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.

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

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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 activity – 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 demethylating 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-^{35}S]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 ($\sim$1 kb) promoter of the gene contains an unsequenced 'gap' region (Fig. 2*a*). 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 bidirectionally 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'-GAAAGCCAGTGAGTAGGGAT-3; promoter reverse: 5'-CTTGGATGAGCAGAAGGAAC-3'; exon 1 forward: 5'-GGCAGGAAGGGATGGGTCGG-3'; exon 1 reverse: 5'-GGGGCGAGGCACTAGCGAA-AC-3'; exon 2 forward: 5'-GGTAAACCCCACATTC-TGAG-3'; exon 2 reverse: 5'-GAGGAACTAGCTTAC-TGAGC-3'; exon 3 forward: 5'-CTAAGACTGGAGC-CGAAGGA-3'; exon 3 reverse: 5'-GGAGATTGCCC-TGTGGTATGC-3'. The gap region was sequenced twice unidirectionally with two separate reverse primers – gap forward 1: 5'-TGTCCCTGGAGACCG-GCAAAGT-3'; gap reverse 1: 5'-TCCGGAGTGGGA-GGAGCCTGGA-3'; gap forward 2: 5'-AGGAAGGG-CGAGGCGCTGTA-3'; gap reverse 2: 5'-TCGCGCGT-CTTTGTACGCGG-3'. Genetic variation was assessed using freely available bioinformatics software for sequence alignment (Geneious, Biomatters Ltd; Drummond *et al.* 2010).

DNA/RNA extraction and reverse transcription

DNA was isolated from $\sim$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 $\sim$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 different 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 Biosystems) 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 (C_t) values. Sample to sample variation in RNA quality and RT efficiency was corrected by normalizing target gene C_t values against the endogenous control (*Gapdh*) C_t values. The relative gene expression, presented as arbitrary units, was calculated according to the comparative C_t method using the formula: $2^{-\Delta\Delta C_t}$, where $\Delta C_t = C_t$ target $- C_t$ endogenous control, and $\Delta\Delta C_t = \Delta C_t$ target $- \Delta C_t$ 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 predicting *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 ($\sim$1 kb upstream region), (*b*) TFBS should contain at least one CpG dinucleotide, thus rendering the site susceptible to DNA methylation 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 pyrosequencing 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′-AGGTGGTTAAGTTGAGATTTGTT-3′; reverse biotinylated primer: 5′-CCTTCCCACTCACTC-TACTAACAT-3′; and sequencing primer: 5′-TTAAG-TTGAGATTTGTTTAG-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 ($\sim 2 \mu$g) 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–Wilk 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. 1*a*, $p = 0.042$). This finding was also confirmed at the protein level, as Western blotting showed a P11 deficit present in FSL animals (Fig. 1*b*, $p = 0.021$).

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. 2*a* and the gap-region sequencing results are shown in Fig. 2*b*. 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. 2*a*). 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. 2*c*, $p = 0.164$) 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. 3*a*: 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. 3*a*).

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
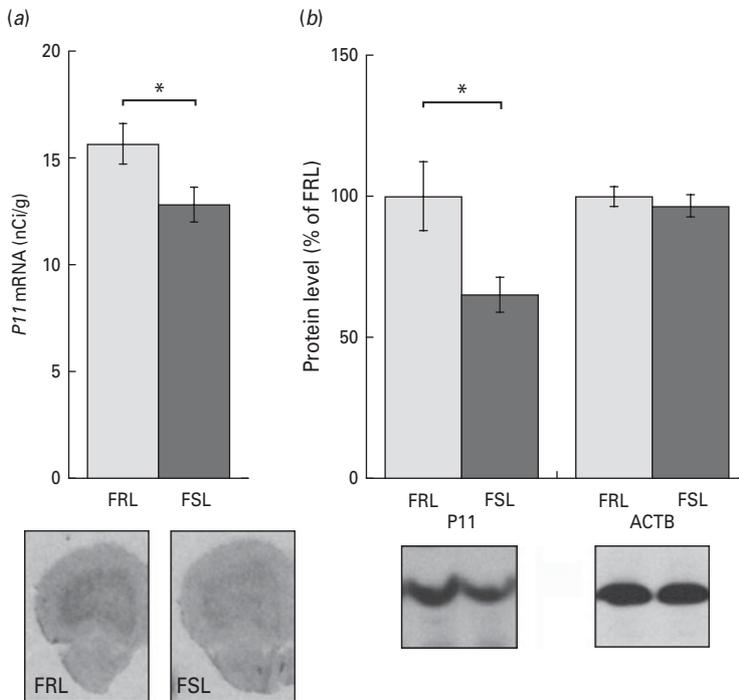
Fig. 1. P11 levels in the prefrontal cortex of untreated FSL and control (FRL) animals. (*a*) *P11* mRNA levels measured by *in-situ* hybridization were reduced in the FSL model ($n = 8$ FRL, $n = 9$ FSL; $n = 1$ FSL outlier excluded). Representative *in-situ* images of medial frontal cortex of FRL and FSL are shown. (*b*) P11 protein levels measured by Western blotting were reduced in the FSL model ($n = 9$ FRL, $n = 9$ FSL; ACTB as control). Representative Western blot images of P11 and ACTB are shown. * $p < 0.05$.

