Monoamine oxidase A gene DNA hypomethylation – a risk factor for panic disorder?

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Abstract

The monoamine oxidase A (MAOA) gene has been suggested as a prime candidate in the pathogenesis of panic disorder. In the present study, DNA methylation patterns in the MAOA regulatory and exon 1/intron 1 region were investigated for association with panic disorder with particular attention to possible effects of gender and environmental factors.

Sixty-five patients with panic disorder (44 females, 21 males) and 65 healthy controls were analysed for DNA methylation status at 42 MAOA CpG sites via direct sequencing of sodium bisulfate treated DNA extracted from blood cells. The occurrence of recent positive and negative life events was ascertained. Male subjects showed no or only very minor methylation with some evidence for relative hypomethylation at one CpG site in intron 1 in patients compared to controls. Female patients exhibited significantly lower methylation than healthy controls at 10 MAOA CpG sites in the promoter as well as in exon/intron 1, with significance surviving correction for multiple testing at four CpG sites (p < 0.001). Furthermore, in female subjects the occurrence of negative life events was associated with relatively decreased methylation, while positive life events were associated with increased methylation. The present pilot data suggest a potential role of MAOA gene hypomethylation in the pathogenesis of panic disorder particularly in female patients, possibly mediating a detrimental influence of negative life events. Future studies are warranted to replicate the present finding in independent samples, preferably in a longitudinal design.

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Key words: Anxiety, epigenetics, gender, MAOA, methylation.

Introduction

Panic disorder (MIM %167870) is an anxiety disorder characterized by sudden, unexpected attacks of intense fear and anticipatory anxiety often associated with agoraphobia and a life-time prevalence of 1–3%, with women being affected approximately twice as often as men (Weissman et al. 1997). Family and twin studies indicate a strong genetic contribution to the pathogenesis of panic disorder with an estimated heritability of up to 48% (Hettema et al. 2001).

Among a variety of genes that have been identified as potential risk genes for panic disorder (cf. Jacob et al. 2010), the monoamine oxidase A (MAOA) gene has been suggested as one of the prime candidates: MAOA plays a pivotal role in the degradation of biogenic amines such as epinephrine, norepinephrine and serotonin by oxidative deamination (Shih & Chen, 1999), presumably to a high degree also in the human brain (Meyer et al. 2006). MAOA inhibitors such as phenelzine or moclobemide have been proven to be effective in the treatment of panic disorder
Methylation patterns in the regulatory region of epigenetic factors in panic disorder by investigating time we attempted to elucidate potential effects of yet. Of our knowledge no such studies have been conducted.

This lack of consistency in genetic association studies might reflect the low power of individual studies to detect a small effect, aetiological heterogeneity, high complexity of the clinically defined phenotype or random error in the absence of a true effect. Additionally, epigenetic processes such as methylation or acetylation critically influencing gene regulation and mediating adaptation to environmental factors might account for inconsistent association findings. Particularly, methylation of the cytosine pyrimidine ring in CpG-dinucleotides has been shown to be of major functional significance by mainly silencing DNA transcription (cf. Jaenisch & Bird, 2003). First landmark epigenetic studies with regard to psychiatric phenotypes have been published reporting, for example, association of MB-COMT promoter hypomethylation with schizophrenia and bipolar disorder (Abdolmaleky et al. 2006), hypermethylation of the 5-HTT promoter region in major depression (Philibert et al. 2008a,b), hypermethylation of RELN in schizophrenia (Abdolmaleky et al. 2005; Grayson et al. 2005), hypermethylation of the DAT promoter to be associated with decreased alcohol craving (Hillemacher et al. 2009), methylation changes in the BDNF gene in major psychoses (Mill et al. 2008) and correlation of MAOA methylation patterns with alcohol and nicotine dependence (Philibert et al. 2008a,b). In panic disorder, however, to the best of our knowledge no such studies have been conducted yet.

Therefore, in the present pilot study, for the first time we attempted to elucidate potential effects of epigenetic factors in panic disorder by investigating methylation patterns in the regulatory region of the MAOA gene in a sample of 65 patients with panic disorder as compared to 65 age- and gender-matched healthy controls. Particular attention was paid to possible gender effects as well as to the potential influence of environmental factors. In analogy to previous female-specific association findings of the longer, more active MAOA VNTR alleles with panic disorder, MAOA promoter DNA hypomethylation possibly conferring higher transcriptional activity was hypothesized to be associated with panic disorder particularly in female patients.

