Antidepressant treatment is associated with epigenetic alterations in the promoter of P11 in a genetic model of depression

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Abstract

P11 (S100A10) has been associated with the pathophysiology of depression both in human and rodent models. Different types of antidepressants have been shown to increase P11 levels in distinct brain regions and P11 gene therapy was recently proven effective in reversing depressive-like behaviours in mice. However, the molecular mechanisms that govern P11 gene expression in response to antidepressants still remain elusive. In this study we report decreased levels of P11, associated with higher DNA methylation in the promoter region, in the prefrontal cortex of the Flinders Sensitive Line (FSL) genetic rodent model of depression. This hypermethylated pattern was reversed to normal, as indicated by the control line, after chronic administration of escitalopram (a selective serotonin reuptake inhibitor; SSRI). The escitalopram-induced hypomethylation was associated with both an increase in P11 gene expression and a reduction in mRNA levels of two DNA methyltransferases that have been shown to maintain DNA methylation in adult forebrain neurons (Dnmt1 and Dnmt3a). In conclusion, our data further support a role for P11 in depression-like states and suggest that this gene is controlled by epigenetic mechanisms that can be affected by antidepressant treatment.

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Introduction

P11, also known as S100A10, has an established function in the intracellular trafficking of transmembrane proteins to the cell surface (Rescher & Gerke, 2008). P11 has also been recognized as a key modulator of neuronal function, with a novel role implicated in the pathophysiology of depression (Svenningsson & Greengard, 2007). The latter is corroborated by evidence showing that P11 levels are decreased in post-mortem brain tissues of depressed subjects and that P11 knockout (KO) mice display a depression-like phenotype (Anisman et al. 2008; Svenningsson et al. 2006). Tricyclic antidepressants, monoamine oxidase inhibitors (MAOIs) and electroconvulsive therapy (ECT) are antidepressant therapies that have been shown to increase P11 levels in the frontal cortex of rodents (Svenningsson et al. 2006). More recently, selective serotonin reuptake inhibitors (SSRIs) were also added to the pharmacological interventions that affect this gene (Egeland et al. 2010; Warner-Schmidt et al. 2010). In addition, P11 gene therapy in mice is effective in reversing depressed behaviours (Alexander et al. 2010).

In contrast to the above-mentioned biochemical and pharmacological literature, genetic association studies of P11 with depressive disorders have yielded...
inconclusive results (Tzang et al. 2008; Verma et al. 2007). This is, however, not a surprising outcome given the recent poor replication of genetic loci involved in depression (Bosker et al. 2011). Nonetheless, in the absence of DNA nucleotide substitutions, gene expression aberrations can still be observed and accounted for by epigenetic phenomena. Studies of brain tumour cell lines have demonstrated that DNA methylation — a core epigenetic regulator of gene expression — plays a key role in the transcriptional control of P11 (Dudley et al. 2008; Lindsey et al. 2007).

DNA methylation is a heritable epigenetic mark which in vertebrates primarily occurs at CpG dinucleotides. Two main ways by which DNA methylation can affect gene expression are illustrated by the ‘single’ and the ‘bulk’ CpG methylation models, respectively (Sharma et al. 2010). The first model takes into account distinct DNA elements [usually transcription factor (TF)-binding sites] whose methylation or demethylation usually leads to gene silencing or activation, respectively. The second model collectively investigates numerous CpG sites and regards high mean methylation levels as a determinant of inactive chromatin structure that negatively modifies gene expression (Riggs et al. 1998).

DNA methylation in mammals is catalysed by three main enzymes: DNMT1, DNMT3a and DNMT3b (Bestor et al. 1988; Okano et al. 1999). It was recently shown that DNA methylation in adult forebrain neurons of mice is maintained specifically through the action of DNMT1 and DNMT3a (Feng et al. 2010). Active DNA demethylation, on the contrary, is still a mechanistically controversial topic (Wu & Zhang, 2010). However, different proteins have been implicated in promoting DNA demethylation and GADD45b is currently among the best documented deactivating candidates acting in the adult neuronal system (Ma et al. 2009).

