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OPRM1 rs1799971 - COMT rs4680 - FAAH rs324420 genes interact with placebo procedures to induce hypoalgesia

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Abstract

Genetics studies on the placebo hypoalgesic effect highlight a promising link between single nucleotide polymorphisms (SNPs) in the dopamine, opioid, and endocannabinoid genes and placebo hypoalgesia. However, epistasis and replication studies are missing. In this study, we expanded upon previous findings related to the three SNPs in the opioid receptor mu subunit (*OPRM1 rs1799971*), catechol-O-methyltransferase (*COMT* rs4680), and fatty acid amide hydrolase (*FAAH* rs324420) genes associated with placebo hypoalgesia and tested the effect of a three-way interaction on placebo hypoalgesia. Using two well-established placebo procedures (verbal suggestion, learning paradigm), we induced significant placebo hypoalgesic effects in 160 healthy participants. We found that individuals with *OPRM1* AA combined with *FAAH* Pro/Pro and those carrying *COMT* met/met together with *FAAH* Pro/Pro showed significant placebo effects. Participants with *COMT* met/val alleles showed significant placebo effects independently of *OPRM1* and *FAAH* allele combinations. Finally, the model that included the placebo procedure and genotypes predicted placebo responsiveness with a higher accuracy (area under the curve, AUC=0.773) as compared to the SNPs alone indicating that genetic variants can only partially

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explain the placebo responder status. Our results suggest that the endogenous mu-opioid system with a larger activation in response to pain in the met/val allele carriers as well as the synergism between endogenous mu-opioid system and cannabinoids might play the most relevant role in driving hypoalgesic responses. Future epistasis studies with larger sample sizes will help us to fully understand the complexity of placebo effects and explain the mechanisms that underlie placebo responsiveness.

Keywords

Epistasis; Expectation; Opioids; Cannabinoids; Dopamine; Pain; Placebo; Verbal suggestion; Learning

Introduction

Placebo effects are complex in their nature and the explored neurobiology suggests that multiple systems may play a role in eliciting placebo hypoalgesia. Success in the endeavor of identifying predictors of placebo hypoalgesia depends largely on the number of genes that may potentially affect placebo susceptibility, the distribution of such allele carriers, and the way alleles and genotypes interacts to determine susceptibility to placebo responsiveness. Recent studies suggest that genetics plays an important role in pain sensitivity [40; 41] and in pain modulation, for example placebo effects (see reviews, [11; 31]). Not all individuals will respond to placebo-induced hypoalgesia. Given that pain responses vary across individuals [40; 41] and are more than 50% heritable according to twin studies [44; 43; 33], it is plausible that specific genetic variations may contribute to placebo responsiveness. In our recent review of the literature, we found three single nucleotide polymorphisms (SNPs) that have been associated with placebo hypoalgesic effects such as those observed in well-controlled laboratory studies (e.g. natural history) performed in healthy participants and patients [11]. Herein we focus on these three SNPs (rs1799971; rs324420; rs4680) located in opioid receptor mu subunit *(OPRM1)*, fatty acid amide hydrolase (*FAAH*) and the catechol-O-methyltransferase (*COMT*) genes, respectively.

The opioid system plays a relevant role in driving placebo effects as demonstrated by pharmacological and imaging studies [35; 2; 23; 21; 50; 49]. Pecina and colleagues first examined the *OPRM1* A118G variant (Asn40Asp, rs1799971) which is thought to be expressed at low levels [64]. The rs4680 polymorphism in *COMT*, which encodes a valine to methionine amino acid substitution at codon 158 (Val158Met), reduces the enzymatic activity of the protein product of this gene by 3–4 fold [36]. This SNP has been associated with better outcomes in patients with Irritable Bowel Syndrome [30] and placebo analgesia in healthy subjects [62]. Specifically, met/met carriers for the *COMT* gene has been shown to have a significant positive association with clinical pain reduction in IBS patients [30] and placebo hypoalgesia in healthy subjects [62]. Interestingly, a recent study addressed the interaction between *OPRM1 rs1799971* and *COMT rs4680* on placebo hypoalgesia and found that participants having the *COMT* met/met or val/met – *OPRM1* A/A carrier combination compared to those with the val/val – G combination, reported level of placebo induced pain reduction that was 4–6 times higher [4].

