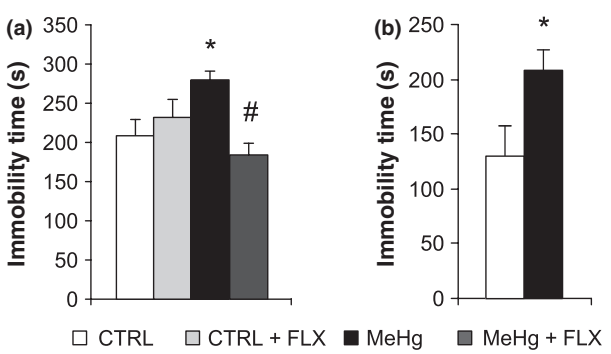


**Fig. 1** Depression-like behavior of MeHg-exposed mice in the forced swim test performed at the age of 12 weeks (a, $n = 9$–12) and 14 months (b, $n = 6$). Fluoxetine (FLX) treatment significantly decreased immobility time only in MeHg-exposed mice, while antidepressant administration had no significant effect in control mice. Two-way ANOVA of immobility time confirmed significant interaction between the MeHg-exposure and fluoxetine treatment [$F(1,37) = 11.37$, $p = 0.002$).*$p < 0.05$ compared to control; #$p < 0.05$ compared to MeHg-exposed group, which did not receive fluoxetine.

### Effects on hippocampal BDNF mRNA levels

BDNF is implicated in the pathophysiology of depression and anxiety and is regulated by antidepressant treatments (Nestler *et al.* 2002; Castrén *et al.* 2007). We therefore studied BDNF mRNA expression and distribution in the hippocampal formation of 12-week-old control and MeHg-exposed mice (Fig. 2a). The analysis revealed that MeHg-exposure led to a significant decrease in BDNF mRNA level in the dentate gyrus [$F(3,22) = 3.96$, $p = 0.004$ vs. control, one-way ANOVA followed by Fisher's PLSD test] but not in CA1 and CA3 regions (Fig. 2b). Consistent with these observations, BDNF mRNA levels were 20% lower in the dentate gyrus of MeHg-exposed 14-month-old mice compared with control animals [$F(1,10) = 15.93$, $p = 0.003$; one-way ANOVA] (Fig. 2c). Chronic fluoxetine treatment restored BDNF mRNA expression in the dentate gyrus of MeHg-exposed animals to normal levels [$F(3,22) = 3.96$, $p = 0.017$ vs. MeHg, $p = 0.827$ vs. control, one-way ANOVA followed by Fisher's PLSD test] (Fig. 2b).

There were no differences in TrkB mRNA expression in the hippocampal formation between control and MeHg-exposed animals at any age tested (Fig. S1).

### The effect of perinatal MeHg-exposure and chronic fluoxetine treatment on the epigenetic state of the BDNF gene

We speculated that the perinatal exposure to MeHg might induce a transcriptional program that leads to repression of plasticity-related genes. Histone modifications, which have been implicated in transcriptional regulation of gene expression, have recently been associated with synaptic plasticity and depression-like behavior (Levenson *et al.* 2004; Kumar *et al.* 2005; Tsankova *et al.* 2006). We focused on the chromatin state of the BDNF promoter IV, which is known to be regulated in an activity-dependent manner at the mRNA and chromatin level (Tao *et al.* 1998; Martinowich *et al.* 2003; Tsankova *et al.* 2006). The level of histone H3K27 tri-methylation was significantly increased in the 12-week-old MeHg-exposed mice compared to controls [$F(3,14) = 5.99$, $p = 0.01$, one-way ANOVA followed by Fisher's PLSD test] (Fig. 3a). Furthermore, increased levels of histone H3K27 tri-methylation at BDNF promoter IV persisted in 14-month-old MeHg-exposed mice [$F(1,10) = 13.53$, $p = 0.004$; one-way ANOVA] (Fig. 3c). The H3K27 tri-methylation is implicated in silencing of gene expression (Bernstein *et al.* 2007; Kouzarides 2007) which is consistent with the reduction in BDNF mRNA levels in the hippocampus. Chronic treatment with fluoxetine did not significantly alter the level of tri-methylated histone H3K27 in either