The first aim of this study was to investigate the levels of P11 in the prefrontal cortex (PFC) of a genetic rodent model of depression. As P11 has been shown to specifically interact with serotonin receptors (Svenningsson et al. 2006; Warner-Schmidt et al. 2009), we worked with the Flinders Sensitive Line (FSL) and with its controls (Flinders Resistant Line; FRL) as this model exhibits changes consistent with the serotonergic hypothesis of depression (Overstreet et al. 2005). Second, after observing decreased P11 levels in FSL, we hypothesized that P11 regulation is influenced by epigenetic modifications. In agreement with this hypothesis, DNA methylation analyses revealed a hypermethylated pattern in the promoter region of FSL. Third, we looked for P11 expression and DNA methylation changes in response to chronic administration of escitalopram. Escitalopram is an antidepressant acting as a SSRI that has already been shown to have antidepressant effects in the FSL model of depression (El Khoury et al. 2006). As predicted, the antidepressant treatment led to an increase in P11 mRNA levels in FSL that was associated with a reduction in promoter DNA methylation. Interestingly, this hypomethylation occurred in combination with a decrease in the expression of two genes encoding enzymes known to maintain DNA methylation in adult forebrain neurons (Dnmt1 and Dnmt3a).

Materials and methods

Tissue samples

PFC regions from female FSL and FRL rats (age range 8–15 months) were dissected and immediately stored at −70°C until subsequent experimental analyses. Samples were obtained from naive rats (FSL and FRL) and escitalopram-treated rats (FSL-Esc and FRL-Esc). Escitalopram was administered in food pellets as described previously (El Khoury et al. 2006) for a period of 3 wk prior to euthanasia. All experiments were approved by the Ethical Committee for Protection of Animals at the Karolinska Institute.

Gene expression by in-situ hybridization

To measure P11 mRNA levels in the PFC of untreated animals, radiolabelled riboprobe in-situ hybridization experiments were performed as described previously (Egeland et al. 2010; Svenningsson et al. 1997, 2006). Briefly, 12-μm-thick sections were incubated with a [α-35S]UTP-labelled riboprobe that was made by in-vitro transcription from DNA corresponding to nucleotides 1–287 of the coding sequence of the rat P11 gene. After hybridization and washing, autoradiography was performed using Kodak Biomax MR film (Rochester, USA) and optical density was measured with the NIH ImageJ software.

Protein expression by Western blotting

To detect P11 protein levels in the PFC of naive animals, Western blotting experiments were performed as described previously (Svenningsson et al. 2006; Warner-Schmidt et al. 2009). In brief, brain samples were homogenized on ice and sonicated in 1% SDS. Protein concentrations were measured and, after separation, samples were transferred to PVDF membrane and blocked. Immunoblotting was then performed with primary polyclonal antibodies against P11 (anti-S100A10, 1:200, R&D Systems, USA) and
polyclonal antibodies against actin (anti-β-actin, 1:2000) overnight at 4 °C followed by incubation in 
HRP-conjugated secondary antibodies (1:5000) for 1 h at 4 °C. Immunoreactive bands were visualized using 
ECL Western blotting reagent (PerkinElmer, USA), exposed to Kodak Biomax XAR film (Rochester, USA) and 
optical densities were quantified using NIH ImageJ software.