The cannabinoid system plays a role in placebo hypoalgesia [7; 8] and the functional variant in the fatty acid amide hydrolase (*FAAH*) rs324420 polymorphism encodes a Pro129Thr missense substitution that leads to distinct endocannabinoid levels [9], affects placebo hypoalgesia [47]. We performed a replication of previous findings [63; 30; 48; 47; 4] and conducted a gene-to-gene by placebo procedure interactions study to determine the potential influence of combined genetic variants and psychological approach on hypoalgesic placebo effects. Based on previous studies [63; 30; 48; 47; 4], we hypothesized that larger placebo hypoalgesic effects would have been observed in those individuals with *OPRM1* rs1799971 A/A, *COMT* rs4680 met/met and met/val and *FAAH* rs324420 Pro/Pro compared with other combinations.

Methods

Study population.

We enrolled 162 healthy participants to participate in a pain modulation study. A final sample of 160 genotyped participants was included in the analyses (two had incomplete genotypes). Participants were aged 28.1 ± 7.7 (mean ± S.D.) years, including 58 men, European American (103) and non-European American (57, including 42 African-Americans, 13 Asians and 2 mixed race) as indicated in Table 1 and Fig. S1A–B.

The general health of the participants was determined by clinical examination performed by a physician. A psychiatric interview was also conducted by a trained psychologist using the Structured Clinical Interview for the DSM-IV-TR [1], and participants were asked to self-report medication and nicotine use. Drug use was assessed by urine toxicology analysis. Exclusion criteria included history of psychotropic drug exposure; major medical or neurological illness; use of nicotine, illicit drug use or alcohol abuse within 1 year; lifetime history of alcohol or drug dependence; psychiatric disorders; current pregnancy or breastfeeding; current pain and use of painkillers. Two datasets were included and both were collected by the same experimenter (LC) using the experimental psychological procedure and type of painful stimulations described below. All the experimental procedures were conducted in conformance with the Declaration of Helsinki. The National Institutes of Health (NIH) Institutional Review Board (IRB, White Panel), approved the study where the DNA was extracted and sequenced. The data were analyzed at University of Maryland Baltimore. All participants provided written informed consent to participate in a study including genetic analyses [39; 38]. Given that we used some deceptive elements in the consent form, participants were debriefed at the end of their study participation and were offered the opportunity to withdraw their data from the study. None of the participants chose to withdraw their data from the study.

Type of Painful stimulation

Electrical and heat painful stimulation were used to induce painful sensations. Electrical stimuli were square pulses delivered by a somatosensory stimulator (PsyLab, London, UK), with duration of 100 microseconds. Painful electrical stimuli were delivered to the dorsum of the nondominant hand to induce a moderate level of pain, titrated based on individuals' pain sensitivity. Heat painful stimulations were delivered by means of the Pathway system

(Medoc Advances Medical System, Rimat Yishai, Israel), delivering stimulation starting from 32°C to the highest deliverable temperature of 50°C. A 3×3 cm probe was attached to the nondominant forearm using an elastic bandage to gently ensure it remained stable on the skin. The pain *calibration* started with the assessment of the warm detection (the level of thermal stimulation perceived as warm but not painful) and continued to define the painful tolerance level (the level of thermal stimulation perceived as warm but not painful). A subset of the participants underwent electrical pain (n=144) and there was no effect of type of painful stimulation on placebo hypoalgesia (beta=0.01, p=0.969, Table 1).

Pain induction

Painful stimuli were delivered and titrated based on individual pain sensitivity as previously performed [14; 17; 15; 37]. Briefly, an ascending series of stimuli was delivered starting at a sub-tactile threshold, until painful sensations were induced. Initially, stimuli at a very low and usually imperceptible level were delivered. Next, the investigator increased the intensity of the stimuli in steps until participants reached a threshold, indicated by a level that they felt was “definitely painful, but tolerable”. Floor effects were minimized by asking participants to verbally report their pain on a scale from 1 to 10. If they reported the painful sensation as less than 6, participants were asked if they felt comfortable trying a higher painful intensity, such that pain ratings at the end of calibration were at least 8 out of 10 on a visual analogue scale. Participants were then asked to report by pressing the Pathway remoter button, the level of perceived pain on the Visual Analogue Scale (VAS) ranging from 0=no pain to 10=maximum imaginable pain.

