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PHARMACOGENETICS

A candidate gene study of serotonergic pathway genes and pain relief during treatment with escitalopram in patients with neuropathic pain shows significant association to serotonin receptor2C (HTR2C)

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Abstract
Purpose Previous studies have shown that a small fraction of patients with peripheral neuropathic pain experiences >50% pain relief during treatment with selective serotonin reuptake inhibitors (SSRIs), whereas most patients have no or only slight relief. The aim of this study was to investigate the association between polymorphisms in genes involved in the serotonergic pathway and the effect of escitalopram on peripheral neuropathic pain.

Methods We genotyped 34 participants from a placebo-controlled trial of escitalopram in peripheral neuropathic pain for polymorphisms in five genes: the serotonin receptor 2A (*HTR2A*) gene, the serotonin receptor 2C (*HTR2C*) gene, the *ABCB1* gene encoding for the P-glycoprotein, the *CYP2C19* gene, and the serotonin transporter gene (*SLC6A4*).
Results The SNP rs6318 (Cys23Ser) in the *HTR2C* gene showed significant association with treatment response in men (p=0.047), with 75% carrying the C allele being responders. The same tendency was seen in women. Similarly, carriership of the C allele at rs6318 was associated with better pain relief during treatment with escitalopram [odds ratio (OR) 15.5, p=0.014)] Furthermore, there was a tendency of better relief with increasing number of short alleles for the 5-HTTLPR polymorphism of the serotonin transporter (OR 5.7, p=0.057). None of the other polymorphisms showed a significant association with treatment response to escitalopram.
Conclusion This study indicates that variation in the *HTR2C* gene is associated to the pain-relieving effect of escitalopram in patients with painful polyneuropathy.

Keywords Pharmacogenetics · Neuropathic pain · SSRI treatment · Treatment response

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Introduction

Antidepressants and anticonvulsants play a key role in treating peripheral neuropathic pain [1]. Within the antidepressant drug class, both tricyclic antidepressants and serotonin (5-HT)/noradrenaline reuptake inhibitors are recognized for their evidence-based clinically relevant pain relief [1, 2]. The

efficacy of selective serotonin reuptake inhibitors (SSRIs) for treating peripheral neuropathic pain is controversial. Approximately 20 years ago, two small clinical trials found slight but statistically significant pain relief with the SSRIs paroxetine and citalopram in diabetic neuropathic pain [3, 4]. However, a larger study with fluoxetine failed to find any effect [5]. Recently, we reported that escitalopram, which is the pharmacologically active S-enantiomer of citalopram, had a small, albeit statistically significant, effect on painful polyneuropathy [6]. Thus, taken together, data suggests that SSRIs do have some impact on peripheral neuropathic pain.

The antidepressants are believed to relieve neuropathic pain by enhancing descending monoaminergic inhibitory neuron systems modulating signaling in the pain pathway at its passage through the dorsal horn of the spinal cord [2]. The contribution of serotonergic neurons in pain modulation has been a matter of debate [7, 8]. It has been indicated that some serotonergic connections are antinociceptive whereas others are pronociceptive, and it is believed that 5-HT_1 receptors are related to antinociception whereas 5-HT_2 and 5-HT_3 receptors are related to pronociception [8]. Data from clinical trials on SSRIs in peripheral neuropathic pain indicate that the net effect of enhancing serotonergic neurotransmission is antinociception. If data from the trials is analyzed in more detail, it is found that the small statistically significant effect of SSRIs does not come from a small effect in all or most patients but from a larger effect in a small fraction of patients, with approximately 25% of patients experiencing >50% pain relief [1]. This could indicate that response to SSRIs in peripheral neuropathic pain is seen only in a genetic subgroup of patients.

This is a posthoc follow-up study on a recent randomized clinical trial on escitalopram in patients with neuropathic pain [6]. The aim was to test whether the pain-relieving effect of escitalopram in patients with peripheral neuropathic pain could be associated with polymorphisms in genes involved in the serotonin system or those involved in transport or elimination of escitalopram. We tested polymorphisms in five genes encoding the serotonin receptor 2A and −2C (*HTR2A*, *HTR2C*), the serotonin transporter (*SLC6A4*), the adenosine triphosphatase (ATP)-dependent drug efflux pump (*ABCB1* or P-glycoprotein 1), and the enzyme cytochrome P450 2C19 (*CYP2C19*). Polymorphisms were chosen based on having an impact on protein structure or function and with a frequency in the Caucasian population that justified analysis in a small sample.

