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ORIGINAL ARTICLE

# Interaction between variation in the D2 dopamine receptor (*DRD2*) and the neuronal calcium sensor-1 (*FREQ*) genes in predicting response to nicotine replacement therapy for tobacco dependence

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We have previously demonstrated that a functional dopamine D2 receptor promoter variant (*DRD2* −141 *Ins/Del*) predicts response to nicotine replacement therapy (NRT). The present study extends this finding in the same population of 363 NRT-treated subjects, by examining variation in the gene encoding the neuronal calcium sensor-1 protein (*FREQ*), which functions to regulate D2 receptor desensitization. The results indicate a statistically significant interaction effect of *DRD2−141* and *FREQ* genotypes on abstinence at the end of the NRT treatment phase; 62% of the smokers with at least one copy of the *DRD2* −141 *Del* allele and two copies of the *FREQ* rs1054879 A allele were abstinent from smoking, compared to 29–38% abstinence rates for other smokers in the trial. This result suggests that the interaction between variation in the *DRD2* and *FREQ* genes, which both encode components of the D2 dopamine receptor signal transduction pathway, impacts the efficacy of NRT.
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## Introduction

There are six FDA-approved medications for the treatment of nicotine dependence, including the various forms of nicotine replacement therapy (NRT) and bupropion.[1] Yet, the majority of smokers are unable to quit smoking and maintain abstinence with these medications.[2,3] Although a considerable amount of data from both open-label and placebo-controlled clinical trials support the efficacy of NRT and bupropion in the treatment of nicotine dependence, there is a great deal of variability in how patients respond to each of these treatments (reviewed by Lerman *et al.*[4]). Previously, we examined the role of two putative functional variations in the *DRD2* gene, −141 *Ins/Del* and C957T, in the therapeutic outcomes of bupropion and NRT treatment for nicotine dependence.[5] Our findings indicate that smokers homozygous for the *DRD2* −141 *Ins* benefit more from bupropion treatment and persons with *Ins/Del* or *Del/*

*Del* genotypes at the *DRD2*−141 locus respond better to behavioral counseling or NRT.[5] Results from this study and others[6–8] may, in the future, help practitioners maximize the therapeutic benefit of pharmacological treatments for nicotine dependence.

Although most pharmacogenetic studies of the dopaminergic neurotransmitter system in nicotine dependence have focused on genes encoding dopamine G-protein-coupled receptors (GPCRs) or genes encoding dopamine-metabolizing enzymes, dopaminergic neurotransmission is not solely dependent on these components of dopamine-mediated signal transduction pathways. During the past several decades, advancements in proteomic- and yeast two-hybrid-based methods have given rise to a growing list of intracellular GPCR interacting proteins (GIPs) (reviewed by Bockaert *et al*.[9]). Studies show that GIPs regulate key aspects of GPCR signaling, including receptor trafficking and effector coupling.[9] Based on their ability to regulate signaling through dopamine receptors, it has recently been postulated that dopamine receptor interacting proteins (DRIPs) may contribute to the pathology and/or treatment of psychiatric disorders.[10]

One DRIP of particular relevance to the biology of addiction disorders is neuronal calcium sensor-1 (NCS-1). NCS-1 is an evolutionarily conserved EF hand calcium binding protein involved in the regulation of a wide variety of neuronal functions including membrane traffic, cell survival, and ion channel and receptor signaling (reviewed by Burgoyne *et al*.[11]). In mammalian cells, NCS-1 has been shown to promote exocytosis from dense core vesicles of neuroendocrine cells and plays a prominent role in D2 dopamine receptor desensitization via a direct interaction with the receptor.[12] Given the established role for NCS-1 in regulating D2 receptor signaling, these studies present the intriguing possibility that alterations in NCS-1 activity and/or expression may contribute to the dopaminergic pathophysiology of nicotine dependence and response to nicotine replacement medications.

In this brief report, we extend our previous finding that genetic variation in a component of the dopaminergic signaling pathway, the D2 dopamine receptor, may be useful in predicting the response to pharmacological treatments for nicotine dependence. DNA samples from participants in the bupropion and NRT trials were genotyped for two SNPs in the gene encoding the NCS-1 protein (*FREQ*) to determine if variation in genes encoding multiple components of the dopaminergic signal transduction pathway could have additional value in predicting therapeutic response. Here, we report a novel finding from the open-label randomized trial comparing nicotine nasal spray (NS) and transdermal nicotine (TN).