Method

Samples

The overall sample comprised 65 German patients with panic disorder (44 females; 21 males; age: 33.3 ± 9.7 yr ± S.D.). The diagnosis of panic disorder was ascertained by experienced psychiatrists on the basis of medical records and structured clinical interviews according to the criteria of DSM-IV (Wittchen, 1997). Patients with mental retardation, neurological or neurodegenerative disorders impairing psychiatric evaluation were not included in this analysis. Medication with antidepressants was recorded (see Table 1). The control group consisted of 65 healthy subjects matched to the patient group by gender and age (44 females, 21 males; age 33.1 ± 9.5 yr ± S.D.; T = 0.11, p = 0.91). Absence of mental Axis 1 disorders was established by experienced psychologists on the basis of a structured clinical interview (Mini International Neuropsychiatric Interview) according to the criteria of DSM-IV (Wittchen, 1997). Cases as well as controls were of Caucasian origin.

As smoking has been shown to be associated with lower methylation frequency of the MAOA as well as MAOB promoter, possibly due to cigarette smoke-induced increase in nucleic acid demethylase activity (Launay et al. 2009; Philibert et al. 2010), smoking status was ascertained in patient and control samples (see Table 1; missing data for three female patients). Data regarding the occurrence of life events subjectively rated as positive, negative or neutral during 6 months preceding disease onset (for patients) or 12 months prior to inclusion in the present study (for controls) were collected using a comprehensive scale derived from several established life event scales (Brown & Harris, 1978; Holmes & Rahe, 1967; Paykel et al. 1975; Rahe, 1979; Yeaworth et al. 1992; M. Rietschel, personal communication), with life event data missing for four female patients (see Table 1). To allow for comparability between patients and controls, the percentage of positive, negative or neutral life...
events relative to the total number of reported events in the respective time period was calculated and used for further analysis.

The study was approved by the Ethics Committee of the University of Muenster, Germany, written informed consent was obtained from all participating subjects and the study was conducted according to the ethical principles of the Helsinki Declaration.

Isolation of DNA

DNA was isolated from human whole blood using FlexiGene DNA Kit (Qiagen, Germany) according to the manufacturer’s instructions. In brief, lysis buffer was added to the samples and cell nuclei and mitochondria were pelletized by centrifugation. The pellet was re-suspended and incubated in denaturation buffer, which contains a chaotropic salt and protease. DNA was precipitated by addition of isopropanol and washed in 70% ethanol. The dried pellet was re-suspended in 25 mM Tris–HCl hydration buffer (pH 7.8). DNA concentration was determined by measuring at 260 and 280 nm (GENios Pro, Germany).

Bisulfite sequencing

Treatment of DNA with sodium bisulfite converts non-methylated cytosine to uracil, whereas methylated cytosine remains unaltered. Aliquots of isolated DNA were treated with sodium bisulfite using the EZ DNA Methylation-Direct TM Kit (Zymo Research, HiSS Diagnostics GmbH, Germany) according to the manufacturer’s protocol for all samples in one batch. Briefly, 450 ng DNA was submitted to bisulfite treatment for 7 h at 64°C in a thermal cycler (Mastercyler ep, Germany). DNA was eluted after desulfonation and cleaned up using Zymo-Spin IC columns (Zymo Research, HiSS Diagnostics GmbH). As a control, commercially available fully methylated and fully non-methylated DNA was used in all experiments.