DNA sequencing

The rat P11 gene (NCBI Gene ID no. 81778) is 8.65 kb long and consists of three exons. The proximal (~1 kb) 
promoter of the gene contains an unsequenced ‘gap’ region (Fig. 2a). To investigate whether basal P11 differences 
between rat strains were caused by nucleotide sequence differences, PCR products of all exons and 
the known proximal region were sequenced bi-directionally on an ABI 3730 DNA Analyzer (Applied 
Biosystems, USA) using the BigDye Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems). The 
following primers were used for sequencing. Promoter forward: 5'-GAAAGCCAGTGATGGGAT-3'; 
promoter reverse: 5'-CTTGGATGACGAAAGGAAC-3'; exon 1 forward: 5'-GCCAGAAGGGATGGTCGG-3'; 
exon 1 reverse: 5'-GGGCGAGGCACTAGCGAA-3'; exon 2 forward: 5'-GGTTAACCCCACATTCGTGAG-3'; 
exon 2 reverse: 5'-GAGGAACATGCTTAC-TGACC-3'; exon 3 forward: 5'-CTAAGACTGGAGCGCAAAAGGA-3'; 
exon 3 reverse: 5'-GGAGATTGCCCTTGTTGTATGC-3'. The gap region was sequenced twice unidirectionally with two separate reverse 
primers – gap forward 1: 5'-TGTTCCCTGGAGACCGGGCAAAGTG-3'; gap 
reverse 1: 5'-TGGGCGGTGCCATACCGGAAAC-3'; exon 2 forward: 5'-AGGAAGGGGAGGCCGCTGTA-3'; 
exon 2 reverse: 5'-CGAGGCGCTGTA-3; exon 3 forward: 5'-GAGGAACATGCTTAC-TGACC-3'; exon 3 reverse: 5'-GGAGATTGCCCTTGTTGTATGC-3'. The gap region was sequenced twice unidirectionally with two separate reverse 
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reverse 1: 5'-TGGGCGGTGCCATACCGGAAAC-3'; exon 2 forward: 5'-AGGAAGGGGAGGCCGCTGTA-3'; 
exon 2 reverse: 5'-CGAGGCGCTGTA-3; exon 3 forward: 5'-GAGGAACATGCTTAC-TGACC-3'; exon 3 reverse: 5'-GGAGATTGCCCTTGTTGTATGC-3'. The gap region was sequenced twice unidirectionally with two separate reverse 
primers – gap forward 1: 5'-TGTTCCCTGGAGACCGGGCAAAGTG-3'; gap 
reverse 1: 5'-TGGGCGGTGCCATACCGGAAAC-3'; exon 2 forward: 5'-AGGAAGGGGAGGCCGCTGTA-3'; 
exon 2 reverse: 5'-CGAGGCGCTGTA-3; exon 3 forward: 5'-GAGGAACATGCTTAC-TGACC-3'; exon 3 reverse: 5'-GGAGATTGCCCTTGTTGTATGC-3'.

DNA/RNA extraction and reverse transcription

DNA was isolated from ~30 mg frozen tissue using the QIAamp DNA Mini kit or the AllPrep DNA/RNA 
Mini kit (Qiagen GmbH, Germany). Total RNA was isolated from ~30 mg frozen tissue using the RNeasy 
Lipid Tissue Mini kit or the AllPrep DNA/RNA Mini kit (Qiagen) and was treated with DNase I (Qiagen) or 
TURBO DNA-free (Ambion, USA) to eliminate contaminating DNA. Equal numbers of FRL and FSL 
animals were processed with each method to avoid any potential systematic bias due to the usage of dif 
ferent extraction kits. The quality of the extracted RNA was examined using agarose gel electrophoresis 
and total DNA/RNA concentrations were determined spectrophotometrically with the NanoDrop ND-1000 
Spectrophotometer (NanoDrop Technologies Inc., USA). cDNA was synthesized by reverse transcription of 
total RNA with random hexamers, using SuperScript III First-Strand Synthesis System for 
RT–PCR (Invitrogen, USA). DNA/cDNA was stored at −20 °C and RNA at −70 °C, until further usage.

Gene expression by quantitative real-time 
PCR (qRT-PCR)

TaqMan Gene Expression Assays (Applied Bio 
systems) were used for real-time quantification of 
cDNA corresponding to mRNA of target genes and 
endogenous control genes. The assays used were for 
P11 (Applied Biosystems Assay ID: Rn01409218_m1), Ar 
(Rn00560747_m1), Dnmt1 (Rn00709664_m1), 
Dnmt3a (Rn01469994_g1), Dnmt3b (Rn01536414_g1), 
Gadd45b (Rn01452530), Mbd2 (Rn01491487_m1), Mbd4 
(Rn01459459_m1), Aid (Rn01492306_m1) and Gapdh 
(Rn99999916_s1). Gene amplifications were performed in 
triplicate on an ABI PRISM 7900 HT Sequence 
Detection System (Applied Biosystems). Data were 
obtained as threshold cycle (Ct) values. Sample to 
sample variation in RNA quality and RT efficiency 
was corrected by normalizing target gene Ct values 
against the endogenous control (Gapdh) Ct values. 
The relative gene expression, presented as arbitrary 
units, was calculated according to the comparative 
Ct method using the formula: 2^-ΔCt, where ΔCt = Ct 
target – Ct endogenous control, and ΔCt = ΔCt target – ΔCt 
calibrator.