## Results

### *Descriptive data*

Of the 363 participants included in the analyses, 53% were male, 50% were college graduates, and the mean age was 46.7 (s.d. = 11.25) years. On average, participants smoked 24 cigarettes per day (s.d. = 9.33). The mean score on the Fagerström Test for Nicotine Dependence was 5.59 (s.d. = 2.22). For the *FREQ* rs3780715 SNP, 100 (27%) participants were homozygous for the A allele (AA), 181 (50%) were heterozygous (AG), and 82 (23%) were homozygous for the G allele (GG). For the *FREQ* rs1054879 SNP, 111 (31%) participants were homozygous for the A allele (AA), 164 (45%) were heterozygous (AG), and 88 (24%) were homozygous for the G allele (GG). The $D'$ estimate for these two SNPs is 0.22. For the −141C *Ins/Del* SNP, 293 (81%) participants were homozygous for the C allele (CC), 67 (18%) were heterozygous (CN), and three (1%) were homozygous for the N allele (NN).

A total of 176 (48%) participants were randomized to the TN condition and 187 (52%) to the NS condition. There were no significant differences between the treatment groups on genotype or other baseline variables, with the exception of a trend for a sex difference (58% male in TN group, 49% male in NS group; Pearson $\chi^2(1) = 3.54$, $P = 0.06$).

Of the 363 eligible participants, 344 (95%) provided data at end of treatment (EOT) and 347 (96%) provided data at 6-month follow-up. Compared to participants lost to follow-up at EOT, those who provided data at EOT had marginally higher educational levels (Mantel–Haenszel $\chi^2(1) = 3.36$, $P = 0.067$) and were more likely to have two A alleles at the *FREQ* rs3780715 SNP (Mantel–Haenszel $\chi^2(1) = 3.92$, $P = 0.048$). Compared to participants lost to follow-up at 6 months, those who provided data at 6 months were more likely to be in the NS group (Pearson $\chi^2(1) = 4.71$, $P = 0.030$) and to have two A alleles at the *FREQ* rs3780715 SNP (Mantel–Haenszel $\chi^2(1) = 6.03$, $P = 0.014$). There were no other significant differences in genotype or baseline variables by completion status.

### *Treatment outcome*

At EOT, 125 (34%) participants were abstinent (38% of TN group vs 32% of NS group; Pearson $\chi^2(1) = 1.42$, $P = 0.23$). At the 6-month follow-up, 72 (19.8%) were abstinent (19.9% of TN group vs 19.8% of NS group; Pearson $\chi^2(1) = 0.001$, $P = 0.98$). Abstinence rates at EOT by *FREQ* rs3780715 genotype were 40, 34, and 28% among cases with A/A, A/G, and G/G genotypes, respectively (Mantel–Haenszel $\chi^2(1) = 2.85$, $P = 0.092$). For *FREQ* rs1054879, abstinence rates at EOT were 35, 33, and 36% among cases with A/A, A/G, and G/G genotypes (Mantel–Haenszel $\chi^2(1) = 0.02$, $P = 0.89$). Neither *FREQ* SNP was significantly associated with abstinence at 6 months.

A longitudinal logistic regression of abstinence at EOT and 6 months was performed (Table 1). A significant interaction between *FREQ* rs1054879 and *DRD2* −141 *Ins/Del* was found (OR = 2.32, 95% CI = (1.13, 4.79); Z = 2.28, $P = 0.022$). The form of the interaction, based on the bivariate analysis, is illustrated in Figure 1. There was no evidence from the bivariate analysis for an interaction between the *FREQ* rs3780715 SNP and *DRD2*−141. As shown in Table 1, there was a significant effect of time point, with a lower odds of