Three amplicons covering the MAOA promoter region as well as exon 1 and parts of intron 1 were chosen for further analysis in analogy to a previous study on MAOA gene methylation (Philibert et al. 2008a, b). These amplicons begin at nucleotide position –1590 upstream to the start codon and contain 59 CpGs sites (amplicon A: 18 CpGs, amplicon B: 27 CpGs and amplicon C: 14 CpGs). Due to technical failures, particularly at the 3’ and 5’ ends of each amplicon, electropherograms were robustly readable for only 42 CpG sites in all patients/probands. These 42 CpG sites were therefore included in all further analyses (amplicon A: 11 CpGs, amplicon B: 18 CpGs and amplicon C: 13 CpGs; see Fig. 1a, b). Amplicons were polymerase chain reaction (PCR)-amplified using three sets of oligonucleotide primers (amplicon A: 5’-TAAAGAATGAAAGTATTAGGTTGAGAGTT-3’

### Table 1. Characteristics of female patients with panic disorder and healthy controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Female patients (n = 44)</th>
<th>Female controls (n = 44)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr ± s.d.)</td>
<td>34.8 ± 9.6</td>
<td>34.6 ± 9.5</td>
</tr>
<tr>
<td>MAOA VNTR genotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(24/33/34/35 vs. 44/45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking status (yes vs. no)</td>
<td>12 vs. 29</td>
<td>11 vs. 33</td>
</tr>
<tr>
<td>Medication (yes vs. no)</td>
<td>16 vs. 28</td>
<td>n.a.</td>
</tr>
<tr>
<td>Positive life events (% ± s.d.)</td>
<td>7.3 ± 17.6***</td>
<td>32.4 ± 39.3***</td>
</tr>
<tr>
<td>Negative life events (% ± s.d.)</td>
<td>61.1 ± 42.7***</td>
<td>26.8 ± 36.2***</td>
</tr>
</tbody>
</table>

VNTR, Variable number tandem repeat; n.a., not applicable.

a n.s.; T = –0.12, p = 0.90.
b n.s.; Fisher’s exact test = 5.29; p = 0.32.
c n.s.; χ² = 1.73, p = 0.19.
d n.s.; χ² = 0.20, p = 0.66 (data missing for three patients).
e Selective serotonin reuptake inhibitors: n = 14; opipramol: n = 1; fluspirilene: n = 1.
f % of positive life events (see Method); T = 3.84, p < 0.0005 (data missing for four patients).
g % of negative life events (see Method); T = –3.98, p < 0.0005 (data missing for four patients).
*** Significant at significance level of p < 0.001.
and 5'-ATACCCACTCTTTAAAAACCAAACCACCC-3', length: 238 bp; amplicon B: 5'-GGGTGTTGAATTTTGAGGAGAAG-3' and 5'-AAACACAACTACCCAAATCCC-3', length: 429 bp; and amplicon C: 5'-GGGGAGTTGATAGAAGGGTTTTTTTTAT-3' and 5'-TATATCTACCTCCCCCAATCACACC-3', length: 353 bp) (see Philibert et al. 2008a, b, 2010) under the following PCR conditions using Hot Start Taq polymerase (Zymo Research, HiSS Diagnostics GmbH): denaturation at 94.5 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min (40 cycles).

Amplified PCR products were purified with MultiScreen HTS Filter Plates (Millipore GmbH, Germany). DNA sequencing was performed using Big Dye Terminator chemistry (version 3.1; Applied Biosystems by Life Technologies, Germany) on a 3730xl DNA analyser sequencing platform (Applied Biosystems by Life Technologies). The resulting electropherograms allowed the robust identification of methylation status of 42 individual CpG sites in the MAOA promoter/exon 1/intron 1 region using Chromas (Technelysium Pty Ltd, Australia) and Sequence Scanner software (Applied Biosystems by Life Technologies).

The obtained sequences were quantitatively analysed by determining relative peak heights (C/C +T) from the normalized sequence trace files using
Epigenetic Sequencing Methylation analysis software (ESME) specifically designed and evaluated for artificially generated single nucleotide polymorphisms (C/T and G/A), which performs quality control, normalizes signals, corrects for incomplete bisulfite conversion and aligns generated bisulfite sequence and reference sequence to compare C to T peak heights at CpG sites (Lewin et al. 2004) as successfully used to analyse methylation profiles in other psychiatric phenotypes (e.g. Heberlein et al. 1999; Syagailo et al. 2010). To demonstrate the reproducibility of our results, six DNA samples (three patient and three control samples) were submitted to four independent bisulfite conversions and six to eight independent direct sequencing reactions, yielding 92% of the standard deviations of the mean methylation rates at the 42 analysed CpG sites as calculated by the ESME software to be <0.05.