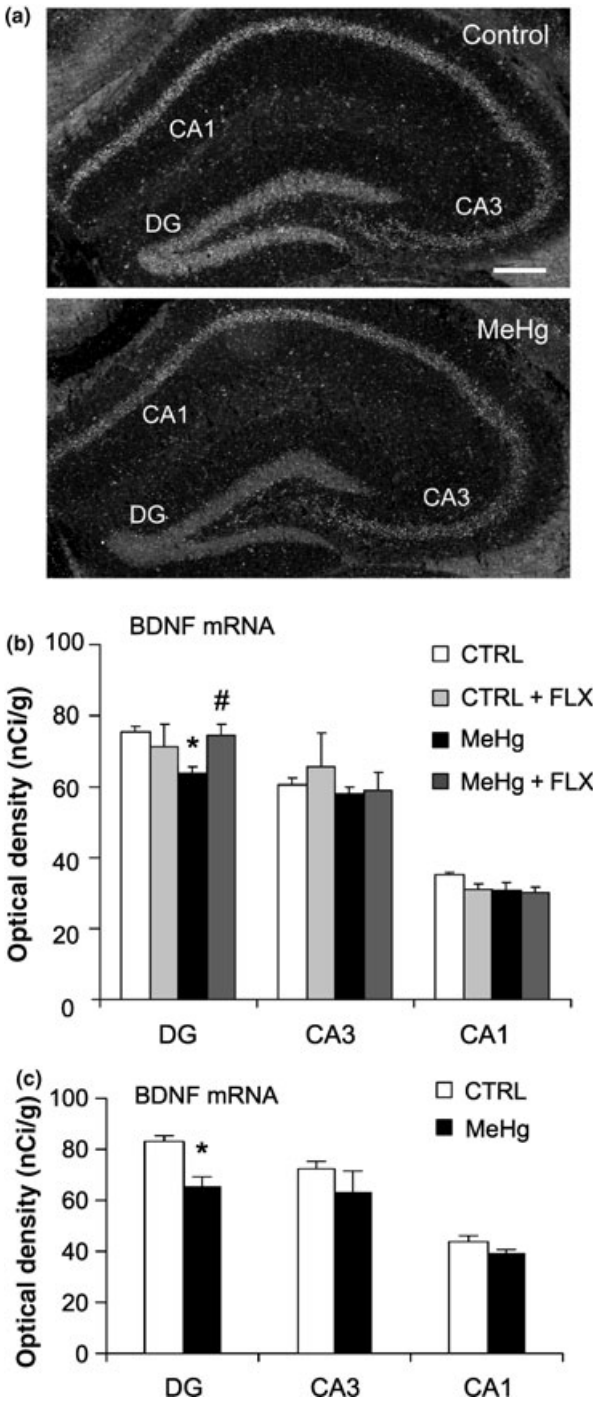


**Fig. 2** (a) Dark-field photomicrographs of coronal hippocampal sections of 12-week-old control and MeHg-exposed mice. Scale bar: 200 μm. (b) Quantitative evaluation of BDNF mRNA expression in the hippocampal formation (dentate gyrus (DG), CA1 and CA3 regions) showed a decreased expression in the in the DG of MeHg-exposed mice that was restored after fluoxetine (FLX) treatment ($n = 4$–8)).*$p < 0.05$ compared to control; #$p < 0.05$ compared to MeHg-exposed group, which did not receive fluoxetine. (c) Similarly, BDNF mRNA level was significantly lower in the DG of 14-month-old MeHg-exposed mice than in controls ($n = 6$). *$p < 0.05$.

control or MeHg-exposed group (Fig. 3a). Two-way ANOVA analysis confirmed the significant effect of the perinatal MeHg exposure [$F(1,14) = 16.46$, $p = 0.001$] and the absence of interaction between MeHg and chronic fluoxetine treatments on the H3-tmK27 level at the BDNF promoter IV [$F(1,14) = 0.12$, $p = 0.73$]. We did not find any significant regulation of H3-tmK27 at the BDNF promoters VII (Fig. 3e) and II (data not shown), which are known to be less regulated by neuronal activity than the promoter IV (Tsankova *et al.* 2006).