was a basal hypermethylated pattern in the naive FSL animals relative to the controls (Fig. 3*b*: 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. 3*b*: 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. 3*b*). 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. 2*a*), spanning a 90-bp region, were studied. Interestingly, the group differences found were the same as for the putative AR binding site (Fig. 3*c*: 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. 3*c*) compared to the 'critical-site' approach (FSL 23.4% *vs.* FRL 20.9%, Fig. 3*b*). 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. 3*d*), 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. 4*a*: FSL *vs.* FSL-Esc, $p < 0.001$, and *Dnmt3a*; Fig. 4*b*: FSL *vs.* FSL-Esc, $p = 0.003$). This was in accord with the FSL-Esc group's reduction in DNA methylation levels (Fig. 3*b*, *c*). No statistically significant group difference was present for *Mbd2* (Fig. 4*c*), *Mbd4* (Fig. 4*d*) or *Gadd45b* (Fig. 4*e*) mRNA levels. However, an observed trend for reduced *Gadd45b* mRNA levels in the hypermethylated untreated depressed animals (Fig. 4*e*: 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.
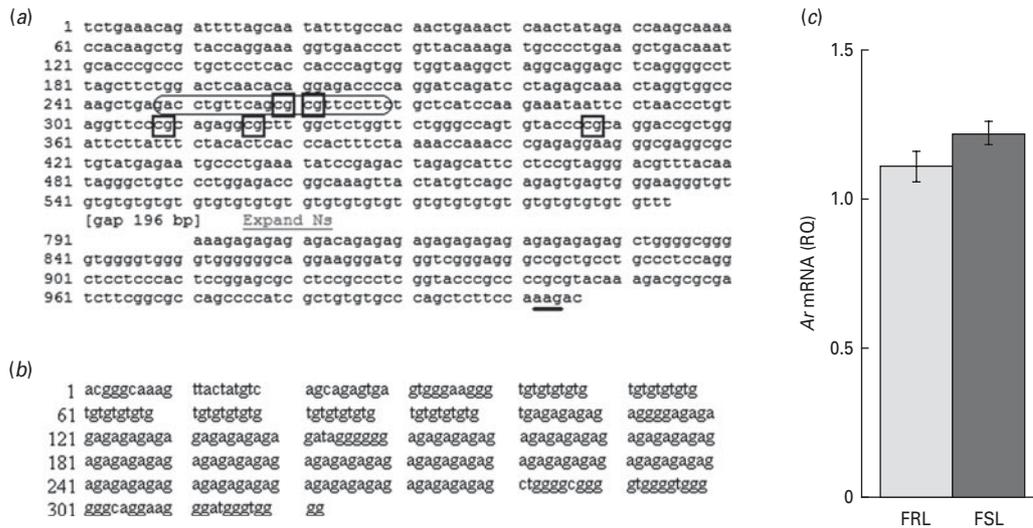
(a)

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121 gcaccogocc tgctoctcac cacccagtgg tggtaaggct aggcaggagc tcaggggcct
181 tagcttctgg actcaacaca ggagaccca ggatcagatc ctagagcaaa ctaggtggcc
241 aagctgagac ctgttcagcg cggttccttgt gctcatccaa gaaataattc ctaaccctgt
301 aggttccgcg agaggcggtt ggctctggtt ctgggccagt gtaccccgca ggaccgctgg
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481 tagggctgtc cctggagacc ggcaaagtta ctatgtcagc agagtgagtg ggaagggtgt
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961 tcttcggcgc cagcccatc gctgtgtgcc cagctcttcc aaagac
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(b)