In-silico screen for TF-binding sites

As the rat promoter of P11 is not well-characterized, a 
region of interest was specified based on putative 
transcription factor-binding sites (TFBS) generated by 
ConSite; a freely available web-based tool for pre 
dicting cis-regulatory elements (Sandelin et al. 2004). 
The search was restricted according to the following 
criteria: (a) data entry should be the putative proximal 
promoter of the P11 gene (~1 kb upstream region), 
(b) TFBS should contain at least one CpG dinucleotide, 
thus rendering the site susceptible to DNA methyl 
ation that can affect TF binding affinity, and (c) priority 
should be given to generated TFs with an hypothesized 
role in the pathophysiology of depression.

Site-specific DNA methylation quantification

DNA methylation levels were quantified using pyro 
sequencing technology on a PyroMark Q24 (Qiagen)
following the manufacturer’s protocol. Bisulfite treatment of the DNA was performed using the EZ DNA Methylation-Gold kit (Zymo Research Corporation, USA). The following primers were used – forward primer: 5′-AGTTGGTAAAGTGAATTGTTT-3′; reverse biotinylated primer: 5′-CCTTCCCCACTCACTCTATAAATCAT-3′; and sequencing primer: 5′-TTAAGTTGAGATTGTTTAG-3′. Successful amplification of desired PCR products was checked on a 1% agarose gel. The assay was designed using the PSQ Assay Design software (Qiagen) and was evaluated by testing with commercially available fully methylated and unmethylated genomic rat DNA (EpigenDx Inc., USA). For confirmation reasons, DNA methylation analyses were performed in duplicate in 81% of the samples.

Whole-genome methylation analysis
Global DNA methylation was assessed using LUMinometric Methylation Assay (LUMA) as described in a detailed protocol by Karimi et al. (2006). In brief, reactions were performed in duplicate and DNA samples (~2 μg) were cleaved using methylation-sensitive or methylation-insensitive restriction enzymes. After 4 h of incubation at 37°C, annealing buffer was added and a Pyrosequencing PSQ 96MA (Qiagen) instrument was programmed to add dNTPs, substrate, enzyme and water. Peak heights were calculated using PSQ 96MA software (Qiagen) and ratios, used for semi-quantitative analysis of whole-genome DNA methylation, were calculated as described in the protocol of Karimi et al. (2006).

Statistical analyses
For most analyses, data are presented as mean values and vertical error bars represent ±1 standard error of the mean (S.E.M.). In the case of Western blotting, data are shown as a percentage of that of the control (FRL) group. The Shapiro–Wilks and Levene’s test were used for testing the normality of the data and the homogeneity of variance, respectively. Depending on the normality of the data, either the parametric Student’s t test or the non-parametric Mann–Whitney U test was used for group comparisons. When only one comparison was applicable (i.e. FSL vs. FRL), statistical significance was set at p < 0.05. When four comparisons were of interest (i.e. FSL vs. FRL, FSL vs. FSL-Esc, FRL vs. FRL-Esc, and FRL vs. FSL-Esc), the p value was Bonferroni-adjusted and set at p < 0.013. All analyses were performed using SPSS software version 17.0 (SPSS Inc., USA). Outliers belonging to the inter-quartile range-based flagged observations generated by SPSS were excluded from the analyses.

Results

P11 mRNA and protein levels in FSL brain tissue
We first examined P11 mRNA expression in the PFC of untreated FSL and FRL rats in order to assess whether there was a basal difference between the two strains. In-situ hybridization revealed significantly reduced P11 mRNA levels in the FSL rodent model of depression (Fig. 1a, p = 0.042). This finding was also confirmed at the protein level, as Western blotting showed a P11 deficit present in FSL animals (Fig. 1b, p = 0.021).