Genotyping of MAOA VNTR

Isolated DNA was genotyped for the MAOA VNTR according to published protocols with minor modifications (Deckert et al. 1999). DNA was amplified by PCR (60 s at 94 °C, 60 s at 52 °C, 120 s at 72 °C for 35 cycles) using oligonucleotide primers F: 5'-AGGCTGCTCCAGAAAC and R: 5'-GGACCTGGG-CAGTTGTCG flanking the polymorphic region. PCR products were run on a 15% polyacrylamide gel and visualized by silver staining. Genotypes were determined by investigators blinded for clinical diagnoses.

Statistical analysis

Categorical data were analysed using the χ² test or the Fisher’s exact test, as appropriate. Differences in continuous variables were evaluated by student’s t test and multivariate analysis of variance. Correlations between dimensional variables were calculated by means of correlation or regression analysis, respectively. Owing to the fact, that the MAOA gene is X-chromosomally located, statistical analyses were performed stratified for gender. Also, according to functionality and previous molecular genetic studies (Deckert et al. 1999; Syagailo et al. 2001), MAOA VNTR alleles were divided into two groups containing either low-activity (2 and 3 repeats) or high-activity (3.5, 4 and 5 repeats) conferring alleles. Transcription factor binding sites were identified using ALGGEN-PROMO search algorithm based on Transfac (version 8.3; Farré et al. 2003; Messeguer et al. 2002).

Results

Sample characteristics

Sixty-five patients with panic disorder and 65 controls were matched for gender (1:1) and age (see Method). However, females were significantly older than males (females, n = 88: 34.7±9.5 yr ± S.D.; males, n = 42: 30.2±9.2 yr ± S.D.; T=2.56, p = 0.01). Due to non-sufficient quality of sequencing data for methylation analysis with ESME software, sample size was reduced to n = 61 (41 females, 20 males) for MAOA amplicon B and to n = 62 (43 females, 19 males) for MAOA amplicon C with complete methylation status at all 42 CpG sites being available for n = 40 female patients and n = 40 matched healthy probands. Given the X-chromosomal location of the MAOA gene, all further analyses were carried out separately for female and male subjects. Characteristics of the female subsamples regarding age, MAOA VNTR status, smoking status, medication and positive or negative life events are given in Table 1. Male patients (n = 21) and controls (n = 21) did not differ with regard to age (p = 0.987), MAOA VNTR status (patients: 3 alleles: n = 7; 4 alleles: n = 14; controls: 3 alleles: n = 5; 4 alleles: n = 16; p = 0.734) or smoking status (p = 0.130).

DNA methylation status of MAOA

In males, all CpG sites in MAOA amplicons A and B were fully non-methylated. Across CpG sites 1–3 and 5–11 in amplicon C, overall methylation in male subjects was low (0.038–0.172) and did not differ between patients and controls (data not shown). At CpGs 4 and 13 in amplicon C, overall methylation was somewhat higher ranging from 0.270 to 0.361, again without any statistical difference between patients and controls (data not shown). At CpG 12 in amplicon C, male patients showed significantly lower methylation than healthy controls [patients (n = 19): mean = 0.738, s.d. = 0.064; controls (n = 19): mean = 0.777, s.d. = 0.051; F = 4.47, p = 0.042]. Females showed high variability in methylation status across all CpG sites in amplicons A–C. Average methylation across all 42 CpG sites in amplicons A–C was significantly lower in female patients as compared...
to healthy female controls (patients \((n=40)\): mean = 0.378, \(S.D. = 0.022\); controls \((n=40)\): mean = 0.393, \(S.D. = 0.018\); \(T = 3.194, p = 0.002\)). When performing multivariate analysis of variance for single CpG sites, at specifically 10 sites in amplicons A (CpG 3, promoter), B (CpGs 13, 14 and 16, promoter) and C (CpG 2, promoter; CpGs 6, 7 and 9, exon 1; CpGs 10 and 11, intron 1) (see Fig. 1b) significantly lower methylation was detected in female patients with panic disorder than in healthy female controls with \(p\) values ranging from 0.04 to < 0.001. After Bonferroni’s correction for multiple testing (42 CpG sites resulting in a corrected \(p\) value of 0.001), associations of hypomethylation at CpG 14 in amplicon B and at CpGs 6, 7 and 10 in amplicon C remain significant. A descriptive overview as well as the statistical analysis of MAOA promoter/exon 1/intron 1 CpG sites differentially methylated between female patients with panic disorder and healthy female controls is provided in Table 2.