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  1 acgggcaaag ttactatgtc agcagagtga gtgggaaggg tgtgtgtgtg tgtgtgtgtg
 61 tgtgtgtgtg tgtgtgtgtg tgtgtgtgtg tgtgtgtgtg tgagagagag aggggagagag
121 gagagagaga gagagagaga gatagggggg agagagagag agagagagag agagagagag
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241 agagagagag agagagagag agagagagag agagagagag ctggggcggg gtggggtggg
301 gggcaggaag ggatgggtgg gg
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(c)

Fig. 2. (*a*) *P11*'s proximal (∼1 kb) DNA sequence. The region corresponds to bases 186644673–186645678 and is a screenshot of NCBI's reference sequence NC_005101.2 at the time of submission. The putative binding site of the androgen receptor (AR) is encircled (consensus sequence: gacctgttcag**CGCG**ttccttc) and includes two CpG sites shown in boxes. The three additional CpG sites that were studied downstream of the AR putative site, are also shown in boxes. *P11*'s transcription start site is underlined. (*b*) The sequencing results of the gap region. This sequence corresponds to bases 498–877 of the sequence shown in panel (*a*). The sequencing reactions were performed using two separate reverse primers in FRL ($n=8$) and FSL ($n=8$). (*c*) Gene expression of the androgen receptor (*Ar*) in the prefrontal cortex of FSL/FRL as measured by quantitative real-time PCR. No difference between strains was found ($n=7$ FRL, $n=10$ FSL). $p>0.05$. RQ, Relative quantification.

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. 3*a*). 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. 1*a*) 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. 3*a*). 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
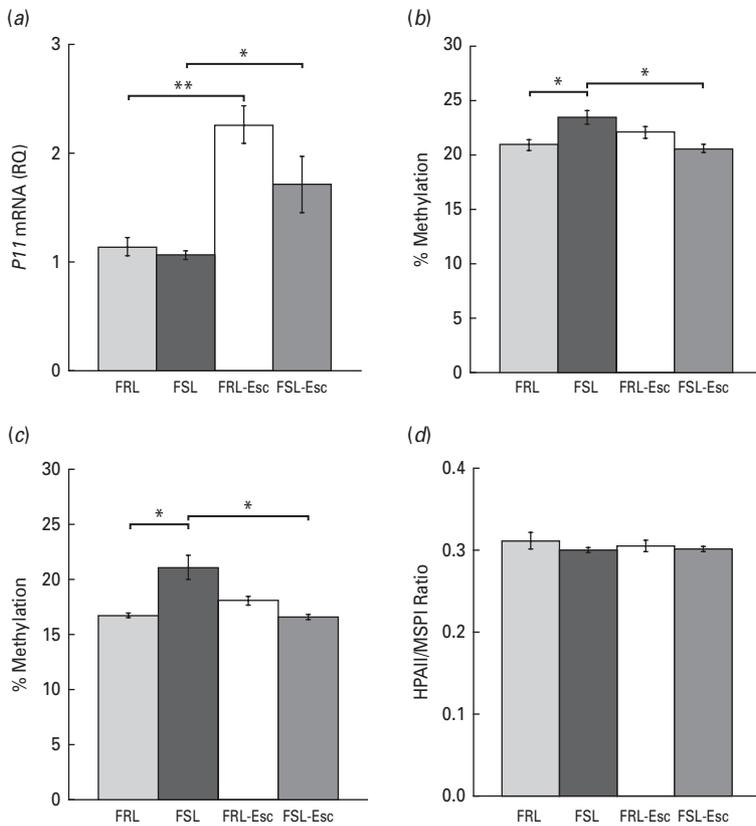
Fig. 3. Gene expression following escitalopram treatment and methylation analyses of *P11* in the prefrontal cortex of FSL and control (FRL) rats. (*a*) *P11* mRNA levels were measured by quantitative real-time PCR and were shown to be increased after antidepressant treatment in both strains ($n=13$ 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. 2*a*) 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.