We next investigated the effect of perinatal MeHg and adult fluoxetine treatments on the acetylation of histone H3 (K9 + K14), a marker of the active chromatin state (Bernstein *et al.* 2007; Kouzarides 2007). Consistent with the transcriptional repression of the BDNF gene, the level of H3 histone acetylation was decreased at the BDNF promoter IV in the exposed 12-week-old animals compared to controls [$F(3,14) = 11.43$, $p = 0.007$, one-way ANOVA followed by Fisher's PLSD test] (Fig. 3b). Moreover, long-lasting decrease in histone H3 acetylation produced by the perinatal exposure to MeHg persisted in 14-month-old animals [$F(1,10) = 16.11$, $p = 0.002$; one-way ANOVA] (Fig. 3d) and correlated with decreased BDNF mRNA level in the hippocampus. Importantly, chronic fluoxetine treatment significantly increased histone H3 acetylation at the BDNF promoter IV in the MeHg-exposed mice [$F(3,14) = 11.43$, $p < 0.0001$ vs. MeHg, one-way ANOVA followed by Fisher's PLSD test], while no significant effect of fluoxetine was observed in control mice (Fig. 3b). The significant interaction of perinatal MeHg-exposure and chronic fluoxetine treatment was confirmed by two-way ANOVA [$F(1,14) = 17.92$, $p = 0.0008$]. No regulation of H3K27 tri-methylation or H3 acetylation was observed at the BDNF promoters VII (Fig. 3e) and II (data not shown), or at the transcriptionally active housekeeping β-actin gene promoter (Fig. 3e). Taken together, these data suggest that a perinatal MeHg exposure induces a long-lasting reduction in BDNF expression in the hippocampus, which correlates with a repressive chromatin state produced by the increased H3-tmK27 and decreased acH3 histone levels at the BDNF promoter IV, and that chronic fluoxetine treatment overcomes this repression by up-regulating H3 acetylation at the BDNF promoter IV.

Histone methylation facilitates the DNA methylation at specific promoter regions, leading to repression of gene expression (Lachner and Jenuwein 2002; Volkel and Angrand 2007). To find out whether the increased level of H3K27 tri-methylation at the BDNF promoter IV correlates with an increase in DNA methylation at CpG sites within this promoter (Fig. 4a), we identified the methylation status of several cytosines, using sodium bisulfite mapping and

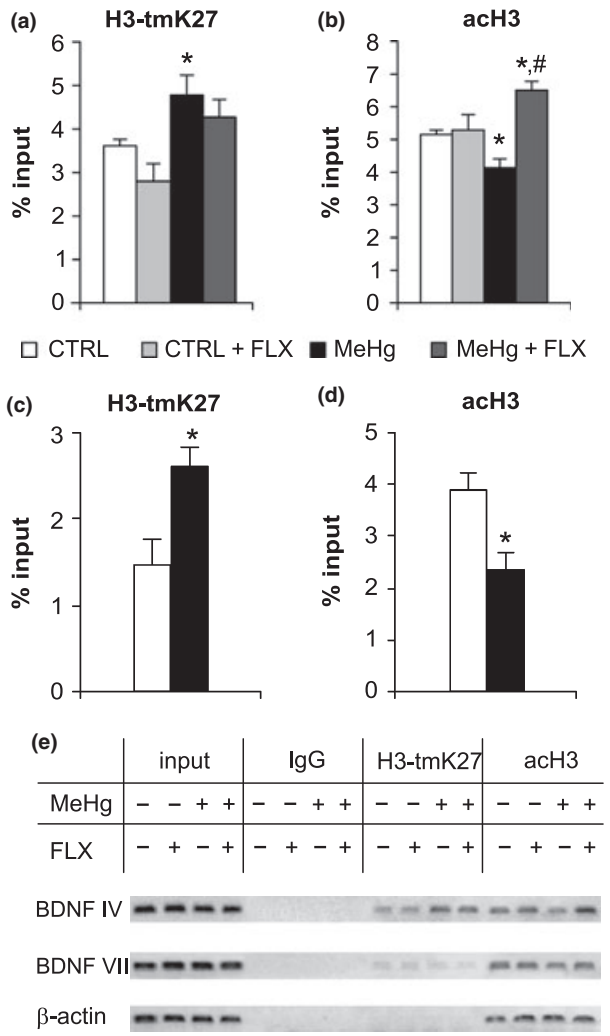


**Fig. 3** Histone modifications in hippocampus of adult males after perinatal exposure to MeHg and adult chronic fluoxetine treatment. (a–d) Levels of histone H3 modifications at BDNF promoter IV were quantified by RT-PCR ($n$ = 3–9). (a and b) In 12-week-old mice, histone H3K27 tri-methylation was significantly increased (a, * $p < 0.05$ vs. control) and H3 acetylation was decreased (b, * $p < 0.01$ vs. control) in the MeHg-exposed group. Chronic fluoxetine treatment did not change H3-tmK27 level (a), but acH3 was significantly enriched (b, # $p < 0.0001$ vs. MeHg) only in MeHg-exposed animals. (c and d) In 14-month-old MeHg-exposed mice, the level of H3 methylated K27 was increased (c, *$p < 0.05$), whereas H3 acetylation was decreased (d, *$p < 0.05$). (e) Representative ChIP analysis of the histone level at the promoters BDNF IV, BDNF VII and β-actin in 12-week-old mice. As a control for ChIP assay, samples were immunoprecipitated with non-immune rabbit IgG. Histone H3 modifications were not reportedly changed at the promoters BDNF VII and β-actin gene, which were used as control.

