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SOCIAL ISOLATION-INDUCED EPIGENETIC CHANGES IN MIDBRAIN OF ADULT MICE

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Social isolation and loneliness increase the risk of death as much as well-established risk factors for mortality such as cigarette smoking and alcohol consumption. The underlying molecular mechanisms are poorly understood. In the present study, 3 months old male C57BL/6 mice were socially isolated by individual housing for another 3 months. At the age of 6 months, epigenetic changes were analyzed in midbrain. Social isolation of male adult mice led to an increased global DNA methylation, which was associated with enhanced activity of DNA methyltransferase. Di- and tri-methylation of global histone H3 lysine 4 (H3K4) were increased in midbrain of socially isolated mice, accompanied by enhanced H3K4 histone methyltransferase activity. In addition, social isolation of adult mice led to activation of histone acetyltransferases as well as of histone deacetylases (HDAC) resulting in a net enhancement of histone H3 lysine 9 (H3K9) acetylation. Gene-specific effects were observed for *Hdac1*, *Hdac3* and the serotonin transporter *Slc6a4*. Social isolation led to an up-regulation of *Hdac1* and *Hdac3*, associated with decreased DNA methylation in the CpG island of the respective genes. On the contrary, the *Slc6a4* gene was down-regulated, which was associated with enhanced DNA methylation. Collectively, the results from the present study demonstrate for the first time that social isolation of adult mice leads to a wide range of global epigenetic changes and these effects may have profound impact on gene expression pattern and phenotype of the socially isolated animals.

Key words: *social isolation, brain, epigenetics, DNA methylation, serotonin, serotonin transporter, schizophrenia, depression*

INTRODUCTION

Epigenetics refers to chromatin-based mechanisms important in the regulation of gene expression that do not involve changes to the DNA sequence *per se*. These include DNA methylation, histone density and posttranslational modifications, and RNA-based mechanisms (1). Epigenetic mechanisms are responsible for tissue-specific gene expression during differentiation and these mechanisms underlie the processes of developmental plasticity. Widespread epigenetic reprogramming occurs after fertilization to ensure totipotency of the developing embryo, with methylation patterns associated with genomic imprinting being maintained (2). Developmentally induced epigenetic modifications of DNA are generally stable during the mitotic cell divisions that continue throughout a lifetime (2). On the other hand, the epigenome is also dynamic and responds to changes of the environment (3, 4). Gene regulation by epigenetic mechanisms occurs not only in the developing brain but in the adult brain as well (5). Epigenetic mechanisms may be activated by environmental factors, such as diet, behavior, medication and social interactions/social relationships (6).

The modern way of life in industrialized countries is greatly reducing the quantity and quality of social relationships (7). Many people live far away from their relatives. More and more people of all ages are living alone, and loneliness is becoming increasingly common (7). A recent meta-analysis of 148 studies involving 308849 participants demonstrates that individuals with adequate social relationships have a 50% greater likelihood of survival compared to those with poor or insufficient social relationships (7). Social isolation and loneliness increase the risk of death as much as well-established risk factors for mortality such as smoking and alcohol consumption and exceed the influence of other risk factors such as physical inactivity and obesity (7).

It should be distinguished between two types of social isolation: social isolation in the early-life and social isolation in the adult age. Exposure of mammals to early-life social isolation or maternal separation profoundly affects brain development and adult behavior, and may facilitate the occurrence of psychiatric disorders, such as depression and schizophrenia (8). These effects are, at least in part, mediated by epigenetic mechanisms (9). The epigenetic changes in response to early-life social isolation are in agreement with the concept that pre- and post-natal periods are sensitive phases of developmental plasticity mediated by epigenetic modifications (2).

In contrast, whether social isolation and loneliness in adults also cause epigenetic changes is less clear (3-6). Importantly, the

majority of the lonely participants enrolled in the aforementioned meta-analysis are individuals suffering from loneliness in their adult age. Therefore, we designed the present study to address this question by using adult mice. We used individual housing as a mouse model of social isolation and studied the epigenetic changes in the brain. Particularly, we focused on midbrain because it controls the visual and auditory system, eye movement, and mediates emotional processing (10). The *raphe* nuclei in the midbrain represent the principal source of serotonergic innervation (11). It is believed that selective serotonin reuptake inhibitors, the most widely prescribed antidepressants in the treatment of depression, act in these nuclei. The largest group of serotonergic neurons in the brain originates from the dorsal raphe nucleus in the midbrain and pons. From here, the serotonergic neurons project to nearly every area of the forebrain (12). The dorsal raphe nucleus also receives inputs from some of its projection regions, forming feedback loops that are critical for stress response and emotion regulation (12, 13). We hypothesized that social isolation of adult mice will lead to a wide range of epigenetic changes.

MATERIAL AND METHODS

Animals

Male C57BL/6 mice were housed in groups after weaning. At the age of 3 months, the mice were either left in group (as control) or assigned to social isolation by individually housing for another 3 months. The animal experiment was approved by the responsible regulatory authority (Landesuntersuchungsamt Rheinland-Pfalz; 23 177-07/G 12-1-021) and was performed in accordance with the German animal protection law and the guidelines for the use of experimental animals as stipulated by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Blood pressure measurement

Systolic blood pressure, diastolic blood pressure, and mean blood pressure were measured noninvasively in conscious animals by determining the tail blood volume with a volume-pressure recording sensor and an occlusion tail-cuff using a computerized system (CODA System, Kent Scientific) (14, 15). Animals were placed in individual holders. The occlusion cuff and the volume-pressure recording cuff were placed close to the base of the tail. After a habituation period of 30 min on a warm pad, the tail cuff was inflated 20 times before measurements were performed. The mean of a minimum of 6 recordings on each occasion was taken, and the mean data were compared between the groups.