<table>
<thead>
<tr>
<th>Amplicon A</th>
<th>Patients ((n=44))</th>
<th>Controls ((n=44))</th>
<th>(F)</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG3</td>
<td>0.439 (0.047)</td>
<td>0.462 (0.040)</td>
<td>5.77</td>
<td>0.018</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amplicon B</th>
<th>Patients ((n=41))</th>
<th>Controls ((n=41))</th>
<th>(F)</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG13</td>
<td>0.397 (0.031)</td>
<td>0.415 (0.047)</td>
<td>4.47</td>
<td>0.038</td>
</tr>
<tr>
<td>CpG14</td>
<td>0.362 (0.043)</td>
<td>0.392 (0.039)</td>
<td>11.73</td>
<td>0.001*</td>
</tr>
<tr>
<td>CpG16</td>
<td>0.412 (0.042)</td>
<td>0.438 (0.051)</td>
<td>6.16</td>
<td>0.015</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amplicon C</th>
<th>Patients ((n=43))</th>
<th>Controls ((n=43))</th>
<th>(F)</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG2</td>
<td>0.372 (0.074)</td>
<td>0.401 (0.052)</td>
<td>4.24</td>
<td>0.043</td>
</tr>
<tr>
<td>CpG6</td>
<td>0.378 (0.066)</td>
<td>0.426 (0.058)</td>
<td>12.71</td>
<td>0.001*</td>
</tr>
<tr>
<td>CpG7</td>
<td>0.473 (0.042)</td>
<td>0.505 (0.046)</td>
<td>11.67</td>
<td>0.001*</td>
</tr>
<tr>
<td>CpG9</td>
<td>0.468 (0.051)</td>
<td>0.493 (0.043)</td>
<td>6.48</td>
<td>0.013</td>
</tr>
<tr>
<td>CpG10</td>
<td>0.487 (0.039)</td>
<td>0.518 (0.036)</td>
<td>14.18</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>CpG11</td>
<td>0.286 (0.061)</td>
<td>0.322 (0.046)</td>
<td>9.43</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Mean, Mean methylation; \(F\), multivariate analysis of variance; all results significant at significance level of \(p<0.05\).
* \(p\) values remaining significant after Bonferroni’s correction for multiple testing.

Table 2. Significant MAOA gene methylation differences between female patients with panic disorder and healthy controls

\[ n = 40 \text{ patients and } n = 40 \text{ probands} \]

Given this predominantly female-specific main effect, all further analyses were conducted in the female subgroups of patients and controls only.

**Influence of age, smoking status, medication and MAOA VNTR on MAOA methylation**

Age was neither different between female patients and female controls (see Table 1) nor did it influence average methylation across all CpG sites (available for \(n=40\) patients and \(n=40\) probands) in the female subsample (\(r=0.159, p=0.159\)), which also held true when considering patients (\(r=0.288, p=0.072\)) and controls (\(r=0.035, p=0.839\)) separately.

No difference in smoking status could be discerned between female patients and controls (see Table 1). Also, smoking status did not significantly influence average methylation across all CpG sites in the female subsample [smokers (\(n=23\)): mean overall methylation = 0.389, \(S.D. = 0.019\); non-smokers (\(n=54\)): mean overall methylation = 0.385, \(S.D. = 0.022\); \(T = -0.813, p = 0.419\)], which also applied when analysing patients (\(p = 0.945\)) and controls (\(p = 0.132\)) separately.

Sixteen female patients (35.4%) were medicated with antidepressants, mainly with SSRIs (see Table 1). None of the controls was taking any kind of medication. Medication status did not influence average methylation across all CpG sites (available for \(n=40\) patients) in the female patient sample [medicated (\(n=15\)): mean overall methylation = 0.374, \(S.D. = 0.027\); non-medicated (\(n=25\)): mean overall methylation = 0.381, \(S.D. = 0.019\); \(T = 0.883, p = 0.383\)].