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 ($\sim 32\%$ affected males and $\sim 68\%$ 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. 2*c*). Post-translational modifications can, of course, not be ruled out.
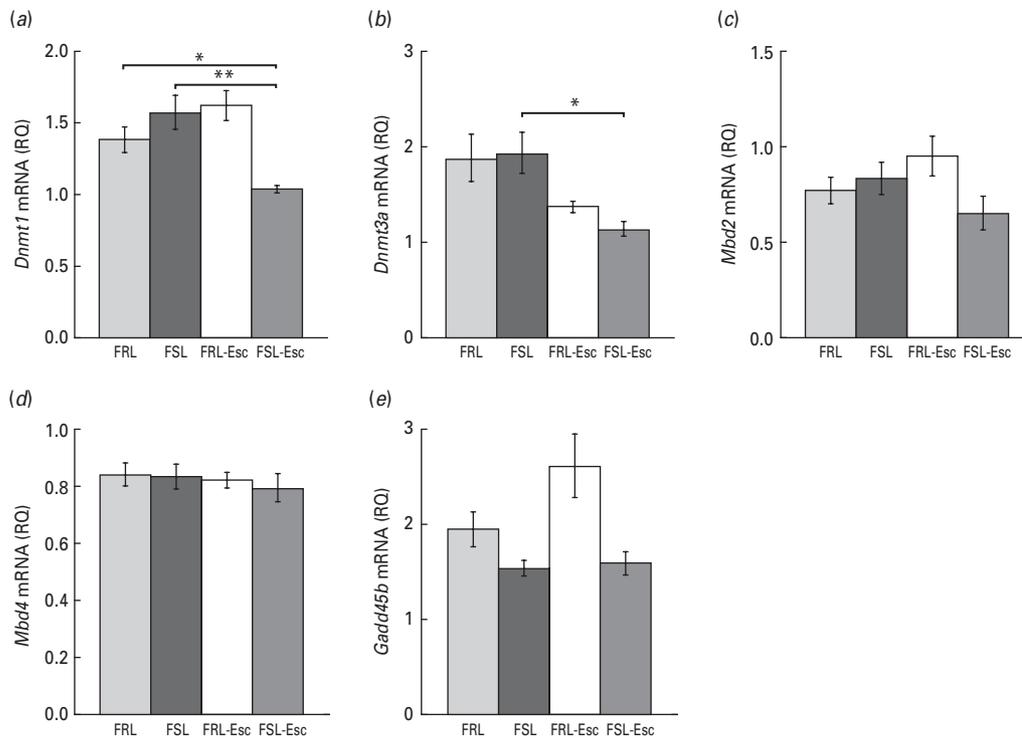
Fig. 4. Gene expression analyses by quantitative RT-PCR of DNA methylation-associated enzymes in the prefrontal cortex of the FSL/FRL strains following escitalopram administration. (*a*, *b*) Both DNA methyltransferase 1 (*Dnmt1*) and DNA methyltransferase 3a (*Dnmt3a*) mRNA levels were reduced in the FSL strain after antidepressant treatment ($n = 12$ FRL, $n = 14$ FSL, $n = 6$ FRL-Esc, $n = 9$ FSL-Esc; $n = 1$ FSL-Esc *Dnmt1* outlier). This coupled decrease in the FSL-Esc group is not only in accordance with its reduction in DNA methylation levels after treatment, but also with DNMT1's and DNMT3a's known role in maintaining DNA methylation in adult forebrain neurons. (*c*–*e*) Gene expression levels of three enzymes known to promote DNA demethylation (*Mbd2*, *Mbd4*, *Gadd45b*) did not reveal any significant differences between animal groups ($n = 12$ FRL, $n = 14$ FSL, $n = 6$ FRL-Esc, $n = 9$ FSL-Esc; $n = 1$ FSL and $n = 1$ FSL-Esc *Gadd45b* outliers). However, a trend for decreased *Gadd45b* levels in FSL animals (FSL *vs.* FRL, $p = 0.06$) is in line with their basal hypermethylated pattern and is supported by GADD45b's demethylating role in the adult brain. * $p < 0.013$, ** $p < 0.003$. RQ, Relative quantification.

Interestingly, we detected elevated methylation levels at the putative binding site of AR in the FSL strain (Fig. 3*b*). 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. 3*c*). 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. 3*b*, *c*). As indicated

by our global DNA methylation analysis (Fig. 3*d*), 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 CCpGG sites which represent a minor fraction ($\sim$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. 4*a*). 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. 4*b*). 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 (C_t 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. 4*c–e*), there was a trend for decreased *Gadd45b* levels in FSL animals (Fig. 4*e*: FSL *vs.* FRL, $p = 0.06$). The same trend was observed between the two treated groups (Fig. 4*e*: 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. 3*b, c*). It should also be mentioned that mRNA levels of *Aid* 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. 3*a*) 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.

Acknowledgements

Statement of Interest

None.

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