P11 DNA sequencing and putative TFs
DNA sequencing of FSL and FRL did not reveal any nucleotide substitutions that could account for the basal differences in P11 levels. The P11 promoter region is shown in Fig. 2a and the gap-region sequencing results are shown in Fig. 2b. The in-silico screen for putative TFs based on the criteria set out in the Methods section resulted in the identification of an androgen receptor (AR) consensus sequence that includes two CpG sites (Fig. 2a). According to the species link at the ConSite database, the sequences used for the matrix model were from the genus Rattus. As reduced Ar mRNA levels have previously been reported in post-mortem brains of depressed individuals (Wang et al. 2008), we performed qRT-PCR to compare endogenous Ar mRNA levels between FSL and FRL. However, no significant difference was found (Fig. 2c, p = 0.164) suggesting that aberrant Ar mRNA levels do not underlie the depressed phenotype.

Chronic escitalopram administration and P11 mRNA expression
Using qRT-PCR, we studied the effect of chronic escitalopram administration on P11 mRNA expression in the PFC of the FSL/FRL model. Bonferroni-adjusted comparisons revealed that P11 mRNA levels were significantly increased in the escitalopram-treated groups relative to the untreated ones (Fig. 3a: FSL vs. FSL-Esc, p = 0.004; and FRL vs. FRL-Esc, p < 0.001). Following escitalopram administration, the mRNA level of the FSL-Esc group was not statistically different from that of the control (FRL) group (Fig. 3a).

DNA methylation in the P11 promoter
DNA methylation was first assessed at the region corresponding to the putative AR binding site. Bonferroni-adjusted comparisons showed that there
was a basal hypermethylated pattern in the naive FSL animals relative to the controls (Fig. 3b: FSL vs. FRL, p = 0.006). In addition, escitalopram administration was associated with a decrease in DNA methylation in the FSL-Esc group compared to the corresponding untreated (FSL) group (Fig. 3b: FSL vs. FSL-Esc, p = 0.002). The methylated pattern in the FSL-Esc group was now statistically similar to that of the controls (FRL) (Fig. 3b).

Subsequently, to address the bulk methylation model, we added the DNA methylation information deriving from three downstream CpG sites. Thus five consecutive CpGs (shown in Fig. 2a), spanning a 90-bp region, were studied. Interestingly, the group differences found were the same as for the putative AR binding site (Fig. 3c: FSL vs. FRL, p = 0.004; FSL vs. FSL-Esc, p = 0.003). It is also noteworthy that the absolute methylation difference between FSL and controls was higher in the ‘bulk’ analysis (FSL 21% vs. FRL 16.7%, Fig. 3c) compared to the ‘critical-site’ approach (FSL 23.4% vs. FRL 20.9%, Fig. 3b). Whole-genome methylation analyses were performed to investigate whether global methylation level was associated with escitalopram treatment. No effect on whole-genome methylation was found (Fig. 3d), supporting gene specificity of the P11 methylation results.

**Gene expression of DNA methylation-associated enzymes**

As an exploratory analysis of candidate enzymes that could be associated with the observed DNA methylation changes, we examined the mRNA levels of three DNA methyltransferases (Dnmt1, Dnmt3a, Dnmt3b) and four genes encoding proteins that are suggested to be involved in DNA demethylation (Mbd2, Mbd4, Gadd45b, Aid) using qRT-PCR. Bonferroni-adjusted comparisons showed that Dnmt1 and Dnmt3a levels were significantly decreased following treatment with escitalopram in the FSL group (Dnmt1; Fig. 4a: FSL vs. FSL-Esc, p < 0.001, and Dnmt3a; Fig. 4b: FSL vs. FSL-Esc, p = 0.003). This was in accord with the FSL-Esc group’s reduction in DNA methylation levels (Fig. 3b, c). No statistically significant group difference was present for Mbd2 (Fig. 4c), Mbd4 (Fig. 4d) or Gadd45b (Fig. 4e) mRNA levels. However, an observed trend for reduced Gadd45b mRNA levels in the hypermethylated untreated depressed animals (Fig. 4e: FSL vs. FRL, p = 0.06) is in line with GADD45b’s demethylating role in the adult brain (Ma et al. 2009). No statistical analysis was performed on Dnmt3b and Aid mRNA data as C_t values were >35 or undetectable, respectively.
Discussion