MS-SNuPE assay (Frommer *et al.* 1992; Clark *et al.* 1994). Previous studies have shown that decreased CpG methylation at these sites correlates with increased DNA binding of transcription factors such as cAMP response element-binding protein (Martinowich *et al.* 2003). Our results showed the significant increase in hippocampal DNA methylation at BDNF promoter IV in 14-month-old mice exposed to MeHg during early development (Fig. 4b). Direct sequencing approach confirmed the increased DNA methylation state at this promoter (data not shown). These data suggest that perinatal MeHg exposure produces a long-lasting increase in DNA methylation in the BDNF promoter IV region and a concomitant increase in the repressive H3K27 tri-methylation.

## Discussion

Here we show that developmental exposure to MeHg can lead to long-lasting depression-like behavioral alterations associated with repressive epigenetic changes in the regulation of the BDNF gene expression. We further show that chronic antidepressant treatment of adult mice reverses the effects of perinatal MeHg on behavior and BDNF gene expression at least partially through increased histone acetylation at the BDNF promoter area.

The etiology of depression is considered to be multifactorial, and this includes neurodevelopmental aspects. Susceptibility factors of genetic and/or environmental origin may increase the risk of mood disorders later in life by affecting the development of brain pathways involved in emotional behavior (see Ansorge *et al.* 2007). To elucidate the mechanisms behind the interaction between environmental and genetic factors on brain maturation, we need standardized animal models where quantitative developmental exposures reproducibly bring about behavioral effects that mimic these disorders. We have previously observed that exposure to low levels of MeHg during early stages of development induces long-term behavioral effects that are compatible with depression in rodent animal models (Onishchenko *et al.* 2007). Our current data confirm that MeHg-exposed mice show a longer immobility response in the FST in adult. We further found that fluoxetine treatment for 21 days in adult mice perinatally exposed to MeHg reverses the depression-like behavior in the FST. Despite certain limitations, FST has been proven to be an effective tool for estimating depression-like behavior and antidepressant activity in laboratory rodents (Cryan *et al.* 2002) and it has been extensively used in behavioral neuroscience for more than three decades (Porsolt *et al.* 1977; Porsolt 2000). Even if for depression-related behavior the FST has predictive validity to evaluate responses to antidepressant, one should always consider the obvious limits that rodent models have in relation to human affective disorders. Our data indicate that perinatal MeHg exposure, presumably through adverse interferences during critical developmental stages, produces a long-lasting predisposition to depression-like behavior, which can be ameliorated by adult antidepressant treatment.

Prospective studies on children prenatally exposed to low concentrations of MeHg have shown dose-related impair-