The distribution of MAOA VNTR genotypes did not differ across female patient and control samples (24/33/34/35 vs. 44/45; \(\chi^2 = 1.73, p = 0.19\); for details see Table 1). However, while MAOA VNTR genotype distribution (24/33/34/35 vs. 44/45) did not influence average methylation across all CpG sites in female patients (\(p = 0.723\)), in female controls the more active genotypes (44/45) were associated with
In general, female patients with panic disorder had suffered from a significantly higher percentage of negative life events than female healthy controls (see Table 1). While in the overall sample of female subjects the percentage of negative, positive or neutral life events was not associated with average methylation across all CpG sites, the percentage of negative life events was associated with a relatively decreased methylation status specifically at two CpG sites (data missing for four patients, n=76; amplicon B, CpG 14: \( r = -0.198, p = 0.043 \) (see Fig. 2); amplicon C, CpG 6: \( r = -0.209, p = 0.035 \)). Reciprocally, the percentage of positive life events was found to be associated with a relatively increased methylation status at four CpG sites (data missing for four patients, n=76; amplicon B, CpG 13: \( r = 0.192, p = 0.048 \); amplicon B, CpG 14: \( r = 0.262, p = 0.011 \) (see Fig. 2); amplicon B, CpG 16: \( r = 0.218, p = 0.029 \); amplicon C, CpG 11: \( r = 0.263, p = 0.011 \)). The percentage of neutral life events was not correlated with methylation status at any single CpG site (data not shown). When considering patient and control samples separately, in the patient group two results remained significant: association of a higher percentage of negative life events with a relatively decreased methylation status at CpG 6 in amplicon C (data missing for four patients, n=36: \( r = -0.316, p = 0.030 \)) and association of a higher percentage of positive life events with a relatively increased methylation status at CpG 13 in amplicon B (data missing for four patients, n=36: \( r = 0.289, p = 0.044 \)). No significant association of life events with methylation status could be detected in the control group.

**Discussion**

First, at 39 out of 42 investigated MAOA promoter/exon 1/intron 1 CpG sites all male subjects were found to be non-methylated or marginally methylated, while females displayed variable levels of MAOA DNA methylation. This gender difference in DNA methylation within the MAOA gene possibly due to its X-chromosomal location has been described before in several studies (e.g. Philibert et al. 2008a,b; Pinsonneault et al. 2006; Wong et al. 2010) and therefore confirms gender-dependent methylation at the MAOA locus.

Second, the present study, for the first time, suggests altered methylation patterns in the MAOA gene to be correlated with panic disorder. In female subjects, a general hypomethylation across all 42 investigated CpG sites was observed in patients as compared to controls. Relative hypomethylation was particularly evident at 10 CpG sites (amplicon A CpG 3; amplicon B CpGs 13, 14 and 16, amplicon C CpG 2, 6, 7, 9, 10 and 11) in the MAOA promoter region as well as in exon 1 and intron 1. This finding might have functional consequences as mRNA expression analysis from autopsy samples of human brains suggested MAOA promoter methylation status to modulate MAOA transcription (Pinsonneault et al. 2006). Also, an *in silico* analysis provided support for epigenetic
processes to drive MAOA expression (Shumay & Fowler, 2010). As decreased methylation is generally assumed to rather activate gene transcription, the present observed MAOA promoter hypomethylation in female patients with panic disorder might result in increased expression of MAOA, leading to a decreased availability of epinephrine, norepinephrine and serotonin in the synaptic cleft. This hypothesis would be in line with previous reports of the longer MAOA VNTR alleles also conferring higher MAOA activity to be in line with previous reports of the longer MAOA VNTR alleles also conferring higher MAOA activity to be associated with panic disorder (Deckert et al. 1999; Maron et al. 2005; Samochowiec et al. 2004). Additionally, analysis of individual CpG sites for their relevance regarding potential transcription factor binding revealed that differential methylation at MAOA promoter CpG sites associated with panic disorder might interfere with the action of the following transcription factors: amplicon A CpG 3: ENKTF-1; amplicon B CpGs 13/14: c-Myb; amplicon B CpG 16: XBP-1, AR; amplicon C CpG 2: c-Jun, TFII-I; amplicon C CpGs 10/11: E2F-1; TFII-I. However, the functional consequence of MAOA promoter hypomethylation remains highly speculative and warrants thorough investigation in future studies.