Isolation of midbrain

At the age of 6 months, the mice were sacrificed with an overdose of pentobarbital. The skull was opened; the brain was removed and rinsed in ice-cold dissection buffer (Hank's balanced salt solution). The olfactory bulb and cerebellum tissues were removed, and the pons and medulla oblongata were carefully separated to expose the midbrain. After separation of the cortex and hippocampus, the midbrain was gained as previously described (16).

Isolation of genomic DNA, histones and nuclear extracts

The midbrain samples were homogenized in a TissueLyser (Qiagen). Genomic DNA, histones and nuclear extracts were isolated using the QIAamp DNA Micro Kit (56304, Qiagen), the EpiQuik Total Histone Extraction Kit (OP-0006, Epigentek) and the Nuclear Extraction Kit II (OP-0022, Epigentek), respectively.

Measurement of epigenetic changes

Global DNA methylation, global histone H3K4 methylation, global histone K3K9 acetylation were measured using the MethylFlash™ Methylated DNA Quantification Kit (P-1034, Epigentek), the EpiQuik Global Di-Methyl Histone H3K4 Quantification Kit (P-3022, Epigentek), the EpiQuik Global Tri-Methyl Histone H3K4 Quantification Kit (P-3026, Epigentek), and the EpiQuik Global Acetyl Histone H3K9 Quantification Kit (P-4010, Epigentek), respectively. In these kits, DNA, methylated H3K4 or the acetylated H3K9 were captured to the provided microplate due to a high DNA affinity or with specific antibodies, respectively. Then, the amount of methylated H3K4 and acetylated H3K9 was measured through an ELISA-like reaction by reading the absorbance. The values in control groups were set 100% and changes in the isolated animals were compared to the control values.

Measurement of activity of DNA methyltransferase, histone methyltransferase, histone acetyltransferase and histone deacetylase

Activity of DNA methyltransferase (DNMT) activity, histone methyltransferase (HMT), histone acetyltransferase (HAT) and histone deacetylase (HDAC) was measured using the EpiQuik Histone Methyltransferase Activity/Inhibition Assay Kit (H3K4) (P-3002, Epigentek), the EpiQuik™ HAT Activity/Inhibitor Assay Kit (P-4003, Epigentek), and the Epigenase HDAC Activity/Inhibition Direct Assay Kit (P-4034, Epigentek), respectively. In these kits, the provided microplates contain the respective substrates stably coated onto the wells. Midbrain samples (histones or nuclear extracts) were added to catalyze to the intended reaction. The products were then detected in ELISA-like reactions using specific antibodies. Activity of the enzymes in the control animals was set 100% and changes in the isolated animals were compared to these values.

Immunohistochemistry

Midbrain samples were embedded in Tissue-Tek® OCT™ Compound (4583, Sakura). Cyrostat sections (7 µm) were stained for acetylated histone H3 lysine 9 (H3K9ac) using the rabbit anti-acetyl-H3K9 (H9286, Sigma-Aldrich; diluted 1:1000) as the primary antibody. The specimens were then incubated with biotinylated anti-rabbit secondary antibody (BA-1000, Vector Laboratories) and then stained with the VECTASTAIN® ABC-Reagent (PK-6100, Vector Laboratories). Counterstaining was performed with hematoxylin (MHS32, Sigma-Aldrich). Staining quantification was performed using the TMARKER software as described by the developers (17). In brief images were loaded into the software and background was subtracted for each picture by the software. Afterwards the ratio *r* was adjusted to fit the labeled nuclei in the image. All other settings are left as default and the labeled nuclei were counted by the software.

Real time qPCR

RNA of midbrain samples was isolated using peqGOLD TriFast™ (30-2010, PEQLAB). Brain tissue was placed in 2 ml Safe-Lock Tubes™ (0030 120.094, Eppendorf) together with Percellys ceramic beads (91-PCS-CKM, Peqlab) and 500 ml TriFast™ solution. The tissue was lysed at 20.000 Hz for 4 minutes in a TissueLyser (Qiagen). RNA was isolated according

to manufacturer's protocol. cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (4368813, Applied Biosystems). Quantitative real time qPCR reactions were performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich). Relative mRNA levels of target genes were quantified using comparative threshold C_T normalized to housekeeping gene glycerinaldehyd-3-phosphat-dehydrogenase (Gapdh). mRNA expression in control animals was set 100%. Primers were designed as described previously (18). The qPCR primer sequences were as follows: Gapdh_forward: ctc aac tac atg gtc tac atg ttc ca, Gapdh_reverse: cca ttc tcg gcc ttg act gt, Hdac1_forward: ttc caa cat gac caa cca ga, Hdac1_reverse: ggc agc atc ctc aag ttc tc, Hdac3_forward: tgc ttc aat ctc agc att cg, Hdac3_reverse: gta gcc acc acc tcc cag ta, Slc6a4_forward: ggc tga gat gag gaa cga ag and Slc6a4_reverse: atg tgg atg ctg gca tgt ta.