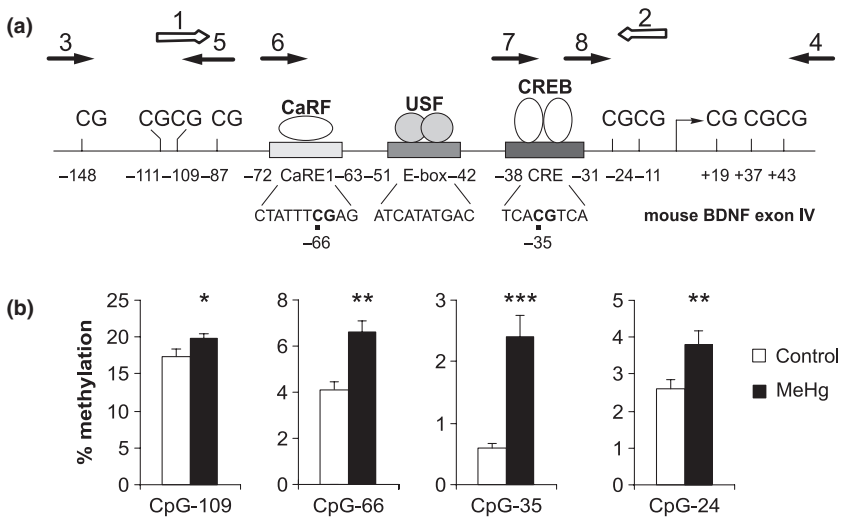


**Fig. 4** (a) A schematic presentation of the CpG sites and the regulatory elements within the mouse BDNF promoter IV (modified from Martinowich *et al.* 2003). Empty arrows 1 and 2: primers, used for ChIP assay. Black arrows: primers, used for DNA methylation analysis -3: Methyl-Fwd, 4: Methyl-Rev, 5: CpG-109, 6: CpG-66, 7: CpG-35 and 8: CpG-24. (b) Long-lasting changes in DNA methylation at the activity-dependent BDNF promoter IV in 14-month-old mice after perinatal exposure to MeHg. MS-SNuPE assay showed a significant increase in DNA methylation at the −109, −66, −35 and −24 CpG dinucleotides at the BDNF promoter IV in MeHg-exposed animals ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ments in memory, attention, language, and visuospatial perception (see Grandjean and Landrigan 2006). So far, there are no reports on depressive syndrome in subjects exposed *in utero* to low levels of MeHg. However, it should be considered that impairments in attention in children are often associated with mood disorders and that antidepressant treatment significantly improves the decrease in attention (Quintana *et al.* 2007). In addition, attention deficits in childhood have been reported in adults affected by depression (Alpert *et al.* 1996). It is also worth to mention that depressive syndromes have been reported in humans later in life after previous occupational exposure to mercury (Kobal Grum *et al.* 2006).

BDNF and its tyrosine kinase receptor TrkB have been proposed as key players in the regulation of mood disorders and antidepressant effects (see Castrén *et al.* 2007). BDNF levels have been shown to be reduced in hippocampus and serum of depressed patients and antidepressant treatment restores brain and blood BDNF levels to the normal range (Chen *et al.* 2001; Knable *et al.* 2004; Karege *et al.* 2005). In addition, the Val-66-Met polymorphism in the BDNF coding region that influences the activity-dependent BDNF release has been found to be associated with mood disorders in some, albeit not all populations (Post 2007). Furthermore, decreased expression of hippocampal BDNF mRNA and protein, especially in the dentate gyrus, has been shown in stress-induced animal models of depression (Smith *et al.* 1995; Grønli *et al.* 2006). Our *in situ* hybridization experiments detected a decrease in BDNF mRNA expression in the dentate gyrus of MeHg-exposed mice, which was reversed by chronic fluoxetine treatment. The correlation between hippocampal BDNF levels and behavioral response in the FST in our model further emphasizes the role of BDNF in the pathophysiology of mood disorders and antidepressant treatment.

It is known that conditions inducing BDNF expression also increase neurogenesis in the subgranular zone of the dentate gyrus and that antidepressants, as well as stress, co-regulate BDNF levels and subgranular zone neurogenesis (see Duman and Monteggia 2006). These facts point to the possibility that the lowering of dentate gyrus BDNF induced by MeHg may be associated to a decreased neurogenesis, which could also be restored by fluoxetine.

Fluoxetine treatment did not affect hippocampal BDNF mRNA level in control mice; although antidepressants have been shown to increase BDNF mRNA in most studies, many studies have not seen any significant effect (see Duman and Monteggia 2006).

The long-term regulation of gene expression through epigenetic mechanisms is presumed to be one of the molecular mechanisms that can mediate permanent changes in brain functioning and contribute to the pathogenesis of psychiatric disorders (Tsankova *et al.* 2007). Chromatin status and DNA methylation exerts control over the accessibility of transcription factors to promoters. Acetylation of the lysine residues neutralizes basic charge of the histone tails, thus unfolding chromatin and promoting transcription factor binding to DNA. Histone acetylation at the lysine-9 (K9) residue of H3 histones is a well-established marker of active chromatin. On the contrary, DNA methylation and

methylation of the histone H3-K27 have been implicated in the silencing of gene expression (Bernstein *et al.* 2007; Kouzarides 2007).