**Table 1 Longitudinal logistic regression model of abstinence at EOT and 6-month follow-up[a]**

| *Predictor* | *OR (95% CI)* | P |
|---|---|---|
| Sex (female = 1) | 0.75 (0.48, 1.15) | 0.19 |
| Nicotine dependence | 0.94 (0.83, 1.07) | 0.35 |
| Cigarettes per day | 0.99 (0.96, 1.03) | 0.78 |
| Time point (6 months = 1) | 0.40 (0.29, 0.56) | <0.001 |
| Treatment group (NS = 1) | 0.87 (0.41, 1.83) | 0.72 |
| −141C *Ins/Del* (*Ins/Ins* = 1) | 0.31 (0.14, 0.69) | 0.004 |
| *FREQ* rs3780715 | 0.79 (0.54, 1.16) | 0.23 |
| *FREQ* rs1054879 | 0.67 (0.39, 1.32) | 0.25 |
| *FREQ* rs1054879 by −141 *Ins/Del* | 2.32 (1.12, 4.79) | 0.022 |
| Treatment group by *FREQ* rs3780715 | 1.06 (0.47, 2.39) | 0.89 |
| Treatment group by *FREQ* rs1054879 | 0.57 (0.24, 1.36) | 0.21 |
| Treatment group by time point | 1.34 (0.83, 2.16) | 0.23 |

[a]*FREQ* rs3780715 and *FREQ* rs1054879 are represented in the model as number of G alleles: 0 = A/A, 1 = A/G, 2 = G/G.
OR: odds ratio; CI: confidence interval; NS: nasal spray.

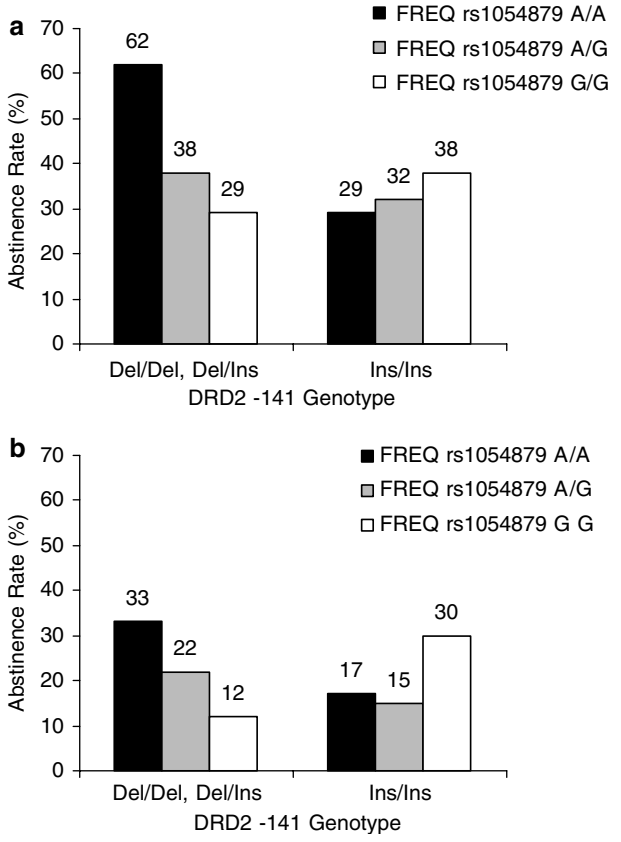


**Figure 1** Abstinence rates by *FREQ* rs1054879 and −141 *Ins/Del* genotypes at (**a**) EOT (*FREQ* rs1054879 in *DRD2 Del/Del, Del/Ins* Mantel–Haenszel $\chi^2(1) = 4.21$, $P = 0.040$, *FREQ* rs1054879 in *DRD2 Ins/Ins* Mantel–Haenszel $\chi^2(1) = 1.46$, $P = 0.23$) and (**b**) 6-month follow-up (*FREQ* rs1054879 in *DRD2 Del/Del, Del/Ins* Mantel–Haenszel $\chi^2(1) = 2.47$, $P = 0.12$, *FREQ* rs1054879 in *DRD2 Ins/Ins* Mantel–Haenszel $\chi^2(1) = 3.74$, $P = 0.053$).