Bisulfite conversion and pyrosequencing

Isolated genomic DNA was bisulfite-converted and cleaned up with the EZ DNA Methylation-Direct™ Kit (D5021, Zymo Research). Bisulfite pyrosequencing was performed on a PSQ96MA Pyrosequencing System (Biotage, Uppsala, Sweden) with the PyroGold SQA reagent kit (Biotage). PCR primers and sequencing primers for bisulfite pyrosequencing were designed using the Pyrosequencing Assay Design Software (Biotage) to study the methylation status of the CpG islands in the promoter regions of *Hdac1* (-172 to -132, 7 CpGs analyzed), *Hdac3* (-272 to -196, 6 CpGs analyzed) and *Slc6a4* (-21 to +13, 9 CpGs analyzed) with following sequences: Hdac1_forward: aga ttt ttt ttt tgg tgt ttt gag t, Hdac1_reverse: biotin-ata act tct cta aac tac cct tac, Hdac1_sequencing: gta tag tta ggg ttt tat ttt aat, Hdac3_forward: gtt ggg ggg att agg ttt, Hdac3_reverse: biotin-acc aac aat ctt cct aac tca cta, Hdac3_sequencing: aga atg taa gag tag ttt tt, Slc6a4_forward: tgt tgt atg tgt agg aat gag tg, Slc6a4_reverse: biotin-act cta aat ctc caa aat ttc cat c and Slc6a4_sequencing: tga ggg gta gta agt ata aat ag.

For the methylation data analysis the Pyro Q-CpG software (Biotage) was used. The mean methylation status of the CpGs was analyzed as described by Schneider *et al*. (19). In brief, methylation status in % of the examined single CpGs was obtained by the Pyro Q-CpG software (Biotage). The single values were summed up and divided by the number of CpGs investigated to get the mean methylation status (%) of the examined part of the CpG islands.

Statistical analysis

Data are expressed as the mean ± S.E.M. Student's t test was used for comparison of the two groups. Values of $P<0.05$ were considered significantly different.

RESULTS

Blood pressure

Social isolation of adult male C57BL/6 mice for 3 months led to a moderate, but significant and reproducible increase in blood pressure (*Table 1*). No significant differences were found in body weight, food intake or water consumption between control and isolated animals (*Table 1*).

DNA methylation and DNA methyltransferase activity

Social isolation of adult male C57BL/6 mice for 3 months led to an increase in global DNA methylation in midbrain (*Fig*.

Table 1. Body weight and blood pressure (BP) at the age of 6 months (n=10).

	Control	Isolation	*P* value
Body weight (g)	34.1±1.3	32.9±0.9	0.45
Systolic BP (mmHg)	98.1±1.0	105.6±2.9	0.02
Diastolic BP (mmHg)	75.7±1.3	80.0±1.9	0.08
Mean BP (mmHg)	82.9±1.2	88.2±2.2	0.04
Food intake (g/day)	3.5±0.1	3.7±0.1	0.34
Water intake (g/day)	3.6 0.2	4.0±0.1	0.17

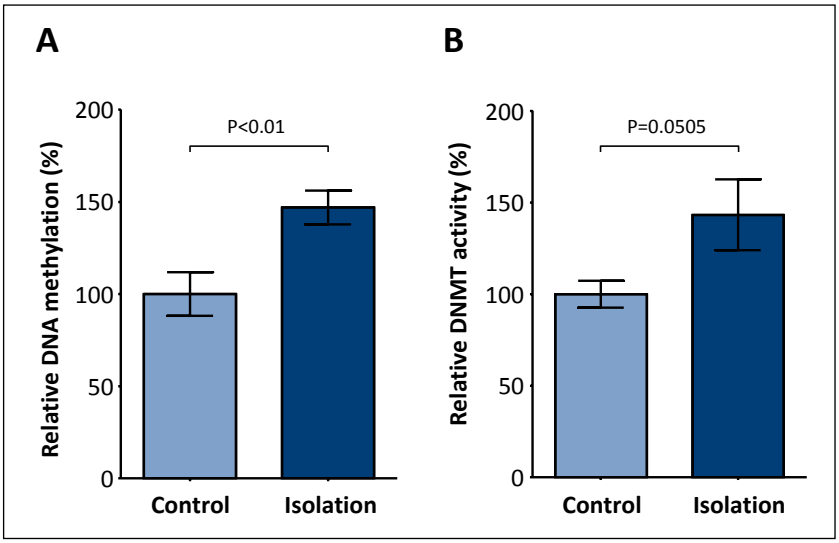


Fig. 1. Social isolation enhances global DNA methylation and DNMT activity in midbrain. Adult (3 months of age) male C57BL/6 mice were either kept in groups (control) or housed individually (isolation) for another 3 months. Global DNA methylation (*A*) and DNA methylatransferase (DNMT) activity (*B*) were analyzed in midbrain. The values shown are relative to Control, which was set 100%. Columns represent mean ±S.E.M., n=7–10.