The involvement of epigenetic mechanisms in the activity-dependent regulation of BDNF gene expression has recently been demonstrated even in complex tissues such as hippocampus consisting of multiple cell types (Chen *et al.* 2003; Martinowich *et al.* 2003; Tsankova *et al.* 2006). Since the promoter IV of the BDNF gene is prominently regulated by neuronal activity and contains binding sites of many activity-regulated transcription factors, such as CaRF (calcium-responsive factor) and cAMP response element-binding protein (Tao *et al.* 1998; Chen *et al.* 2003; Martinowich *et al.* 2003), we focused our analysis on that promoter region. We have found that early exposure to MeHg induces long-lasting repressive changes in the chromatin structure of the BDNF gene in the hippocampus, consisting of an increase in histone H3-K27 tri-methylation and decrease in H3 acetylation at the BDNF promoter IV. This is consistent with the recent observation that social defeat stress, which produces depression-like behavioral changes and decreases BDNF mRNA levels, increases and decreases histone methylation and acetylation, respectively, in BDNF promoter regions (Tsankova *et al.* 2006). Moreover, we detected DNA hypermethylation at the BDNF promoter IV at the binding sites of activity-regulated transcription factors in the hippocampus of MeHg-exposed mice, which also is consistent with a repressed expression of the BDNF gene.

Chronic treatment with fluoxetine did not significantly alter the hypermethylation of H3-K27 in either MeHg-exposed or control mice. However, antidepressant treatment significantly up-regulated H3 acetylation at the BDNF promoter IV in MeHg-exposed mice, but not in control animals, thereby apparently overcoming the repressive chromatin state and contributing to the restoration of BDNF mRNA levels and recovery of behavior in the FST. This finding is in agreement with the recent study reporting that antidepressant treatment failed to counteract the social defeat induced increase in the repressive H3-K27 dimethylation at BDNF promoters, but it significantly increased H3 acetylation only in stressed mice and thereby restored BDNF mRNA levels and the stress-repressed behavior (Tsankova *et al.* 2006). Imipramine selectively down-regulated histone deacetylase 5 (HDAC5) in hippocampus and viral over-expression of HDAC5 counteracted the effects of imipramine (Tsankova *et al.* 2006), suggesting that decreased activity of HDACs may mediate the effects of fluoxetine on histone acetylation. These results suggest that significant changes in chromatin remodeling can be detected also in complex tissues such as hippocampus. Together these data suggest that stress and toxic substances may bring about an epigenetically mediated long-term suppression of BDNF gene expression and thereby contribute to depression-related behavior. Importantly, these epigenetic changes are not permanent, but are subject to alteration by treatments which ameliorate the long-term behavioral symptoms.

Although the actual mechanisms underlying MeHg-induced effects on plasticity and gene expression require further investigation, the results of the present study point to the importance that early environmental factors play in programming gene activity patterns via epigenetic changes. Toxic agents present in nature and recognized as environmental stressors are among the factors that can target epigenome causing long-lasting and even transgenerational effects (Sutherland and Costa 2003; Anway *et al.* 2005). Perinatal MeHg exposure might become a valuable experimental model, which allows the use of standardized and quantifiable exposures to study environment/epigenome interactions leading to long-term behavioral changes. A better understanding of the role that such interactions play in the etiology of complex diseases will help us to identify risk factors and possible preventive strategies.

## Acknowledgments

This work was supported by grants from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), the Swedish Research Council, the European Commission (FOOD-CT-2003- 506143) (SC), and Sigrid Juselius Foundation and Academy of Finland (EC). We are grateful to Salutas Pharma GmbH for providing fluoxetine.

## Supplementary material

The following supplementary material is available for this article:

**Fig. S1** TrkB mRNA levels in the hippocampal formation of MeHg-exposed mice did not differ from control levels either at the age of 12 weeks (a) or 14 months (b).

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1471-4159.2008.05484.x (This link will take you to the article abstract).

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

## References

Aid T., Kazantseva A., Piirsoo M., Palm K. and Timmusk T. (2007) Mouse and rat BDNF gene structure and expression revisited. *J. Neurosci. Res.* **85**, 525–535.

Alpert J. E., Maddocks A., Nierenberg A. A., O'Sullivan R., Pava J. A., Worthington J. J. 3rd, Biederman J., Rosenbaum J. F. and Fava M. (1996) Attention deficit hyperactivity disorder in childhood among adults with major depression. *Psychiatry Res.* **62**, 213–219.

Ansorge M. S., Hen R. and Gingrich J. A. (2007) Neurodevelopmental origins of depressive disorders. *Curr. Opin. Pharmacol.* **7**, 8–17.

Anway M. D., Cupp A. S., Uzumcu M. and Skinner M. K. (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science* **308**, 1466–1469.

Bernstein B. E., Meissner A. and Lander E. S. (2007) The mammalian epigenome. *Cell* **128**, 669–681.