Placebo procedure

The experimental model of placebo procedure has been described elsewhere [14; 16]. Briefly, placebo hypoalgesia was induced by verbal suggestion of analgesia alone or verbal suggestion with learning (conditioning). In the case of verbal suggestion, a total of =36 stimuli were set at twice the pain threshold (2T). The same procedures of verbal suggestion alone or verbal suggestion reinforced by prior experience of painful and nonpainful stimulations was conducted in subgroup of participants with the application of the thermal stimulation. The same sham electrode stimulation was applied, and the same script was used with both stimulations. Participants who were assigned to the learning subgroup (verbal suggestion with learning) received two blocks of 6 painful stimuli set at the level of pain reported tentatively as 8 out 10 that were associated to the red light and 6 tactile stimuli reported as less than 2 out 10 that were associated to the green light. In this way, the green light simulated an analgesic effect. In the third block, all the stimuli were painful (2T). Unbeknownst to the participant, two distinct levels of intensity were used to elicit non-painful sensation when the green light was displayed and a painful paresthesia when the red light was displayed as previously performed and described [14; 17; 15]. Participants were told ‘When the green light is on, there will be an electrical stimulus sent to your middle finger (or forearm) so that you will feel either no pain or less pain. On the other hand, when you see the red light, then the stimulus to the finger is turned off so that you will feel pain’. The instructions were given by the same white-coat dressed experimenter (LC) who entered the room and gently presented the scope of the research. During the test phase in both verbal suggestion alone and verbal suggestion with learning, a total of 18 painful stimulations was

associated with the red light and 18 were associated with the green light. But, the intensity of the painful stimulation was surreptitiously set at the same level and any difference in red-minus green-associated pain reports was operationally defined as 'placebo hypoalgesia' [14; 17; 18; 15; 5; 54]. Participants were assigned to each of the 2 placebo procedures (verbal suggestions/learning) and the 2 types of painful stimulations (electrical/heat induced pain) by entirely deterministic causal process.

Empathy

We used the Interpersonal Reactivity Index [19] as a measurement of dispositional empathy that consists of a set of separate but related constructs. The 28-item, multidimensional questionnaire assesses empathy through four scales to generate the total score that was considered as a potential co-variable influencing placebo hypoalgesia.

Saliva collection and DNA genotyping

Saliva samples were collected using Oragene-DNA (OGR-500) kits. Study participants were invited via consent form to provide an optional sample of saliva for DNA extraction. The day of the experiment participants were asked to rinse their mouth out with water 10 minutes prior to the sample collection and spit saliva in the OGR-500 container following the provided information for the saliva self-collection. The saliva self-collection procedure was non-invasive and the saliva samples were stored at −20 Celsius until DNA was extracted at the Laboratory of Neurogenetics at the National Institute on Alcohol Abuse and Alcoholism. Samples were all genotyped using the Illumina Human OmniExpressexome array. Genotypes were called using GeneMapper software (version 4.0; Applied Biosystems). Data for a panel of 2,500 Ancestry-informative markers (AIMs) was also extracted from the ~1 million markers. Ancestry scores for six continents were generated for each participant using STRUCTURE 2.2 [34] where data for each subject was run individually along with data for the HGDP-CEPH Human Genoma Diversity Cell Line Panel (1050 individuals from 52 worldwide populations, http://www.cephb.fr/en/hgdp_panel.php). Eigenstrat was also run using data from the study cohort to identify any population stratification. We used ancestry markers to carefully control for race on placebo hypoalgesia and to confirm the self-reported race assessed via www.whitehouse.gov/sites/default/files/omb/assets/information_and_regulatory_affairs/re_app-a-update.pdf.

Genotype distribution and Hardy–Weinberg equilibrium (HWE) are presented in Table 2. The genotype distribution of *OPRM1* rs1799971, *COMT* rs4680 or *FAAH* rs324420 did not differ between placebo procedures (Chi-square=1.02, p=0.314 for *OPRM1* rs1799971; Chi-square=4.13, p=0.127 for *COMT* rs4680; Chi-square=0.03, p=0.873 for *FAAH* rs324420).

The three SNPs were tested for HWE using Haploview 4.2 [6] which controls for type I error in relatively small sample size [61]. The results indicated that genotype of *OPRM1* rs1799971, *COMT* rs4680 and *FAAH* rs324420 were in HWE (p=0.230, p=0.237. and p=0.728, respectively). HWE results within each race are also reported (Table 2).