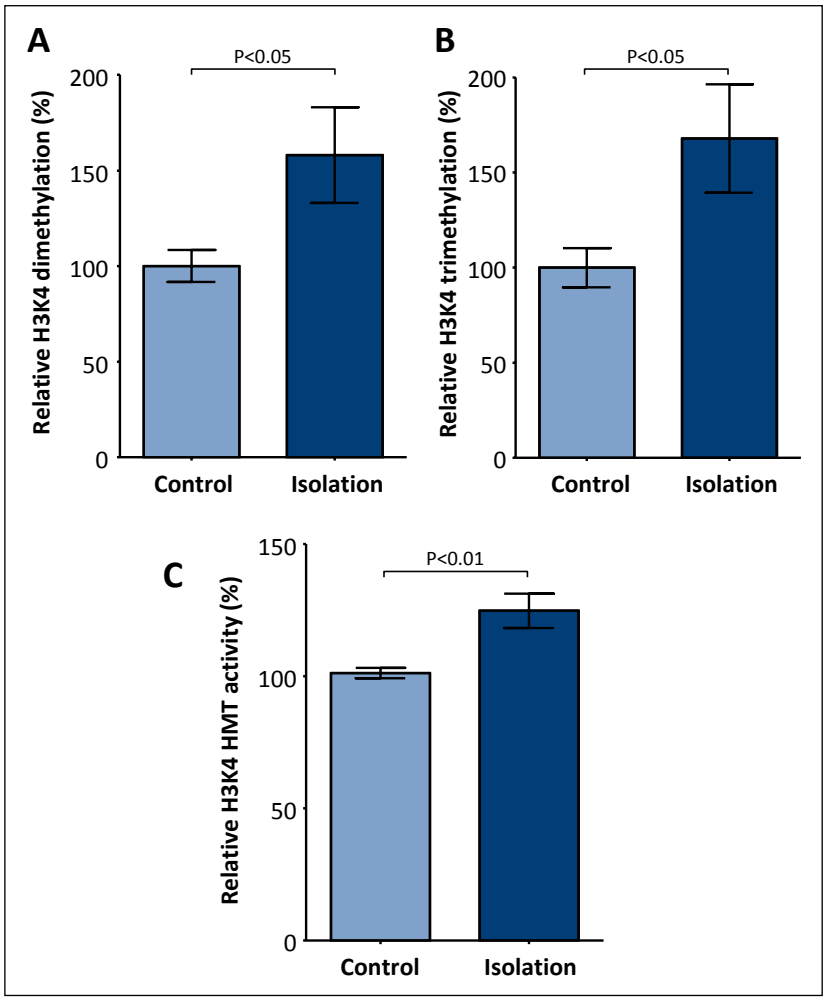


Fig. 2. Social isolation enhances global histone H3K4 methylation and H3K4 HMT activity in midbrain. Adult (3 months of age) male C57BL/6 mice were either kept in groups (control) or housed individually (isolation) for another 3 months. Global histone H3K4 di-methylation (*A*), tri-methylation (*B*) and histone H3K4 methylatransferase (HMT) activity (*C*) were analyzed in midbrain. The values shown are relative to Control, which was set 100%. Columns represent mean ±S.E.M., n=5–6 (*A* and *B*) or 10 (*C*).

1A). Consistently, DNMT activity in midbrain was found enhanced (*Fig. 1B*). It is not known whether social isolation for a time period shorter than 3 months is sufficient to induce epigenetic changes in adult brain, as we did not measure DNA methylation or histone modification at time points prior to 3 months.

H3K4 methylation

Histone posttranslational modifications are important epigenetic marks. Social isolation of adult male C57BL/6 mice for 3 months resulted in increased methylation of H3K4 in midbrain, both H3K4 dimethylation (*Fig. 2A*) and H3K4 trimethylation (*Fig. 2B*). This was associated with enhanced activity of H3K4 HMT (*Fig. 2C*).

H3K9 acetylation

Acetylation of H3K9 is postulated as a positive mark occurring in transcriptionally active euchromatin. Acetylation of the histone tails is carried out by HAT and removed by HDAC. Social isolation of adult male C57BL/6 mice led to an increase in the activity of both HAT (*Fig. 3A*) and HDAC (*Fig. 3B*) in midbrain. The net acetylation of H3K9 was enhanced by social isolation (*Fig. 3C*). The enhanced H3K9 acetylation in midbrain in response to social isolation could also be seen in the immunohistochemical staining using an antibody against acetylated H3K9 (*Fig. 4*). Quantification of the images shows an increase of ≈20% (1497±84.5 positively stained cells in the isolation group compared with 1252±62.2 positive cells in the control group, n=9, P<0.05).

mRNA expression levels and promoter methylation of selected genes

The mRNA expression of histone deacetylases Hdac1 and Hdac3 was significantly increased in the midbrain of socially isolated C57BL/6 mice (*Fig. 5A* and *5C*). This increase in mRNA expression was associated with a decrease in CpG methylation in the promoter region of these genes (*Fig. 5B*).

On the contrary, the mRNA expression of the serotonin transporter Slc6a4 was markedly decreased by social isolation (*Fig. 6A*), which was paralleled by an increase in DNA methylation in the promoter region (*Fig. 6B* and *6C*).