Methods

Patients

The study involved patients with painful previously described polyneuropathy [6]. In brief, the clinical study tested the effect of escitalopram (Cipralex®, H. Lundbeck A/S, Denmark) versus placebo on painful polyneuropathy in a randomized, double-blind, placebo-controlled crossover trial. Patients were carefully selected according to predefined criteria and had painful polyneuropathy. Patients entered a double-blind, crossover treatment sequence for 6 + 6 weeks with escitalopram (20 mg/day) and placebo, separated by a washout period of 2 weeks. They were randomized into two groups to receive escitalopram in either the first or second period. Patients rated pain relief on a 6-point ordinal scale (complete, good, moderate, slight, none, worse, from 6 to 1, respectively) after the fifth week of each treatment. Results for 41 patients were included in the clinical data analysis. Eleven patients reported a moderate or better pain relief during treatment with escitalopram (responders), and 30 reported weak or no pain relief. Serum concentrations of escitalopram did not differ between responders and nonresponders.

After finishing the original study, all 41 patients in the original study were contacted with regard to the posthoc study. Thus, blood samples from 34 of the patients (26 men, 8 women) were included in the pharmacogenetic study reported here. Samples from all 11 responders and 23 of the 30 nonresponders were analyzed. Approval by the Institutional Ethical Review Board was granted, and informed consent was obtained from each patient.

Selection of candidate genes

Five candidate genes were tested: serotonin receptor 2A, serotonin receptor 2C, p-glycoprotein, CYP2C19, and serotonin transporter. Candidate genes were selected based on the fact that they either are obviously involved with the serotonin system or have a known association to escitalopram based on earlier literature. From the candidate genes, polymorphisms were selected by the following inclusion criteria: (1) heterozygosity >0.1 in the Caucasian population (http://www.ncbi.nlm.nih.gov/snp); (2) placed in a coding region; (3) give rise to a missense mutation or influence gene function. This led us to test the following single nucleotide polymorphisms (SNPs): rs6314 (His452Tyr) in the serotonin receptor 2A gene, rs6318 (Cys23Ser) in the serotonin receptor 2C gene, rs2032582 (Ala 893Ser/Thr) in the gene coding for P-glycoprotein 1, rs4244285 (*2) & rs12248560 (*17) in cytochrome P450, subfamily C19 (CYP2C19), and an insertion/deletion polymorphism, 5-HTTLPR, in the promoter region of the serotonin transporter gene (solute carrier family six, member four).

Genotyping

Genomic DNA was isolated from peripheral leukocytes using Maxwell 16 (Promega, Tokyo, Japan). The rs6314 (C1354T) in serotonin receptor 2A, rs4244285 (*2), and