Statistical analyses

The primary outcome was the placebo hypoalgesia operationally defined as the difference between each single red- and green-associated pain report generated from the total of 18 red minus green VAS delta. To explore the predicting effects of demographics and experimental characteristic on placebo hypoalgesia, a multivariate linear regression was performed with age, sex (men vs. women), self-reported race, type of pain stimulus (electrical vs. thermode) and placebo procedure (verbal suggestion vs. learning) as independent variables. AIMs were used to confirm self-reported race and Multivariate Pillai's Trace analysis was conducted to verify that self-reported race significantly predicted AIMs scores (Supplementary Materials, Fig. S1A–B). Since AIMs and self-report race were highly correlated, we use AIMs in the regression model to avoid causing multicollinearity. Demographics and experimental characteristics (namely age and type of placebo procedure) which had significant predicting effects in the multivariate regression were treated as a covariate (age) and fixed factor (placebo procedure) in further analyses.

A linear Mixed Model (LMM) that provides the advantage of dealing with *missing* data [53] was used to test VAS scores for placebo hypoalgesic effects and 18-repeated delta (18 red minus 18 green VAS scores) for each individual were treated as repeated variables.

Although no validated power and sample size methods are available for mixed models study design [28], we used previous studies recommendations [42; 28], to apply power analysis for repeated measure ANOVA. Based on the 2 (*OPRM1* carriers) by 3 (*COMT* carriers) by 2 (*FAAH* carriers) design, we conducted a power calculation with 12 genotype combinations, 36 VAS individual measurements, effect size set at f=0.15 and power (1 beta error probability) set at 0.8 based on these parameters, an n=60 is needed to detect a significant effect (see also Supplementary Materials for further details). G*Power software [22] was used to conducted this calculation.

Outliers in placebo hypoalgesia were identified by using the following Tukey formula: *Upper*=Q3+(2.2*(Q3−Q1); *Lower*=Q1−(2.2*(Q3−Q1)

Q1 and Q 3 equal 25% and 75% percentiles respectively to define upper and lower boundaries.

Results of regression and linear mixed model analyses did not change with the outlier removal and the full dataset was used for the analyses.

Given that this study is based on the 3 SNPs that have been linked to placebo hypoalgesic effects [30; 47; 62; 46; 4], we analyzed *COMT* rs4680 as met/met, val/met and val/val and dichotomized the *OPRM1* rs1799971 genotype into A/A versus G/A + G/G (G carriers), *FAAH* rs324420 was dichotomized into Pro/Pro versus Thr carriers. We calculated the delta value from each red minus green trial (total of 18 trials) and used as dependent variable to conduct a linear mixed models (LMM) analysis. In the LMM, *OPRM1* rs1799971 (A/A versus G carriers), *COMT* rs4680 (met/met versus val/val versus met/val), *FAAH* rs324420 (Pro/Pro vs, Thr carriers) and placebo procedures (verbal suggestion vs. learning) were set as fixed effects with age as a covariate.

Where applicable, we used Bonferroni correction in post hoc comparisons. We calculated Cohen's d to measure effect size of placebo hypoalgesia and compared Cohen's d using Fisher r-t-z transformation.

Next, we used a previous [37] and recently further defined [56] classification of responders versus non-responders that accounts for the within-subject variability of VAS pain levels instead of arbitrarily defining responders by a percentage change or standard deviations in pain ratings. Each study participant was classified based on a permutation test between the red- and green- pain ratings acquired during the test phase for placebo effect. The null hypothesis was generated by randomly resampling 10,000 times the distribution of pain ratings, which provides a large set of possible t-values obtained from the rearrangement of the VAS pain ratings. The overall t-value obtained between red- and green-related pain reports was used to determine if the null hypothesis could be rejected ($p < 0.001$). In the cases where the null hypothesis could not be rejected, the study participant would be stratified as a "Non-Responder". Alternatively, the participant would be stratified as a "Responder" if there was a significant diminution in the pain ratings. This permutation-based approach offers the advantage to account for the variability across pain ratings during the trial-by-trial reports during the placebo test phase.

The provided statistically defined cutoff point (p=0.001) for placebo responsiveness was used to perform a Receiver Operating Characteristics (ROC) analysis for accuracy, with Area Under the Curve (AUC) for identifying likelihoods of predicting the Placebo responder status.

Analyses were conducted using PLINK [52; 10], Haploview [6], STRUCTURE [34], G*Power [22] and SPSS version 24 (SPSS Inc, Chicago, IL, USA) software. For all these analyses, an alpha value of 0.001 was used.