DISCUSSION

Loneliness in humans is associated with an increase in blood pressure and this effect is independent of other risk factors such as age, gender, race or ethnicity, cardiovascular risk factors, medications, or health conditions (20, 21). In the socially isolated mice used in the present study, we found an increase of systolic blood pressure that is ~7 mmHg over that of the control mice (*Table 1*). These results indicate that our mouse social isolation model resembles human loneliness. The isolated mice

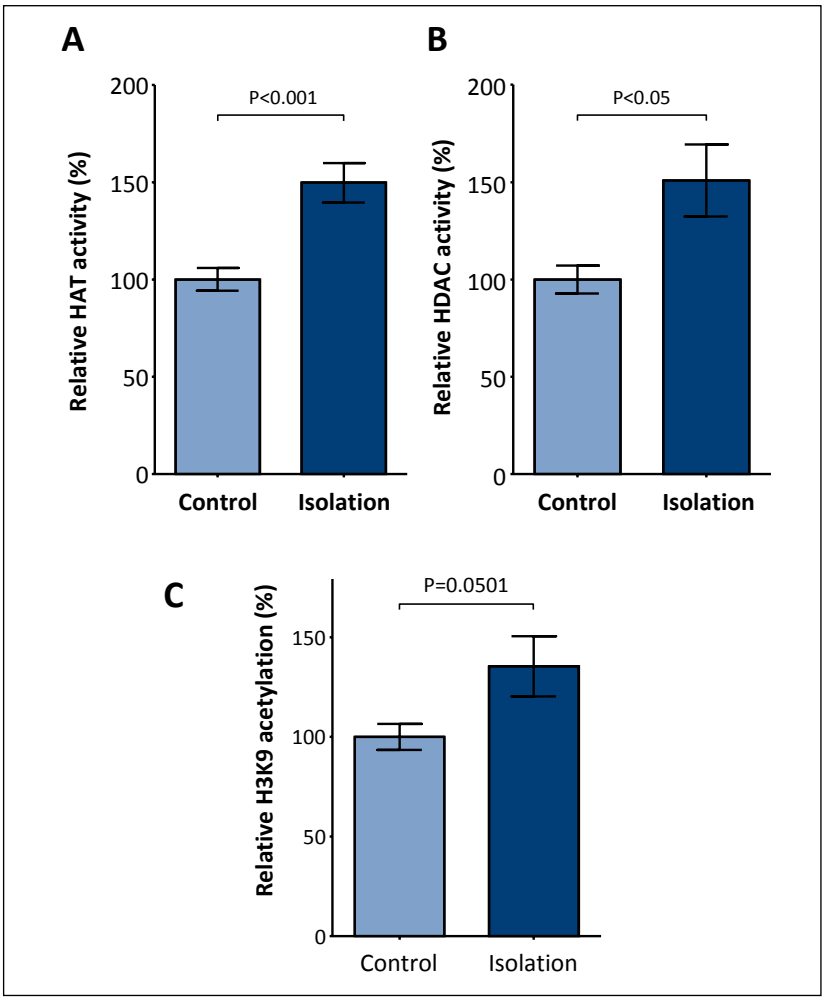


Fig. 3. Social isolation enhances HAT activity, HDAC activity and global histone H3K9 acetylation in midbrain. Adult (3 months of age) male C57BL/6 mice were either kept in groups (control) or housed individually (isolation) for another 3 months. Activity of histone acetyltransferases (HAT), histone deacetylases (HDAC) and global histone H3K9 acetylation (*C*) were analyzed in midbrain. The values shown are relative to Control, which was set 100%. Columns represent mean ±S.E.M., n=9–10 (*A* and *B*) or 8 (*C*).