**Table 2 *FREQ* haplotype by *DRD2-141* association with abstinence**

| *Haplotype (N)*[a] | *Time point* | *% abstinent* DRD2−*141* = CC | *OR:* DRD2−*141* = *N/N or C/N vs CC (95% CI)*[b] |
|---|---|---|---|
| 1_1 (239.69) | EOT | 0.35 | 2.78 (1.44–5.39) |
| 1_2 (141.31) | | 0.36 | 0.52 (0.19–1.43) |
| 2_1 (146.31) | | 0.22 | 2.10 (0.88–5.03) |
| 2_2 (198.69) | | 0.34 | 1.27 (0.62–2.58) |
| 1_1 (239.69) | 6 months | 0.16 | 2.60 (1.26–5.36) |
| 1_2 (141.31) | | 0.22 | 0.60 (0.18–1.97) |
| 2_1 (146.31) | | 0.16 | 1.34 (0.47–3.83) |
| 2_2 (198.69) | | 0.23 | 0.73 (0.31–1.77) |

[a]Haplotype is RS3780715_RS1054879; *N* = number of chromosomes.
[b]OR: odds ratio; CI: confidence interval; EOT = end of treatment.

abstinence at 6 months compared to EOT. As previously reported,[5] the main effect of *DRD2* −141 *Ins/Del* was also significant. There was no evidence for interacting effects of either *FREQ* SNP with treatment type. Finally, as previously reported,[5] analyses of 41 random SNPs provided no evidence of population stratification in this sample.

We also examined the interaction of the *FREQ* haplotypes with *DRD2*−141 in determining outcome using log-linear modeling within the program HAPIPF. The model assumed a saturated interaction between the two *FREQ* SNPs, *DRD2*−141 genotype, and outcome. The test was not significant at EOT (likelihood ratio $\chi^2(9) = 15.07$, $P = 0.09$) or at 6 months (likelihood ratio $\chi^2(9) = 8.63$, $P = 0.5$). As shown in Table 2, the *DRD2*−141 association with outcome appears to be modified only by alleles at the *FREQ* 1045879 locus. Therefore, the haplotype does not increase outcome prediction above the logistic model.

## Discussion

The present study examined the association of variation in the *FREQ* gene with therapeutic response to NRT for the treatment of nicotine dependence. Consistent with the role of NCS-1 in modulating dopamine D2 receptor signaling,[12] we found evidence for a statistically significant interaction between a common variant in the *FREQ* gene (rs1054879) and a functional variant in the *DRD2* promoter, *DRD2* −141 *Ins/Del*. This finding builds upon our previous report indicating that smokers with at least one copy of the *DRD2* −141 *Del* allele have higher abstinence rates when treated with NRT,[5] and suggests that this enhanced response to treatment is present only for the subset of smokers who also are homozygous for the A allele of *FREQ* rs1054879; 62% of these smokers were abstinent at EOT compared with abstinence rates of 29–38% among other groups of smokers. This effect is independent of type of NRT and is present (although diminished) at 6-month follow-up, consistent with a pharmacogenetic effect. Because this open-label randomized trial of NRT did not include a placebo arm, it

is not possible to determine whether the observed epistatic genetic effect reflects an enhanced ability to quit smoking or response to NRT.

It has been previously demonstrated that the *Del* allele of the *DRD2*−141 polymorphism reduces the transcriptional activity of the *DRD2* promoter,[13] which is expected to lead to a lower density of D2 receptors at nerve terminals. Although the function of the *FREQ* rs1054879 polymorphism is unknown, the interaction between the D2 dopamine receptor and the NCS-1 protein has been well characterized. *In vitro* studies have shown that NCS-1 attenuates dopamine-induced D2 receptor desensitization and internalization in a calcium-dependent manner.[12] Genetic variation in the *FREQ* gene leading to altered NCS-1 protein levels or function would be expected to alter the rate of D2 receptor desensitization and internalization. Because nicotine stimulates dopamine release from striatal neurons,[14] changes in D2 signaling associated with genetic variation in *FREQ* could modulate liability to relapse and/or response to NRT.