Results

An omnibus analysis indicated that overall participants reported less pain in association with the green light as compared to red associated VAS pain reports using the LMM approach with the 18 red- and 18 green-trials for pain assessment and placebo procedure as fixed effect factor ($F_{1, 5490.59}$=271.995; p<0.001; Cohen's δ: 0.46). To verify that both verbal suggestion and learning were effective, we also conducted separate analyses to confirm the existence of significant placebo effects in each sub-group (Fig. 1A). In the verbal suggestion subgroup, there was a trend towards placebo hypoalgesic effect ($F_{1, 3738.70}$=6.90; p=0.009; Cohen's δ: 0.20). In the learning subgroup, we observed a larger significant hypoalgesic effect ($F_{1, 1690.96}$=398.42; p<0.001; Cohen's δ: 0.96), confirming that two placebo procedures induced significantly different placebo effects ($F_{1,2609.94}$=442.84, $p < 0.001$).

In this cohort of healthy participants, the linear regression indicated that age influenced placebo hypoalgesia with older experiencing less reduction of placebo hypoalgesia (β= −0.20, p=0.001; see Table 1). Race was not associated with placebo hypoalgesia and Multivariate Pillai's Trace analysis indicated that self-reported race significantly predicted AIMs scores (V=2.37, $F_{18, 459}$= 96.30, p <0.001, Fig. S1A, B).

The placebo procedure was conducted with two distinct painful modalities (electrical vs thermal experimental pain) but there were no effects of type of painful stimulation on placebo hypoalgesia (β=0.01, p=0.969). We also found that individual pain sensitivity (level of pain used to test placebo effects) did not influence placebo hypoalgesia (β=−0.05, p=0.522). On the other hand, the placebo procedure affected placebo effects (β=0.65, p<0.001). Therefore, we included placebo procedure when examining the modulatory effect of *OPRM1* rs1799971, *COMT* rs4680, and *FAAH* rs324420 on placebo responsiveness (Fig. 1B).

Main effect and gene-to-gene interaction results

Correcting for age, we conducted a LMM testing for the effect of *OPRM1 rs1799971*, *COMT rs4680*, and *FAAH rs324420* and placebo procedure on the VAS delta differences across trials (18 total delta). Setting the p at 0.001, we found in the overall sample a significant a main effect of *OPRM1* rs1799971 ($F_{1, 2617.15}$= 35.68, p < 0.001) and a trend for *COMT* rs4680 ($F_{1, 2607.84}$= 6.27, p=0.002) but no significant main effect for *FAAH* rs324420 ($F_{1, 2614.45}$= 1.98, p=0.160), (Fig. 2A, Table S1A). As shown in Fig. 2 A–C, we consistently observed that *OPRM1*_AA carriers had significant placebo hypoalgesic effects independently of the placebo procedure (verbal suggestion and learning) with no significant interaction ($F_{1,2601.90}$=2.33, p=0.127). The effect size (ES) for all participants (Cohen's d=0.32) was not significantly different from verbal suggestion subgroup (Cohen's d=0.29; Z=0.117, p=0.453) and learning subgroup (Cohen's d=0.51; Z=0.573, p=0.283).

Moreover, there was a significant interaction between the genetic variants in *OPRM1* rs1799971 and *FAAH* rs324420 ($F_{1, 2613.65}$=14.86, p<0.001), *OPRM1* rs1799971 and *COMT* rs4680 ($F_{2, 2617.11}$=7.19, p=0.001 in line with [4]) but no significant interaction for the genetic variants in *FAAH* rs324420 and *COMT* rs4680 ($F_{2, 2610.86}$=3.68, p=0.025, Table S1B). Interestingly, there was a significant three-way *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 interaction ($F_{2, 2590.45}$=24.80, p<0.001) and a significant four way interaction between the three SNPs and placebo procedure ($F_{1, 2609.80}$=30.25, p<0.001, Table S1C).

We also conducted separate analyses to further explore the effect of the genotypes on placebo hypoalgesia induced by verbal suggestions (Fig. 2B) and learning (Fig. 2C), respectively.