rs12248560 (*17) in CYP2C19 were genotyped using TaqMan® predesigned SNP genotyping assays by Applied Biosystems (Foster City, CA, USA) (assay ID: C__11696920_20, C_25986767_70, C_469857_10, respectively). SNP rs6318 (G68C) in serotonin receptor 2C was genotyped using allele discriminating primers in a SYBR green real-time polymerase chain reaction (PCR) assay. Primers for allele-specific amplification, ie., two allele-specific primers (ASP) and a locus primer (LSP) were designed using guidelines recommended by Applied Biosystems; ASP: 5′-GGCCTATTGGTTTGGCAATW-3′, LSP: 5′-TCCACCATCGGAGGTATTGAA-3′. DNA was amplified in a total volume of 5 μl containing 2.5 μl SYBR green PCR mix (Applied Biosystems), 900 nmol/L of each primer (DNA Technology, Aarhus, Denmark), and 10 ng genomic DNA. Real-time PCR was performed on StepOnePlus™ real-time PCR system (Applied Biosystems). The rs2032582 (G2677T/A) in ATP-dependent drug efflux pump (ABCB1 or P-glycoprotein 1) is triallelic and was genotyped by sequencing. Forward primer: 5′-AGGTTC CAGGCTTGCTGTAAT-3′, reverse primer: 5′-AGTCCAA GAACTGGCTTTGCT-3′. The primer pair was used for both PCR and sequencing. PCR was performed in a total volume of 10 μl containing 0.2 μM of each primer, 200 μM deoxyribonucleotide triphosphate (dNTP), 1 mmol/ml magnesium chloride ($MgCl_2$), 0.008 U Taq polymerase (Sigma), 1× PCR buffer, and 10 ng genomic DNA. Cycling parameters were 5 min 95°, 94°/59°/72° for 35 cycles and 30 s at each set point, followed by 72° for 7 min. Sequencing reaction was performed using standard procedure and Big Dye Terminator v3.1 cycle sequencing kit, 0.2 μM primer, and 3730XL DNA analyzer (Applied Biosystem). Genotyping of the insertion/deletion polymorphism 5-HTTLPR in the promoter region of the serotonin transporter gene (*SLC6A4*) was performed as previously described [9] and amplified to a 418-bp product (long allele) and a 376-bp product (short allele). PCR was performed in a total volume of 10 μl containing 0.2 μM of each primer, 200 μM dNTP, 1 mmol/ml $MgCl_2$, 0.008 U Taq polymerase (Sigma), 1× PCR buffer and 10 ng genomic DNA. Cycling parameters 5 min 94°, 94°/60°/72° for 30 s for the first two set points and 1 min at 72° for 35 cycles, followed by 72° for 7 min. PCR products were resolved on a 3730XL DNA analyzer (Applied Biosystem).

Data analysis and statistics

Polymorphism in p-glycoprotein1 has two variant alleles (A and T), but as very few patients had the A allele (two nonresponders and one responder, all being heterozygous for AT), we combined AT patients with GT patients in our analyses. Hardy-Weinberg calculations were performed using http://www.genes.org.uk/software/hardy-weinberg.shtml [10]. Escitalopram treatment response was measured as pain relief. Associations to genotypes were analyzed using two pain-relief end points: (1) binary and (2) quantitative relief difference (ordinal scale). Patients were dichotomized based on pain relief during the escitalopram treatment for the binary measure response/no response, as in the original clinical work [6]. If the relief was moderate, good, or complete, patients were categorized as responders, otherwise they were categorized as nonresponders. Association between genotypes and binary pain data was analyzed by Fisher's exact test. Exact logistic regression was used to calculate odds ratios (OR) for treatment response. To add more statistical power, we calculated the difference between pain relief during escitalopram and during placebo to obtain a quantitative measure of treatment response adjusting for individual pain perception. This resulted in values between 3 and −3, with 3 being three categories better pain relief using escitalopram (e.g., moving from no pain relief under placebo to good pain relief under escitalopram), and −3 having three categories better pain relief under placebo compared with escitalopram. We conducted an ordinal logistic regression analysis when analyzing the difference as a categorical variable. Analyzing the difference as a numerical variable, ordinary T test was conducted. As the original study was a crossover study, age and gender did not influence statistics and were not adjusted for. Proportional odds assumption underlying the ordinal logistic regression and the assumption underlying the T test were checked. All statistical analyses were performed using STATA10.1 (Statacorp, TX, USA).

Results

Genotype frequencies for the study population sample are listed in Table 1. Serotonin receptor 2C maps to chromosome X, and data is thus analyzed by gender. The SNP in serotonin receptor 2C (rs6318) shows significant association to treatment response in men ($p=0.047$). Of all the male patients with a G allele, 18% were responders, whereas 75% of patients with the C allele were responders. Too few women were included in the study to conclude anything on their genotype distribution, but the distribution shows the same tendency. When analyzing both men and women in a dominant model GG/G and GC/C (no women had the CC genotype), the association was stronger ($p=0.024$), data not shown.