Third, the present observed female-specific effect of MAOA gene hypomethylation in panic disorder is in line with association findings of MAOA VNTR restricted to the female subgroup of patients with anxiety and affective disorders (Dannlowski et al. 2005; Samochowiec et al. 2004). Therefore, MAOA VNTR hypermethylation in female patients with panic disorder, but not in males, might constitute one of the pathomechanisms contributing to the female-dominant prevalence of panic disorder. Gender effects have previously been suggested in the pathogenesis of panic disorder with female-specific heritability estimates of anxiety sensitivity (Jang et al. 1999) as well as female-specific association findings of candidate genes with the disorder (e.g. Domschke et al. 2007) or neuronal activation correlates of emotional processing (Ohrmann et al. 2010). Reciprocally, male-specific altered serotonin transporter binding potential patterns have been described in panic disorder (Maron et al. 2011) and, mostly, environmental factors have been found to account for increased anxiety sensitivity in men (Jang et al. 1999). Thus, it might be worthwhile to try and further elucidate the potentially gender-specific pathogenetic mechanisms of panic disorder in future studies.

Fourth, we identified a higher ratio of negative and lower ratio of positive life events during the 6 months prior to this study in patients as compared to healthy controls. This finding confirms previous reports of a significant role of life events in the precipitation of panic disorder, particularly in a time-window of 2–3 months before onset of panic disorder (Faravelli 1985; Faravelli & Pallanti, 1989; cf. Klauke et al. 2010) and provides support for a differential role of negative and positive environmental stressors in the pathogenesis of panic disorder. We additionally discerned association of a high percentage of negative life events with a relatively decreased methylation status and reciprocally a higher ratio of positive life events to be associated with increased methylation at certain CpG sites. These effects were particularly true in the patient subgroup, although sample size was very limited. Given the present observed association of decreased methylation with panic disorder, it might be hypothesized that negative life events contribute to a higher disease risk via MAOA gene demethylation, while positive life events might exert a protective effect conferring some resilience to the disease mediated by MAOA gene methylation (cf. Abdolmaleky et al. 2004; Szyf et al. 2008). It has to be noted, however, that the present ascertainment of life events data was purely qualitative and self-descriptive and therefore might not have constituted a valid measure of environmental influences. Nonetheless, the present pilot data on life events potentially influencing epigenetic programming might nourish future, preferably longitudinal studies modelling a pathway including environmental influences, epigenetic alterations and health status or panic disorder vulnerability.

The present findings have to be considered as preliminary and ought to be interpreted in light of some caveats. In general, given the difficulty to obtain relevant brain tissue from panic disorder patients in vivo, DNA isolated from peripheral EDTA-blood was used as an alternative study material, which explicitly does not allow for direct conclusions regarding the respective methylation patterns in brain tissue, but has been suggested as a viable sensor for central processes (see Mill & Petronis, 2007). Several preceding studies have used peripheral biomaterial such as lymphoblast cultures or saliva in epigenetic studies of other psychiatric phenotypes such as Alzheimer’s disease and schizophrenia (see Gladkevich et al. 2004; Murphy et al. 2005; Muschler et al. 2010; Nohesara et al. 2011; Philibert et al. 2007a, b; Terry et al. 2011), with some evidence for highly comparable or even nearly identical DNA methylation patterns in peripheral blood cells/saliva and several brain regions (e.g. Murphy et al. 2005; Nohesara et al. 2011). In general, peripheral biosignatures might, in part, reflect centrally relevant mechanisms as, for instance, Philibert et al. (2007c)
MAOA hypomethylation in panic disorder