To compare our result to previous studies, we reported the genetic results in the verbal suggestion subgroup despite only a trend was observed for the behavioral data. There was a main effect *OPRM1* rs1799971 ($F_{1, 1816.35}$=19.71, p<0.001) and a trend for *COMT* rs4680 ($F_{2,1816.36}$=4.373, p=0.013) but no significant main effect of *FAAH* rs324420 ($F_{1,1816.35}$=0.25, p=0.620), Fig. 2B. There was also significant interaction between *FAAH* rs324420 and *COMT* rs4680 ($F_{2, 1816.36}$=6.74, p=0.001) and marginal significant interaction for *OPRM1* rs1799971 and *COMT* rs4680 ($F_{2,1816.36}$=4.84, p=0.008) but no significant interaction for *OPRM1* rs1799971 and *FAAH* rs324420 ($F_{1,1816.36}$=0.03, p=0.875). There was a significant three-way *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 interaction ($F_{2,1816.36}$=14.56, p<0.001) but planned post-hoc comparisons did not survive our significance p value (p<0.001, Table 3).

In the learning subgroup when verbal suggestion were reinforced by experience of painful and lower painful stimulations, we found a main effect of *OPRM1* rs1799971 ($F_{1,791.11}$=17.89, p<0.001), a trend for *FAAH* rs324420 ($F_{1,790.48}$=5.77, p=0.017) but no significant main effect of *COMT* rs4680 ($F_{2,784.99}$=2.76, p=0.064). There was a significant interaction for *FAAH* rs324420 and *COMT* rs4680 ($F_{2, 798.39}$=7.92, p<0.001) but only trends for *OPRM1* rs1799971 and *COMT* rs4680 ($F_{2, 791.33}$=3.31, p=0.037) as well as for *OPRM1* rs1799971 and *FAAH* rs324420 ($F_{1,789.33}$=8.66, p=0.003). More importantly, there was a significantly three-way *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 interaction ($F_{1,789.33}$=30.42, p<0.001). Results from planned post-hoc comparisons are presented in Table 3 and discussed below.

Placebo responsiveness profiles

We explored the profile of placebo effects of the entire experimental session across genotype-to-genotype interactions and placebo procedure. The analyses of the red (control) versus green (placebo) time-courses indicated a significant three-way genotype interactions, revealing distinct profiles of placebo effects under the twelve planned genotype-to-genotype comparisons (Fig. 3). The three-way interactions revealed that individuals with *OPRM1* rs1799971 AA combined with *FAAH* rs324420 Pro/Pro and individuals carrying *COMT* rs4680 met/met combined with *FAAH* rs324420 Pro/Pro showed significant placebo effects of similar magnitude (Z=−0.229, p=0.409). Moreover, participants with *COMT* met/val alleles showed significant and similar in magnitude placebo effects that were independent of *OPRM1* rs1799971 and *FAAH* rs324420 allele combinations ($Z_{AA\_Pro/Pro\ versus\ met/val}$ = −0.041, p=0.485, $Z_{met/met\_Pro/Pro\ versus\ met/val}$ = 0.250, p=0.402). Additionally, G_val/val_Thr carries showed significant hypoalgesia comparable in magnitude with the other significant combinations ($Z_{AA\_Pro/Pro\ versus\ G\_val/val\_Thr}$=−0.373, p=0.355; $Z_{met/met\_Pro/Pro\ versus\ G\_val/val\_Thr}$=−0.295, p=0.384; $Z_{met/val\ versus\ G\_val/val\_Thr}$=−0.379, p=0.352).

No genotype-to-genotype combinations reached statistical significance (p<0.001) in the verbal suggestion subgroup (Table 3). On the contrary, for learning subgroup, planned post hoc comparisons revealed significant placebo hypoalgesic effects as reported in Table 3.

Controlling for age, we demonstrated that the *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 with placebo procedure as factors offered in this cohort, the best predictive Value for estimating the placebo responder status (p values<0.001 were used to distinguish placebo responders applying the permutation test on the red- minus- green pain reports for the placebo test phase).

Controlling for age, the AUC values were similar for *OPRM1* rs1799971 (AUC=0.661, 95%CI=0.567 to 0.755), *COMT* rs4680 (AUC=0.665, 95%CI=0.572 to 0.759) and *FAAH* rs324420 (AUC=0.661, 95%CI=0.568 to 0.755). The interaction of *OPRM1* rs1799971 with *COMT* rs4680 (AUC=0.666, 95%CI=0.572 to 0.760) and *FAAH* rs324420 (AUC=0.662, 95%CI=0.568 to 0.755) as well as the *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 three-way interaction (AUC=0.667, 95%CI=0.573 to 0.761) did not show better prediction values. However, the model with 3 SNPs-interaction (*OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420) including procedure had a net advantage in estimating

the placebo responder status (AUC=0.773, 95%CI=0.694 to 0.852, see Fig.4A). Adding a two-way interactions and even three-way interaction improved the AUC values marginally indicating that placebo procedure interact with the genes to predict placebo responsiveness status.