Decreased P11 in the PFC of a genetic model of depression

Our data (Fig. 1) support a down-regulation of both mRNA and protein levels of P11 in the PFC of the FSL genetic rodent model of depression compared to its control line (FRL). This finding is consistent with previous publications showing a P11 decrease in the PFC of both depressed patients and a mouse model of depression (Anisman et al. 2008; Svenningsson et al. 2006). It should be noted that the P11 mRNA difference between naive FSL and FRL was not statistically significant when analysed with qRT-PCR (Fig. 3a). However the trend between these two groups in the qRT-PCR analysis (FRL mean = 1.14, FSL mean = 1.06) is in line with the in-situ hybridization mRNA results (Fig. 1a) and the magnitude of P11 mRNA deficit was similar in both types of experiments (1.2-fold lower P11 mRNA in FSL measured with in-situ hybridization vs. 1.1-fold lower measured with qRT-PCR). This statistical discrepancy between the two methods could be due to better cellular resolution with in-situ hybridization, as the samples used to perform qRT-PCR contain heterogeneous cell populations. Technical parameters, like the choice of endogenous control genes in qRT-PCR, could also influence the experimental results; especially when the basal difference in magnitude is known to be small, as in this study (1.1- to 1.2-fold).

Escitalopram administration is associated with increased P11 mRNA levels

We and others have already shown that SSRIs, like escitalopram, have antidepressant/anxiolytic effects in the FSL model of depression (El Khoury et al. 2006; Kanemaru et al. 2009; Overstreet et al. 2004), rendering further behavioural studies not pertinent to the present report. Based on data supporting a better clinical effectiveness of escitalopram compared to other SSRIs (e.g. citalopram or fluoxetine; Cipriani et al. 2009) we considered the study of this drug highly relevant from a psychopharmacological perspective. In our study, 3-wk treatment with this drug was associated with an increase in P11 mRNA expression in the PFC of both FSL and FRL as indicated by qRT-PCR measurements (Fig. 3a). These data are in line with previous findings showing a P11 mRNA increase in rodent brains following a variety of antidepressant treatments (tricyclics, MAOIs, ECT, SSRIs; Svenningsson et al. 2006; Warner-Schmidt et al. 2010). Given the proven interaction specificity of P11 to serotonin receptors (Svenningsson et al. 2006; Warner-Schmidt et al. 2009), our findings suggest a common link between the therapeutic action of SSRIs and the availability of...
certain serotonin receptors on the cell surface (Svenningsson & Greengard, 2007).

FSL are hypermethylated in the P11 promoter region

DNA sequencing indicated that the basal P11 expression difference between naive FSL and control animals was not due to nucleotide substitutions in the analysed, exon and promoter, regions. Based on recent literature describing a close link between DNA methylation and P11 transcriptional activity (Dudley et al. 2008; Lindsey et al. 2007) we hypothesized that the same epigenetic mechanism could account for the P11 deficit observed in FSL.

DNA methylation in the promoter of a gene can affect mRNA expression by altering the binding affinity of TFs. To address this ‘critical site’ hypothesis we performed an in-silico search that resulted in a number of TFs, among which the AR was selected as the most relevant candidate. Based on depression’s pronounced sexual dimorphism (~32% affected males and ~68% affected females; Kaminsky et al. 2006) it has been speculated that sex hormones are involved in the development of the disease. The AR is an androgen-responsive TF which inhibits corticotropin-releasing hormone (CRH) and leads to a dampening of hypothalamic-pituitary-adrenal (HPA) axis activity (Bao et al. 2006). Even if this nuclear receptor has been shown to be transcriptionally down-regulated in post-mortem brains of depressed individuals (Wang et al. 2008), no Ar mRNA difference between FSL and FRL was found in this study (Fig. 2c). Post-translational modifications can, of course, not be ruled out.