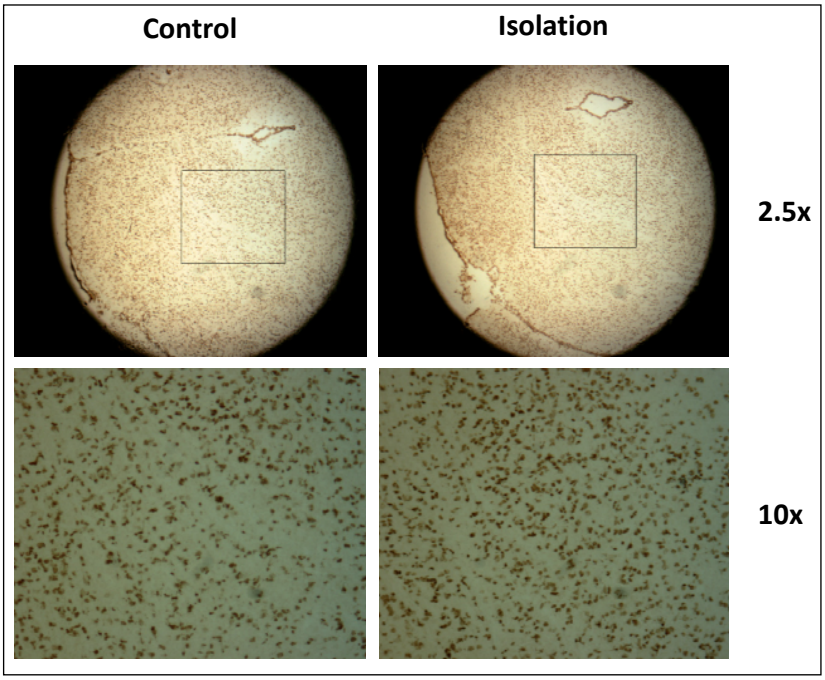
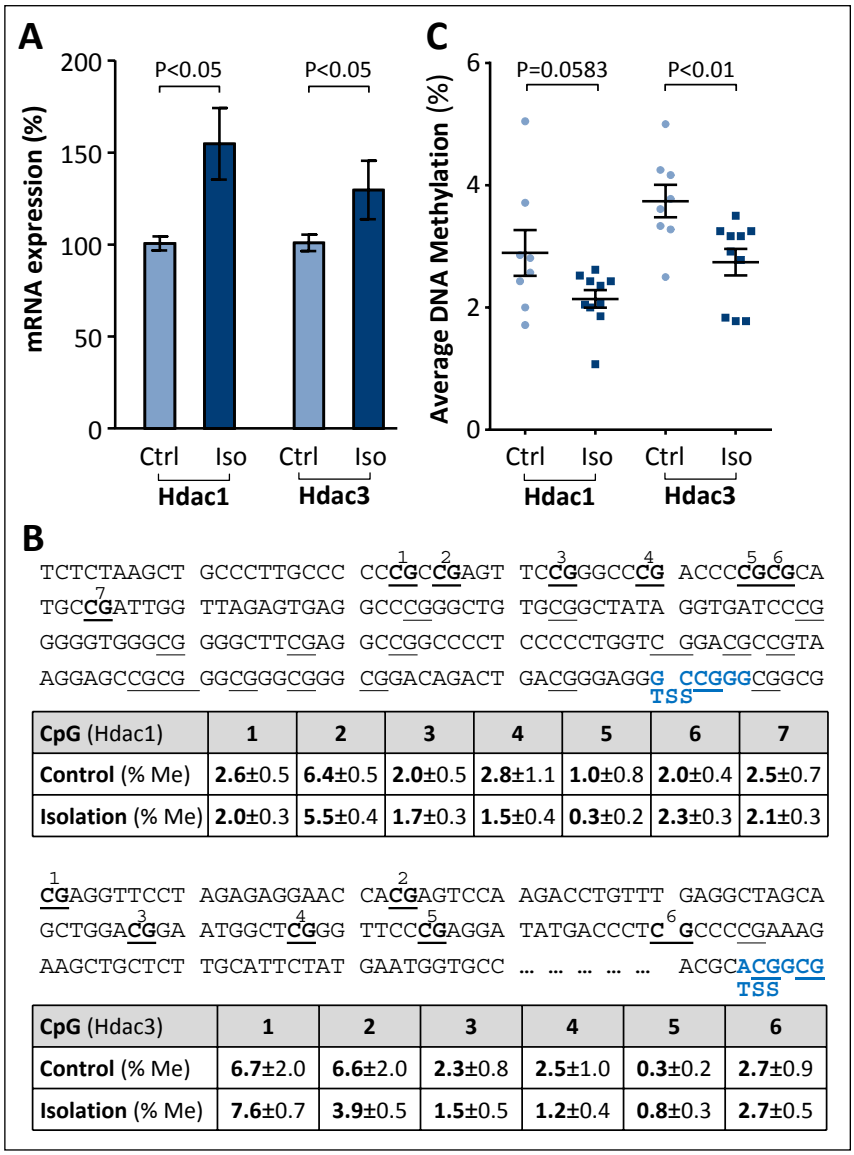


Fig. 4. Social isolation enhances global histone H3K9 acetylation in midbrain. Adult (3 months of age) male C57BL/6 mice were either kept in groups (control) or housed individually (isolation) for another 3 months. Acetylation of histone H3K9 in midbrain was stained with a rabbit anti-acetyl-H3K9 antibody. Upper panels: lower magnification with 2.5× objective. Lower panels: higher magnification with 10× objective. The pictures shown are representative of 9 images with similar results.

could see, hear and smell other mice; without a possibility for intensive interactions. Also this is a similar situation to human loneliness.

A limitation of study is that we measured blood pressure only with the tail-cuff method and not with the radiotelemetry technique. In a validation study by comparison to simultaneous



CpG (Hdac1)	1	2	3	4	5	6	7
Control (% Me)	2.6±0.5	6.4±0.5	2.0±0.5	2.8±1.1	1.0±0.8	2.0±0.4	2.5±0.7
Isolation (% Me)	2.0±0.3	5.5±0.4	1.7±0.3	1.5±0.4	0.3±0.2	2.3±0.3	2.1±0.3

CpG (Hdac3)	1	2	3	4	5	6
Control (% Me)	6.7±2.0	6.6±2.0	2.3±0.8	2.5±1.0	0.3±0.2	2.7±0.9
Isolation (% Me)	7.6±0.7	3.9±0.5	1.5±0.5	1.2±0.4	0.8±0.3	2.7±0.5