*FREQ* rs1054879 is located in the terminal exon of the *FREQ* gene, which encodes the 3′ untranslated region of the *FREQ* mRNA. Because the function of *FREQ* rs1054879 has not been determined, it is possible that *FREQ* rs1054879 is in tight linkage disequilibrium with a functional SNP in the 3′ end of the *FREQ* gene. To investigate the possibility that a functional SNP in *FREQ* modulates the effects of the *DRD2* −141 *Ins/Del* on smoking cessation treatment outcome, it will be necessary to genotype additional known non-synonymous SNPs and to identify novel SNPs in the *FREQ* gene by sequencing the exons and the intron/exon boundaries in the 3′ end of this gene. In addition, based on the results of this study, it will be important to analyze variation in genes encoding additional DRIPs. One interesting candidate DRIP is the gene encoding G-protein-coupled receptor kinase 2 (*ADRBK1*), because this enzyme has been shown to interact with both NCS-1 and DRD2 proteins[12] and functions to regulate DRD2 receptor desensitization.[15,16]

It should be noted that we also examined whether the *FREQ* SNPs modified the effect of the *DRD2* −141 *Ins/Del* on response to bupropion in a placebo-controlled bupropion trial for smoking cessation.[5] In that trial, we found no evidence for an association of the *FREQ* SNPs with smoking cessation or response to bupropion therapy, or modulation of the *DRD2*−141 effect. There are several possible explanations for the lack of a *FREQ* SNP by *DRD2*−141 interaction in the bupropion trial. First, it is possible that the importance of *FREQ* (and NCS-1) in modulating D2 signaling and smoking cessation is specific to NRT; that is, the presence of nicotine and resulting effects on dopamine release may be a necessary condition for this effect to be observed. Alternatively, the different results across the two studies may be attributable to differences in demographic backgrounds, ethnic ancestry, or ascertainment between the two study populations, or the present result may be a false positive.[17] Clearly, the present results should be validated in an independent trial of NRT, preferably a placebo-controlled trial in which the genotype effects on smoking cessation can be separated from effects on response to NRT.

The results of the present study should be considered hypothesis generating, and a few limitations in study design should be considered. First, the absence of a placebo control arm precludes determination of whether the observed effect reflects response to nicotine therapy or a general smoking cessation ability. A second limitation of this study is the incomplete coverage of haplotype blocks within the *FREQ* gene. Given the promising initial evidence for a *DRD2* by *FREQ* interaction, additional investigation of variation across the *FREQ* gene and identification of potentially functional polymorphisms is warranted. Third, we focused the analysis on smokers of European ancestry in order to limit potential bias due to ethnic admixture. We have shown previously that, in this European ancestry sample, there is no evidence for population substructure that could bias study results.[5] Nonetheless, the use of a homogeneous population prevents us from generalizing the results of this study to nicotine-dependent persons of other ethnicities. To confirm the findings of the present study, additional prospective studies using larger populations of nicotine-dependent subjects from multiple ethnic backgrounds are necessary.

Despite these limitations, the present study revealed an intriguing pharmacogenetic interaction between polymorphisms in two genes encoding components of the D2 dopamine receptor signal transduction pathway, *DRD2* and *FREQ,* in a smoking cessation trial with NRT. This finding underscores the importance of examining variation not only in genes encoding neurotransmitter GPCRs, but also in genes encoding proteins that function to regulate intracellular signaling properties of these receptors, such as DRIPs. The results of this study also suggest that genetic variation in DRIPs or other genes encoding proteins that regulate signaling through dopamine receptors may underlie the inconsistent results for *DRD2* polymorphisms in case–control association studies of the smoking phenotype.[18,19] The signal transduction pathway-based approach taken in this study should serve as a biological rationale for studying how variation in genes encoding DRIPs impacts both the pathophysiology of, and the response to pharmacological treatments for, other psychiatric disorders that are thought to result, at least in part, from perturbations in dopaminergic neurotransmission including schizophrenia, bipolar disorder, and cocaine dependence.

## Materials and methods

### *Participants and procedures*

The present study included 363 current cigarette smokers of European ancestry who were aged $\geqslant$18 years and had smoked at least 10 cigarettes per day for the 12 months prior to study enrollment. These individuals were participants in an open-label randomized clinical trial of two forms of NRT for smoking cessation: TN vs nicotine NS. Exclusion criteria included pregnancy or lactation, uncontrolled hypertension, unstable angina, heart attack or stroke within the past 6 months, current treatment or recent diagnosis of cancer,

drug or alcohol dependence, current diagnosis or history of a DSM-IV Axis I psychiatric disorder, and current use of bupropion or nicotine-containing products other than cigarettes.