observed panic disorder specific transcriptional profiles in haemopoetically derived cells and DNA-methyltransferase-1 (DNMT-1) expression in peripheral blood lymphocytes has been reported to mirror neuronal DNMT-1 activity (Zhubi et al. 2009). As for the question whether whole blood analyses might be an apt source for epigenetic studies, we point to a recent study by Philibert et al. (2010), who demonstrated that DNA methylation patterns derived from whole blood as compared to lymphoblasts did not differ significantly. However, it most certainly cannot be excluded that the observed alterations in methylation are due to differing composition of cell subtypes in whole blood, which has not been controlled for in the present study and will have to be subject to further investigation – particularly since MAOA has been shown to be expressed in blood monocytes (Chaitidis et al. 2004), but not in platelets or lymphocytes (Thorpe et al. 1987). Consequently, whole blood as presently analysed might not be the optimal substrate to study MAOA expression patterns based on MAOA DNA methylation. Thus, future studies aiming at experimental determination of MAOA expression related to promoter DNA methylation might want to base their analysis on, for example, fibroblasts, where strong MAOA expression has been observed (e.g. Hotamisligil & Breakefield, 1991). Another caveat is that the present applied method of direct sequencing of bisulphite treated DNA allows for only a semi-quantitative analysis of methylation and might not be valid for small methylation differences. Also, it has to be explicitly stated that the functional consequences of MAOA promoter hypomethylation (see in silico analysis by Shumay & Fowler, 2010) or MAOA VNTR allele distribution (see Deckert et al. 1999) have not been experimentally determined in the present study. Furthermore, the sample under study, while within the range of previously published epigenetic studies in mental disorders, was still very limited in power, particularly in the male subsample. Nevertheless, the sample size, while underpowered to replicate previous categorical association findings of MAOA VNTR with panic disorder, was apparently sufficient to detect methylation effects in the female subgroup, surviving correction for multiple testing at four CpG sites. Additionally, despite the fact that in the present study patients and controls were matched for gender, age and smoking status, additional potentially confounding factors cannot be excluded. In principle, the present observed association of MAOA promoter hypomethylation with panic disorder does not necessarily have to be indicative of a disease state but could, for instance, reflect an environmental exposure arising coincidently in this specific patient population. Furthermore, although 63.6% of the patients were not on any medication and methylation rates did not differ with regard to medication status, the present noted changes could be secondary to antidepressant medication as it has been reported that SSRIs impact epigenetic signatures, e.g. escitalopram has been observed to induce DNA hypomethylation of the PI1 gene promoter region (Melas et al. in press) and fluoxetine was associated with induction of methyl-CpG-binding protein, a transcription factor involved in DNA methylation (Wang et al. 2011). Thus, the present finding warrants replication in a sample of non-medicated subjects. Also, since females were older than men in the present sample, a confounding effect of age regarding the gender-specific effect cannot be excluded, although increasing age has been found to be associated with higher methylation (Philibert et al. 2010) rather than with hypomethylation as presently observed in female patients. Finally, we observed a tendency for the longer, more active MAOA VNTR 4/5 alleles to be associated with increased overall methylation in healthy controls, but not in patients with panic disorder. It could be speculated that increased methylation might account for a ‘neutralization’ of the more active risk alleles as a physiological mechanism in healthy probands. However, this finding is in contrast to previous studies by Philibert et al. (2008, 2010), who report significantly lower methylation in healthy 4-allele homozygotes than in 3/4 and 3/3 genotype carriers, and therefore warrants further investigation in future studies.

Despite the above-mentioned limitations, the present pilot data may be a first hint towards a potential female-specific role of MAOA gene DNA hypomethylation in the pathogenesis of anxiety and anxiety disorders, potentially via a detrimental influence of negative life events. Future longitudinal studies are warranted to replicate the present findings in independent samples, preferably using quantitative methylation analysis techniques, such as pyrosequencing. In addition, the development of an epigenetic murine model allowing analysis of brain tissue is essential to elucidate the functional relevance of MAOA hypomethylation in the pathogenesis of anxiety-related behaviour. Provided there is robust replication, this first evidence for epigenetic factors to be associated with panic disorder is hoped to encourage future molecular genetic studies to take into account epigenetic data, which might aid in reconciling inconsistent association findings and eventually unravelling the ‘hidden heritability’ of panic disorder (cf. Fraga et al. 2005).
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Statement of Interest

None.

References