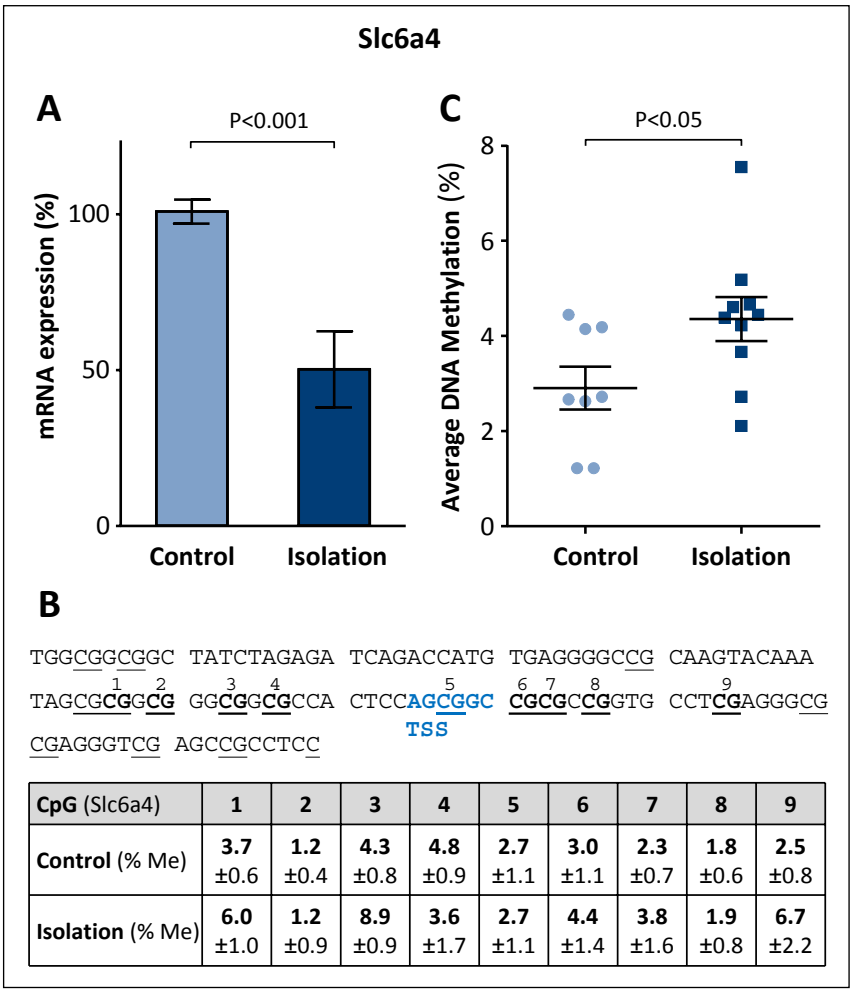
Fig. 5. Social isolation leads to up-regulation of Hdac1 and Hdac3 mRNAs. Adult (3 months of age) male C57BL/6 mice were either kept in groups (control) or housed individually (isolation) for another 3 months. mRNA expression of Hdac1 and Hdac3 was determined in midbrain with quantitative real time RT-PCR (*A*). Partial sequences of the CpG islands of Hdac1 and Hdac3 are shown in panel B. The methylation status of the individual CpGs studied (indicated with numbers) is shown in the tables (*B*). The values of all individual CpGs in each single sample were summed up to get an average methylation value of that animal (*C*). TSS, transcription start site. Columns (*A*) represent mean ±S.E.M. Each point (*C*) represents one animal. n=8–10.

radiotelemetry measurements in mice, the volume-pressure recording tail-cuff system has been demonstrated to provide accurate blood pressure measurements over the physiological range of blood pressure (14).

There is evidence that social environment modulates the progression of atherosclerosis in experimental animals. The Watanabe heritable hyperlipidemic (WHHL) rabbits develop atherosclerotic lesions spontaneously because of a genetic defect in lipoprotein clearance. Individually caged (for 4 months) WHHL rabbits were hyperinsulinemic and showed larger aortic atherosclerosis compared to animals in a stable group, in which littermates were paired daily for the entire study (22). Similar results have been obtained in the atherosclerosis-prone apolipoprotein E-knockout mice. Mice socially deprived by individual housing (for 20 weeks) developed significantly greater atherosclerotic plaque in the innominate artery compared to any other animals housed in groups, regardless with either enriched environment, deprived environment, or enriched environment with exercise (23). In another study, ischemic stroke was induced *via* transient intraluminal middle cerebral artery occlusion (MCAO) in mice. Compared to mice pair-housed with an ovariectomized female, socially isolated (housed individually for 2 weeks before stroke induction) male mice had lower post-stroke survival rate, and developed larger brain infarct and edema (24). These studies are compatible with our results and clearly highlight the detrimental impact of social isolation on cardiovascular health.

The present study was designed to address epigenetic changes in brain as a response to social isolation. The extent of the developmental window for the induction of epigenetic changes is not known, but it appears to extend from the periconceptional period into postnatal life (2). In these time periods, epigenetic modifications directly or indirectly regulate gene expression and are crucial mechanisms on how maternal nutrition during pregnancy, maternal care in the postnatal period and social stress in the early-life affects physical and mental health in later life (6).

Recent studies indicate that the epigenome can also be modified in adult life. Although monozygotic twins are epigenetically indistinguishable during the early years of life, older monozygous twins exhibited remarkable differences in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation, affecting their gene-expression portrait (25). In a recent study, individually housing of 7–9 weeks-old male C57BL/6 mice for 8 weeks led to a change in the relative



CpG (Slc6a4)	1	2	3	4	5	6	7	8	9
Control (% Me)	3.7 ±0.6	1.2 ±0.4	4.3 ±0.8	4.8 ±0.9	2.7 ±1.1	3.0 ±1.1	2.3 ±0.7	1.8 ±0.6	2.5 ±0.8
Isolation (% Me)	6.0 ±1.0	1.2 ±0.9	8.9 ±0.9	3.6 ±1.7	2.7 ±1.1	4.4 ±1.4	3.8 ±1.6	1.9 ±0.8	6.7 ±2.2