Fig. 3. Gene expression following escitalopram treatment and methylation analyses of P11 in the prefrontal cortex of FSL and control (FRL) rats. (a) P11 mRNA levels were measured by quantitative real-time PCR and were shown to be increased after antidepressant treatment in both strains (n = 13 FRL, n = 13 FSL, n = 6 FRL-Esc, n = 9 FSL-Esc; n = 1 FSL outlier excluded). (b) Increased DNA methylation levels were present at the putative androgen receptor site in the naive FSL animals. This hypermethylated pattern was reversed to control levels after escitalopram administration (n = 11 FRL, n = 10 FSL, n = 6 FRL-Esc, n = 9 FSL-Esc). (c) Mean DNA methylation levels were also examined as a total of five consecutive CpGs (see Fig. 2a) to address the ‘bulk’ DNA methylation model (n = 6 FRL, n = 10 FSL, n = 6 FRL-Esc, n = 9 FSL-Esc). The differences were the same as in the ‘single CpG/critical site’ approach shown in panel (b). Interestingly, the absolute methylation difference between FSL and FRL was higher in the bulk approach (bulk 4.3% vs. critical site 2.5%). (d) Global DNA methylation was not altered by the antidepressant treatment suggesting a specificity of the P11 methylation results (n = 5 FRL, n = 4 FSL, n = 6 FRL-Esc, n = 9 FSL-Esc). * p < 0.013, ** p < 0.003. RQ, Relative quantification.
Interestingly, we detected elevated methylation levels at the putative binding site of AR in the FSL strain (Fig. 3b). However as the binding of AR is not confirmed and there is usually a high noise level in the *in-silico* predictions, we also addressed the ‘bulk’ model of DNA methylation that convincingly led to the same difference (Fig. 3c). It can be argued that the magnitude of the methylation difference observed in this study appears rather low (critical site model: 2.5% difference between FSL and FRL; bulk model: 4.3% difference). Recently, however, a comprehensive epigenomic profiling in cortical brain tissue of psychotic patients showed a similar degree of methylation differences between cases and controls (4–8% in the genes under investigation; Mill *et al.* 2008). An acknowledged possible confound in DNA methylation analyses is the usage of whole-tissue homogenates, as distinct cell types within the brain probably possess their own methylation signature (Connor & Akbarian, 2008). As depression-like phenotypes have been suggested to result from a P11 loss specifically within neurons (Alexander *et al.* 2010), studying a mixture of cells might mask methylation differences with higher effect sizes. In addition, it should be pointed out that this study focused on a short region of the P11 promoter and even if AR-binding in this region is proven in the future, the gene’s activity is most probably regulated by a number of other TFs and co-activators whose binding positions may be influenced by DNA methylation. Thus, an in-depth characterization of the P11 promoter and functional experiments are warranted to support these correlative data.

**Escitalopram administration is associated with a DNA methylation decrease**

After chronic treatment with escitalopram, the hypermethylated profile observed in FSL was reversed to a pattern similar to FRL controls (Fig. 3b, c). As indicated...
by our global DNA methylation analysis (Fig. 3d), this SSRI-associated decrease in P11 DNA methylation was not accompanied by a genome-wide hypomethylation. This is in line with previous data showing that ECT leads to site-specific – and not global – DNA demethylation in the brain (Ma et al. 2009). Of course, it should be noted that the utilized method (LUMA) detects methylation at CpG sites which represent a minor fraction (~7–8%) of all CpG sites, as estimated from the mouse genome (Fazzari & Greally, 2004).

**Hypomethylation is associated with decreased Dnmt1 and Dnmt3a levels**

DNMT1 is a DNA methyltransferase that exhibits a maintenance methylation activity by associating with replication foci during the S-phase of the cell cycle (Leonhardt et al. 1992). Here we report a down-regulation of Dnmt1 mRNA that is only present in FSL rats that were chronically treated with escitalopram and showed a significant reduction in P11 methylation (Fig. 4a). The treated control (FRL) rats did not exhibit any change in Dnmt1 levels compared to untreated FRL rats; consistent with their unchanged methylation status. In line with our results, Dnmt1 knockdown and treatment with a DNA methyltransferase inhibitor (5-aza-2’-deoxycytidine) have been associated with an up-regulation of P11 mRNA levels in brain tumour cells (Dudley et al. 2008; Lindsey et al. 2007).