The tested SNP in serotonin receptor 2A (rs 6314) showed a tendency ($p=0.11$) of A allele carriers exhibiting better response to treatment compared with the wildtype allele (56% vs 24%). However, apart from rs6318, none of the genotyped polymorphisms were significantly associated with response/nonresponse during treatment with escitalopram (Table 1). In the ordinal logistic regression analysis,

Table 1 Genotype association to binary treatment data (response/nonresponse) by escitalopram on neuropathic pain

Gene, polymorphism	Genotypes	Number of individuals		*P* value[a]	OR[b] (95% CI)
		Treatment response			
		No	Yes		
Serotonin receptor 2A, rs6314	GG	19 (76.0%)	6 (24.0%)	0.11	1 (ref)
	GA	4 (44.4%)	5 (55.6%)		3.8 (0.6–26.4)
	AA	0 (− %)	0 (− %)		–
CYP2C19, rs4244285 (*2)	GG	16 (64.0%)	9 (36.0%)	1.0	1 (ref)
	GA	5 (71.4%)	2 (28.6%)		0.7 (0.1–5.6)
	AA	1 (100.0%)	0 (0.0%)		1.9 (0–73.7)
CYP2C19, Rs12248560 (*17)	CC	13 (68.4%)	6 (31.6%)	0.62	1 (ref)
	CT	7 (58.3%)	5 (41.7%)		1.5 (0.3–8.8)
	TT	2 (100.0%)	0 (0.0%)		1.0 (0–13.8)
Serotonin receptor 2C, rs6318 -women[c]	GG	3 (60.0%)	2 (40.0%)	1.0	1 (ref)
	GC	1 (33.3%)	2 (66.7%)		2.6 (0.1–235.0)
	CC	0 (− %)	0 (− %)		–
Serotonin receptor 2C, rs6318 –men[c]	G	18 (81.8%)	4 (18.2%)	**0.047**	1 (ref)
	C	1 (25.0%)	3 (75.0%)		11.7 (1.01–371.8)
P-glucoprotein, rs2032582	GG	8 (72.7%)	3 (27.3%)	0.88	1 (ref)
	GT/AT	12 (63.2%)	7 (36.8%)		1.5 (0.2–12.0)
	TT	3 (75.0%)	1 (25.0%)		0.9 (0.01–18.1)
Serotonin transporter, 5-HTTLPR	L/L[d]	8 (72.7%)	3 (27.3%)	0.27	1 (ref)
	L/S	11 (78.6%)	3 (21.4%)		0.7 (0.1–7.0)
	S/S	4 (44.4%)	5 (55.6%)		3.1 (0.4–32.2)

Bold *p*-values indicate significance, $p < 0.05$

CI confidence interval

[a] *P* value for difference in genotype distribution (Fishers exact test)

[b] Odds ratio (OR) was calculated by exact logistic regression

[c] Analyses are stratified by gender for serotonin receptor 2C as the gene maps to the X-chromosome

[d] *L* long allele, *S* short allele

we found a significant association between serotonin receptor 2C, rs6318, genotype CC, and difference in pain relief for men. For carriers of a C allele at rs6318, the expected ordered odds of obtaining a minimum 1 category better pain relief—when treated by escitalopram—increased by 15.5-fold compared with carriers of a G allele (OR 15.5, p=0.014, Table 2). When combining men and women in a dominant model, as described above, we obtained an OR of 10.6 (p=0.010), p value for model=0.007 (data not shown). Analyzing the difference in pain relief using the ordinary T test, we achieved similar significance (results not shown), indicating that the result is independent of statistical modeling. Polymorphism length in the serotonin transporter (5-HTTLPR) showed a tendency toward better pain relief with increasing number of short alleles. This was, however, not significant (OR for being homozygous for the short allele 5.7, p=0.057). Analyzing data in a recessive model for the short allele did not increase the significance (results not shown). We found no association between quantitative pain relief and SNPs in serotonin receptor 2A (rs6314), CYP2C19 (rs4244285, rs12248560), and p-glycoprotein (rs2032582) (Table 2).