The details of subject recruitment as well as the design and the results of the clinical trial have been previously reported.[5,20] Briefly, participants responding to newspaper advertisements were screened for eligibility, completed a pretreatment assessment of demographics and smoking history, and then were randomized to receive either 8 weeks of nicotine NS or 8 weeks of TN patch. Self-report data on smoking status were obtained at EOT and 6-month follow-up, using a validated timeline follow-back method.[21] Participants who reported complete abstinence (not even a puff of a cigarette) for at least the 7 days prior to the assessment were asked to complete an in-person visit for biochemical verification of abstinence using exhaled carbon monoxide (CO) reading (cotinine verification was not used because the treatments contained nicotine).

This study was approved by the Institutional Review Boards at participating institutions and informed consent was obtained from all participants for both the clinical trial and genetic analysis.

*Measures*

*Genotype.* There are no known functional SNPs in the *FREQ* gene. SNPs were selected using the SNPbrowser 2.0 software (Applied Biosystems Inc.). The three SNPs genotyped (rs3780708, rs3780715, rs1054879) all had minor allele frequencies $>0.2$ and spanned the gene. Rs3780708 is in the 5′ end of the first intron, rs3780715 is in the 3′ end of the first intron, and rs1054879 is in exon 8 (the 3′ most exon). There is about 27 kb spanning the first two SNPs and 33 kb between the second and third SNPs (haplotype blocks 1, 2, and 6, respectively). Unfortunately, there were a large number of genotype failures for the rs3780708 SNP due to technical reasons relating to the fidelity of the genotyping assay ($n=48$); therefore, data are presented for rs3780715 and 1054879.

All genotyping was performed using ABI Assays-on-Demand genotyping reagents according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Genotypes were called using the Allelic Discrimination End-Point Analysis function on an ABI Prism® 7300 Sequence Detection System. The accuracy of the *FREQ* genotypes was insured by including 5% duplicate samples on all genotyping plates. All genotype frequencies were analyzed to ensure that there were no significant deviations from those predicted by Hardy–Weinberg equilibrium.

*Covariates.* Sex, education, age, and smoking rate were assessed at baseline. Nicotine dependence was also assessed at baseline using the Fagerström Test for Nicotine Dependence.[22]

*Primary outcome.* Abstinence from smoking was assessed at EOT (8 weeks after the target quit date (TQD)) and at 6 months after the TQD. In accordance with recommendations of the SRNT expert panel,[23,24] participants were classified as abstinent if they self-reported abstinence (not even a puff) for each of the 7 days immediately prior to the follow-up point (i.e., point-prevalence), and provided a CO reading of $\leqslant 10$ p.p.m. Consistent with these recommendations, we presumed that those who failed to complete the follow-up, or failed to provide a CO sample for biochemical verification, had relapsed.

*Statistical analysis*

Descriptive statistics were obtained for all variables in the analyses. $\chi^2$ tests and *t*-tests were used to test differences in baseline variables by treatment assignment and study completion status. $\chi^2$ tests were used to test differences in abstinence rates by treatment group and genotype. We also examined associations of *FREQ* rs3780715 and *FREQ* rs1054879 genotypes with abstinence separately among cases with different −141C *Ins/Del* genotypes, to identify possible gene–gene interaction effects. A longitudinal logistic regression of abstinence at EOT and 6 months was performed, using the general effect estimation procedure (XTGEE) in STATA (STATA Corporation, College Station, TX, USA). The SNP genotypes (*FREQ* rs3780715 and *FREQ* rs1054879) were treated as ordinal variables (i.e., number of G alleles). The model controlled for covariates (smoking history, sex) and included terms for the following two-way interactions, based on the results of bivariate analysis: treatment group by time point; treatment group by *FREQ* genotypes; and *FREQ* rs1054879 genotype by −141 *Ins/Del*.

Analysis of the two-locus haplotype was performed using the HAPIPF routine in STATA.[25,26]

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