Fig. 6. Social isolation leads to down-regulation of Slc6a4 mRNA. Adult (3 months of age) male C57BL/6 mice were either kept in groups (control) or housed individually (isolation) for another 3 months. Slc6a4 mRNA expression was determined in midbrain with quantitative real time RT-PCR (*A*). Partial sequences of the CpG island of Slc6a4 are shown in panel *B*. The methylation status of the individual CpGs studied (indicated with numbers) is shown in the tables (*B*). The values of all individual CpGs in each single sample were summed up to get an average methylation value of that animal (*C*). TSS, transcription start site. Columns (*A*) represent mean ±S.E.M. Each point (*C*) represents one animal. n=8–10.

levels of dimethyl-H3K9/K27 in nucleus accumbens (26). Here we demonstrate that social isolation of adult mice leads to a wide range of epigenetic modifications in the midbrain, including DNA methylation, histone methylation and acetylation.

In mammals, DNA methylation is almost exclusively restricted to cytosine-guanine (CpG) dinucleotides and is catalyzed by DNMT. DNA methylation is an epigenetic modification that is remarkably stable, but not irreversible, although mechanisms responsible for the removal of DNA methylation marks are still poorly understood. We found in the present study that global DNA methylation is enhanced in midbrain of socially isolated adult mice, which is associated with enhanced DNMT activity (*Fig. 1*).

Post-translational modifications of histones are important for the higher order chromatin structure. Di- and trimethylation of H3K4 is associated with transcriptionally active chromatin. In the present study, we show that the di- and trimethylation of H3K4 is increased in midbrain of adult C57BL/6 mice in response to social isolation, which is consistent with the enhanced activity of H3K4 methyltransferases (*Fig. 2*).

Another histone modification associated with transcriptional activation is acetylation of H3K9. Our results show that H3K9 acetylation is enhanced in midbrain of socially isolated mice (*Figs. 3* and *4*). Acetylation of histone residues is controlled by histone HAT and HDAC. In isolated animals, the activity of both HAT and HDAC is increased (*Fig. 3A* and *3B*). These results indicate that social isolation leads to an activation of the histone modification machinery (both HAT and HDAC) and is in agreement with the fact that histone acetylation is a dynamic process (2). Interestingly, the up-regulation of *Hdac1* and *Hdac3* in midbrain of socially isolated mice was accompanied by a reduction of DNA methylation in the promoter region of these genes (*Fig. 5*), indicating that epigenetic-modifier enzymes (*e.g.* HDACs) are themselves under the control of epigenetic mechanisms (*e.g.* DNA methylation).

The serotonin transporter (5-HTT, SERT, Slc6a4) plays an essential role in serotonin homeostasis (27-29). Basal extracellular fluid serotonin concentrations are markedly increased in the striatum and cortex of Slc6a4$^{+/-}$ and Slc6a4$^{-/-}$ mice because of the reduced reuptake of released serotonin. In contrast to the increases in extracellular fluid levels, serotonin concentrations in brain tissue are decreased by 40–60% in Slc6a4$^{-/-}$ mice, as a consequence of the deficient recycling of serotonin by its transporter (summarized in (30)).

The human *SLC6A4* has multiple functional variants, with the serotonin transporter-linked promoter region (5HTTLPR) variant being the most well-studied one (31). The polymorphism consists of two common alleles in European populations, a short (s) variant with 12 copies of a 22 bp repeat element and a long (l) variant, which has 14 copies of the repeat element. The s allele is associated with decreased mRNA transcription, decreased protein production and increased vulnerability to alcohol dependence and major depression (32).

Recent studies demonstrate that both genetic and epigenetic mechanisms are implicated in the regulation of *SLC6A4* expression. Philibert *et al.* discovered CpG islands in the

SLC6A4 gene. They found that CpG methylation is higher and mRNA production is lower in females as compared to males (32). This is reasonable because DNA methylation is generally associated with gene inactivation. In the present study, we observed a marked reduction in *Slc6a4* gene expression at the mRNA level, which was associated with an increase in CpG methylation (*Fig. 6*). These data are consistent with the results of Philibert *et al*. highlighting the crucial role of DNA methylation as a mechanistic control of *Slc6a4* gene transcription.

Interestingly, the 5HTTLPR polymorphism has been shown to modulate the influence of stressful life events on depression, although no *direct* association between the 5HTTLPR genotype and depression is evident (33). Individuals with one or two copies of the short allele of the 5HTTLPR polymorphism exhibit more depressive symptoms, diagnosable depression, and suicidality in relation to stressful life events than individuals homozygous for the long allele (33). In the present study, we observed a significant reduction of Slc6a4 gene expression without any signs of depression in the socially isolated mice. Similar to the situation in individuals carrying the short 5HTTLPR allele, these mice may be more prone to stress events for developing depression. This hypothesis should be tested in further studies.

In conclusion, our study demonstrates for the first time that social isolation of adult mice leads to epigenetic changes in midbrain, including enhanced DNA methylation, increased histone H3K4 methylation and H3K9 acetylation. The significance of such epigenetic mechanisms is further shown for some specific genes as examples. Social isolation leads to up-regulation of Hdac1/3 and down-regulation of Slc6a4. The changes in mRNA expression are inversely correlated with the status of CpG methylation in the promoter region of these genes. These results indicate that epigenetic mechanisms are involved in the changed gene expression pattern and phenotype in socially isolated individuals.

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