Discussion

For a number of drugs, pharmacogenetic studies indicate that the genetics underlying treatment response or adverse drug effects is much simpler than the genetics of the etiology of complex diseases and thus are probably caused by a limited number of genes. Studies on warfarin have shown that polymorphisms in the *CYP2C9* and the *VKOR* genes account for >30% of the variance in dose response [11]. Other studies have described that HLA-B*5701 can identify >55% of clinically diagnosed hypersensitivity reactions to abacavir in Caucasians [12]. For comparison, 80–90% of the variation in height can be attributed to genetic factors, and so far, >40 genetic loci have been

Table 2 Genotype associations to quantitative neuropathic pain relief (differences between escitalopram and placebo)

Gene, polymorphism	Genotypes	OR (p value)[c]	P value for model[d]
Serotonin receptor 2A, rs6314	GG	1 (ref)	0.16
	AG	2.7 (0.17)	
CYP2C19, rs4244285 *2	GG	1 (ref)	0.81
	GA	1.6 (0.52)	
	AA	Too few	
CYP2C19, Rs12248560 *17	CC	1 (ref)	0.31
	CT	2.1 (0.28)	
	TT	Too few	
Serotonin receptor 2C, rs6318 –women[a]	GG	1 (ref)	0.3
	GC	5.7 (0.32)	
Serotonin receptor 2C, rs6318 –men[a]	G	1 (ref)	**0.01**
	C	15.5 (**0.014**)	
P-glucoprotein, rs2032582	GG	1 (ref)	0.55
	GT/AT	2.1 (0.28)	
	TT	1.6 (0.65)	
Serotonin transporter, 5-HTTLPR	L/L[b]	1 (ref)	0.15
	L/S	1.8 (0.4)	
	S/S	5.7 (0.057)	

Bold p-values indicate significance, $p < 0.05$

[a] Analyses are stratified by gender for serotonin receptor 2C as the gene maps to the X-chromosome

[b] *L* long allele, *S* short allele

[c] Ordinal categorical logistic regression was performed with the most frequent genotype as the reference group; p values correspond to two-sided test of the odds ratio (OR) being equal to 1

[d] Log likelihood ratio test if genotypes in the various models cannot predict pain relief

confirmed to influence the phenotype. Notably, these variants account for only about 5% of the total variation in adult height [13].

We previously demonstrated that treatment with escitalopram relieves pain in some patients with polyneuropathy [6]. The study reported here tested the association between pain relief during treatment with escitalopram and six functional polymorphisms in genes involved in the serotoninergic pathway. We found that pain relief with escitalopram in peripheral neuropathic pain was associated with an SNP in serotonin receptor 2C and a trend of a similar association with the 2A receptor. Furthermore, for polymorphism length of the serotonin transporter, there was a tendency to better relief with increasing number of short alleles. We found no association between pain relief after treatment with escitalopram with polymorphisms in p-glycoprotein or the escitalopram metabolizing enzyme CYP2C19.

Due to the small sample size, this study does not have enough power to find small effects. Hence, the insignificant findings might be a false rejection and the effects be clinically relevant. Finding significant results despite sample size indicates that the effect of the SNP in serotonin receptor 2C might be clinical relevant. The small sample size does, however, result in a weak estimate of the effect, and hence, the confidence intervals are large.

Experiments suggest that some serotonergic receptors are antinociceptive whereas others are pronociceptive, and it is believed that 5-HT_2 receptors are related to pronociception [8]. We tested SNPs that give rise to amino acid changes in both 5-HT_{2A} and 5-HT_{2C} receptors. Several pharmacogenetic studies have shown the 5-HT_{2A} receptor to be a putative regulator of the antidepressant response [14]. A 5-HT_{2A} receptor variant has previously been shown to influence the antidepressant response in mood-disorder patients [15]. We found no significant association between rs6314 in the 5-HT_{2A} receptor and pain relief by escitalopram in our study but saw that a tendency toward holding the A allele gives better treatment response compared with the wildtype allele.