Björnberg K. A., Vahter M., Grawe K. P. and Berglund M. (2005) Methyl mercury exposure in Swedish women with high fish consumption. *Sci. Total Environ.* **341**, 45–52.

Castrén E., Voikar V. and Rantamaki T. (2007) Role of neurotrophic factors in depression. *Curr. Opin. Pharmacol.* **7**, 18–21.

Chakrabarti S. K., James J. C. and Mirmira R. G. (2002) Quantitative assessment of gene targeting in vitro and in vivo by the pancreatic transcription factor, Pdx1. Importance of chromatin structure in directing promoter binding. *J. Biol. Chem.* **277**, 13286–13293.

Chen B., Dowlatshahi D., MacQueen G. M., Wang J. F. and Young L. T. (2001) Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. *Biol. Psychiatry* **50**, 260–265.

Chen W. G., Chang Q., Lin Y., Meissner A., West A. E., Griffith E. C., Jaenisch R. and Greenberg M. E. (2003) Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science* **302**, 885–889.

Clark S. J., Harrison J., Paul C. L. and Frommer M. (1994) High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* **22**, 2990–2997.

Colvis C. M., Pollock J. D., Goodman R. H. *et al.* (2005) Epigenetic mechanisms and gene networks in the nervous system. *J. Neurosci.* **25**, 10379–10389.

Crowley J. J., Blendy J. A. and Lucki I. (2005) Strain-dependent antidepressant-like effects of citalopram in the mouse tail suspension test. *Psychopharmacology (Berl.)* **183**, 257–264.

Cryan J. F., Markou A. and Lucki I. (2002) Assessing antidepressant activity in rodents: recent developments and future needs. *Trends Pharmacol. Sci.* **23**, 238–245.

Dopheide J. A. (2006) Recognizing and treating depression in children and adolescents. *Am. J. Health Syst. Pharm.* **63**, 233–243.

Dulawa S. C., Holick K. A., Gundersen B. and Hen R. (2004) Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology* **29**, 1321–1330.

Duman R. S. and Monteggia L. M. (2006) A neurotrophic model for stress-related mood disorders. *Biol. Psychiatry* **59**, 1116–1127.

Frommer M., McDonald L. E., Millar D. S., Collis C. M., Watt F., Grigg G. W., Molloy P. L. and Paul C. L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl Acad. Sci. USA* **89**, 1827–1831.

Gonzalgo M. L. and Jones P. A. (1997) Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). *Nucleic Acids Res.* **25**, 2529–2531.

Grandjean P. and Landrigan P. J. (2006) Developmental neurotoxicity of industrial chemicals. *Lancet* **368**, 2167–2178.

Grønli J., Bramham C., Murison R., Kanhema T., Fiske E., Bjorvatn B., Ursin R. and Portas C. M. (2006) Chronic mild stress inhibits BDNF protein expression and CREB activation in the dentate gyrus but not in the hippocampus proper. *Pharmacol. Biochem. Behav.* **85**, 842–849.

Hofer M., Pagliusi S. R., Hohn A., Leibrock J. and Barde Y. A. (1990) Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. *EMBO J.* **9**, 2459–2464.

Karege F., Vaudan G., Schwald M., Perroud N. and La Harpe R. (2005) Neurotrophin levels in postmortem brains of suicide victims and the effects of antemortem diagnosis and psychotropic drugs. *Brain Res. Mol. Brain Res.* **136**, 29–37.

Knable M. B., Barci B. M., Webster M. J., Meador-Woodruff J. and Torrey E. F. (2004) Molecular abnormalities of the hippocampus in severe psychiatric illness: postmortem findings from the Stanley Neuropathology Consortium. *Mol. Psychiatry* **9**, 609–620.

Kobal Grum D., Kobal A. B., Arneric N., Horvat M., Zenko B., Dzeroski S. and Osredkar J. (2006) Personality traits in miners with past occupational elemental mercury exposure. *Environ. Health Perspect.* **114**, 290–296.

Kouzarides T. (2007) Chromatin modifications and their function. *Cell* **128**, 693–705.

Kumar A., Choi K. H., Renthal W. *et al.* (2005) Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. *Neuron* **48**, 303–314.

Lachner M. and Jenuwein T. (2002) The many faces of histone lysine methylation. *Curr. Opin. Cell Biol.* **14**, 286–298.