However, DNMT1 alone is not sufficient for maintaining DNA methylation but requires two known members from the DNMT3 family (Chen et al. 2003). DNMT3a and DNMT3b represent these two DNA methyltransferases that possess a de-novo DNA methylation activity (Okano et al. 1999). In contrast to Dnmt3b which is highly expressed only during embryonic development, Dnmt3a is expressed in both the developing and the mature central nervous system (Feng et al. 2005). In this study, besides the reduction in Dnmt1 mRNA, we also found a down-regulation of Dnmt3a levels in the escitalopram treated FSL-Esc strain (Fig. 4b). This is in accord with recent data showing that DNA methylation in adult post-mitotic neurons is maintained by both DNMT1 and DNMT3a (Feng et al. 2010). Our Dnmt3b mRNA data revealed low mRNA levels (Ct values >35), which is in line with the enzyme’s role in the early stages of neurogenesis (Okano et al. 1999).

**GADD45b is a suitable demethylase candidate**

Even if the mechanism leading to loss of DNA methylation is still disputed (Wu & Zhang, 2010) a number of proteins have been proposed to be involved for DNA demethylation to occur. Among these proteins are MBD2 (Bhattacharya et al. 1999), MBD4 (Rai et al. 2008), AID (Popp et al. 2010) and GADD45b (Ma et al. 2009). We studied the mRNA levels of the aforementioned genes to test the possibility that their aberrant gene expression could account for the differences in P11 DNA methylation in the FSL/FRL model. Although no statistically significant difference was found (Fig. 4c–e), there was a trend for decreased Gadd45b levels in FSL animals (Fig. 4e: FSL vs. FRL, p = 0.06). The same trend was observed between the two treated groups (Fig. 4e: FSL-Esc vs. FRL-Esc, p = 0.025). Gadd45b is known to be induced by ECT and to be necessary for promoter DNA demethylation in the adult brain (Ma et al. 2009). A decrease in GADD45b levels in the FSL strain could be the answer for the currently unexplained basal hypermethylated pattern observed between untreated groups (FSL vs. FRL; see Fig. 3b, c). It should also be mentioned that mRNA levels of Ail1 were undetectable in all cases, which is in accord with the gene’s function in primordial germ cells (Popp et al. 2010).

**Unexplained increase of P11 mRNA levels in the control line**

In the present study we were unable to explain the up-regulation of P11 gene expression in the escitalopram-treated controls (FRL-Esc, see Fig. 3a) based on the DNA methylation levels of the region under investigation. However, as mentioned before, P11 is most probably regulated by a number of other TFs and co-activators that bind to sites that were not studied in the present report. For example, glucocorticoid response elements have been reported in the rat P11 promoter region (Zhang et al. 2008) and, thus, both the DNA methylation status at other positions, and/or the protein levels of the TFs binding there, could affect P11 transcription in FRL following escitalopram administration.

**Conclusions**

This study, using the FSL genetic model of depression, adds to the existing literature supporting a role for P11 (S100A10) in the pathophysiology of depression. It also confirms earlier tumour data suggesting an epigenetic control of the P11 gene. In particular, the decreased P11 levels in FSL rats were associated with higher DNA methylation levels in the promoter region. Interestingly, after escitalopram treatment, FSL rats not only exhibited higher P11 expression levels but also decreased DNA methylation accompanied with a transcriptional reduction of two genes (Dnmt1 and
Dnmt3a) known to sustain DNA methylation in adult forebrain neurons. However, the present epigenetic data need to be confirmed both in other settings and, most importantly, in humans before any firm conclusions can be drawn regarding SSRI actions.

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Statement of Interest

None.

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de novo methylation and mammalian development.