The gene for 5-HT_{2C} receptor maps to chromosome X, and men and women were thus initially analyzed separately. We found a significant association between the allelic distribution of rs6318 in the 5-HT_{2C} receptor gene in men for both the dichotomized pain relief measure and the quantitative measure. The rs6318 causes an amino acid change known as Cys23Ser. Studies on the functionality of this polymorphism are contradicting; some indicate that a receptor protein with a serine (C allele) shows decreased binding of serotonin but that the Ser23 allele shows higher constitutive activity [16], whereas other studies show no effect of the rs6318 on receptor signaling [17]. Our results indicate that carriers of a C allele (serine) at rs6318 have significantly better pain relief when treated with escitalopram compared with carries of a G allele (cysteine). Overall, this could potentially be explained by less pain due to less pronociception because of malfunction in 5-HT_2 receptors. The balance between anti- and pronociception will thus be shifted toward antinociception. Alternatively, as the effect on receptor function due to rs6318 is debatable, the positive association to pain relief observed in our study could be a consequence of linkage disequilibrium to another SNP. Very few women participated in the study, and we were not able to see an association between pain relief and 5-HT_{2C} receptor in women. However, when combining men and

women and analyzing data in a dominant model, the association was even more significant, which give more robust evidence that having the C allele for rs6318 creates a better response to treatment than does having the G allele.

Escitalopram inhibits serotonin reuptake by inhibiting the serotonin transporter, *SLC6A4*, and this gene was thus an essential candidate to test the effect on pain relief. We tested a length polymorphism 5-HTTLPR in the promoter region of the gene, which has been shown to influence the transcription level. It has been shown that the short allele of 5-HTTLPR results in decreased transcription [18]. Homozygosity for the long allele is associated with a two times more efficient serotonin reuptake compared with the s/l- or s/s-genotype [19]. A recent study found no relationship between 5-HTTLPR and experimentally induced pain/pain perception, either in fibromyalgia patients or healthy controls [20]. We found no significant association between pain relief and the 5-HTTLPR polymorphism but observed a weak tendency that more responders carry the s/s genotype and more nonresponders carry the l/l genotype.

P-glycoprotein plays an important role in controlling the passage of substances between the blood and brain, including the passage of escitalopram. It is encoded by the multidrug-resistance 1 (*MDR1*) gene and belongs to the ATP-binding cassette transporter family (*ABCB1*). The tested SNP, rs2032582, gives rise to an amino acid substitution, and studies have showed that the G allele decreases drug efflux in vitro [21]. However, the impact on protein function is controversial, as both increase and decrease on pharmacokinetic measures have been reported [22]. The rs2032582 has previously shown to be associated with SSRI (paroxetine) treatment response in patients with major depressive disorder, where the TT genotype gave a better response [23]. We found no association between pain relief and p-glycoprotein in our study.

CYP2C19 is a clinically important enzyme that metabolizes a wide variety of drugs, including escitalopram [24]. Escitalopram is metabolized to the inactive demethylescitalopram. The efficacy of CYP2C19 could thus play a role in the pain-relieving effect of escitalopram. The polymorphism rs4244285 is the causative SNP in the haplotype CYP2C19*2, which give rise to a splicing defect in the protein and thus a nonfunctional enzyme. On the contrary, rs12248560, also in CYP2C19, is included in the haplotype CYP2C19*17, which increases enzyme activity by increased transcription of the protein [25]. We found no association between CYP2C19 polymorphisms and pain relief, which correlates with the fact that no difference in escitalopram plasma concentration between responders and nonresponders was found (data not shown).

State-of-the art pharmacological treatment of peripheral neuropathic pain provides only a few patients with complete pain relief, and a substantial fraction of patients are left with minimal or no relief [1]. Individualized therapy may be one of the only ways to improve treatment response, and it may be achieved by targeting pain mechanisms or other individualized factors at play in the individual patient. The findings in this study point to genetic factors for response to a specific pharmacological therapy, i.e., that polymorphisms of the serotonin receptor may be important for the pain-relieving effect of serotonergic drugs. It is important to remember that serotonergic mechanisms of action are also supposed to be important for drugs other than SSRIs; for example, serotonin/noradrenalin reuptake inhibitors (SNRIS). Thus, genotyping for SNPs of the serotonin 2C and maybe the 2A receptor may help identify patients with peripheral neuropathic pain who may have the best chance of obtaining an adequate response on antidepressants.

To conclude, our data suggest that the serotonin receptor 2C is involved in pain relief in patients with neuropathic pain during treatment with escitalopram. However, although prospective studies on larger samples are required to verify this finding, our findings may be a first step toward individualized pharmacological therapy for neuropathic pain.

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