Lapham L. W., Cernichiari E., Cox C., Myers G. J., Baggs R. B., Brewer R., Shamlaye C. F., Davidson P. W. and Clarkson T. W. (1995) An analysis of autopsy brain tissue from infants prenatally exposed to methymercury. *Neurotoxicology* **16**, 689–704.

Levenson J. M., O'Riordan K. J., Brown K. D., Trinh M. A., Molfese D. L. and Sweatt J. D. (2004) Regulation of histone acetylation during memory formation in the hippocampus. *J. Biol. Chem.* **279**, 40545–40559.

Mahaffey K. R. (2005) Mercury exposure: medical and public health issues. *Trans. Am. Clin. Climatol. Assoc.* **116**, 127–153.

Martinowich K., Hattori D., Wu H., Fouse S., He F., Hu Y., Fan G. and Sun Y. E. (2003) DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science* **302**, 890–893.

McEwen B. S. (1999) Stress and hippocampal plasticity. *Annu. Rev. Neurosci.* **22**, 105–122.

Meaney M. J. (2001) Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. *Annu. Rev. Neurosci.* **24**, 1161–1192.

Mendola P., Selevan S. G., Gutter S. and Rice D. (2002) Environmental factors associated with a spectrum of neurodevelopmental deficits. *Ment. Retard. Dev. Disabil. Res. Rev.* **8**, 188–197.

National Research Council (2000) *Scientific frontiers in developmental toxicology and risk assessment*. National Academy Press, Washington, DC.

Nestler E. J., Barrot M., DiLeone R. J., Eisch A. J., Gold S. J. and Monteggia L. M. (2002) Neurobiology of depression. *Neuron* **34**, 13–25.

Onishchenko N., Tamm C., Vahter M., Hokfelt T., Johnson J. A., Johnson D. A. and Ceccatelli S. (2007) Developmental Exposure to Methylmercury Alters Learning and Induces Depression-like Behavior in Male Mice. *Toxicol. Sci.* **97**, 428–437.

Porsolt R. D. (2000) Animal models of depression: utility for transgenic research. *Rev. Neurosci.* **11**, 53–58.

Porsolt R. D., Bertin A. and Jalfre M. (1977) Behavioral despair in mice: a primary screening test for antidepressants. *Arch. Int. Pharmacodyn. Ther.* **229**, 327–336.

Post R. M. (2007) Role of BDNF in bipolar and unipolar disorder: Clinical and theoretical implications. *J. Psychiatr. Res.* **41**, 979–990.

Quintana H., Butterbaugh G. J., Purnell W. and Layman A. K. (2007) Fluoxetine monotherapy in attention-deficit/hyperactivity disorder and comorbid non-bipolar mood disorders in children and adolescents. *Child Psychiatry Hum. Dev.* **37**, 241–253.

Rantamäki T., Hendolin P., Kankaanpää A., Mijatovic J., Piepponen P., Domenici E., Chao M. V., Männistö P. T. and Castrén E. (2007) Pharmacologically diverse antidepressants rapidly activate brain-derived neurotrophic factor receptor trkb and induce phospholi-

pase-Cgamma signaling pathways in mouse brain. *Neuropsychopharmacology* **32**, 2152–2162.

Smith M. A., Makino S., Kvetnansky R. and Post R. M. (1995) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. *J. Neurosci.* **15**, 1768–1777.

Sutherland J. E. and Costa M. (2003) Epigenetics and the environment. *Ann. N Y Acad. Sci.* **983**, 151–160.

Tao X., Finkbeiner S., Arnold D. B., Shaywitz A. J. and Greenberg M. E. (1998) Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. *Neuron* **20**, 709–726.

Tsankova N. M., Berton O., Renthal W., Kumar A., Neve R. L. and Nestler E. J. (2006) Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat. Neurosci.* **9**, 519–525.

Tsankova N., Renthal W., Kumar A. and Nestler E. J. (2007) Epigenetic regulation in psychiatric disorders. *Nat. Rev. Neurosci.* **8**, 355–367.

Volkel P. and Angrand P. O. (2007) The control of histone lysine methylation in epigenetic regulation. *Biochimie* **89**, 1–20.

Wells J. and Farnham P. J. (2002) Characterizing transcription factor binding sites using formaldehyde crosslinking and immunoprecipitation. *Methods* **26**, 48–56.

Wong J. Y., Liberatore G. T., Donnan G. A. and Howells D. W. (1997) Expression of brain-derived neurotrophic factor and TrkB neurotrophin receptors after striatal injury in the mouse. *Exp. Neurol.* **148**, 83–91.