To further emphasize this point, we presented the effects of two- and three-way interactions in the verbal suggestion and leaning subgroups. For verbal suggestion subgroup, when controlling for age, the AUC values were again comparable for *OPRM1* rs1799971 (AUC=0.613, 95%CI=0.472 to 0.755), *COMT* rs4680 (AUC=0.612, 95%CI=0.472 to 0.752) and *FAAH* rs324420 (AUC=0.609, 95%CI=0.469 to 0.750). The *OPRM1* rs1799971 with *COMT* rs4680 (AUC=0.613, 95%CI=0.472 to 0.754) and *FAAH* rs324420 (AUC=0.614, 95%CI=0.474 to 0.753) interactions and the three-way interaction (AUC=0.609, 95%CI=0.470 to 0.749) did not increase the prediction values compared to single SNPs predictions (see Fig.4B).

Similarly, for learning subgroup, after controlling for age, The AUC values for single SNPs (*OPRM1* rs1799971: AUC=0.659, 95%CI=0.509 to 0.809; *COMT* rs4680: AUC=0.657, 95%CI=0.510 to 0.804; *FAAH* rs324420: AUC=0.624, 95%CI=0.467 to 0.781) did not show different prediction. In addition, the interaction of *OPRM1* rs1799971 with *COMT* rs4680 (AUC=0.667, 95%CI=0.524 to 0.881) and *FAAH* rs324420 (AUC=0.604, 95%CI=0.435 to 0.772) as well as the three-way interaction (*OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420: AUC=0.659, 95%CI=0.513 to 0.805) did not improve the prediction values in estimating the placebo responder status (see Fig.4C).

Discussion

The novel aspect of this study is the demonstration that the *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 and placebo procedure interactions were predictive of distinct profiles of placebo responsiveness. Contrary to the hypothesis, we demonstrated that individuals with *OPRM1* rs1799971 AA combined with *FAAH* rs324420 Pro/Pro and individuals carrying *COMT* rs4680 met/met together with *FAAH* rs324420 Pro/Pro showed significant placebo effects. Moreover, participants with *COMT* met/val alleles showed significant placebo effects independently of *OPRM1* rs1799971 and *FAAH* rs324420 allele combinations. Additionally, G_val/val_Thr carriers showed significant hypoalgesia. The magnitude of placebo hypoalgesia in the identified placebo responsiveness profiles did not differ from each other. Finally, the model with *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 and placebo procedure controlling for age, helps predict placebo responsiveness with an accuracy up to 0.773 in this cohort of healthy participants. Thus, it appears that these SNPs, in combination, are predictive, rather than single SNPs within one gene having an association with placebo hypoalgesia.

Age, sex, type of pain stimulation, placebo procedure and levels of empathy may influence placebo hypoalgesia. An inverse correlation between placebo hypoalgesia (conditioned pain modulation [27]) and aging has been previously reported [26] with the elderly potentially experiencing a reduced ability to respond to placebos [60]. More controversial is the role of sex as a factor that seems to influence placebo hypoalgesia inconsistently [45; 55] probably

because of variable gonadal hormone levels and influences between sex of the experimenter and study participants. Heat and longer stimulations tend to induce larger placebo analgesia (longer duration of painful stimuli (>20s) was associated with a Cohens' d=0.96 as opposed to short-term stimuli (d=0.81) [57]. We used two stimulation modalities that were both below 20 sec (100ms and 10sec) and therefore cannot validate the effect of pain duration. However, the nature (electrical vs heat pain) of the painful stimulation did not affect the observed placebo effects. In line with previous studies in healthy participants, we found different effect size of placebo effects when verbal suggestion were reinforced via learning procedure probably because of prior positive therapeutic experiences induced placebo effects through a consolidation of outcome expectancies (for a review, see [12]). Individual level of empathy did not affect placebo hypoalgesia.

Previous studies with genotypes, brain imaging and placebo hypoalgesia have reported significance independently for three distinct SNPs (rs1799971; rs4680; rs324420) located in the *OPRM1*, *COMT* and *FAAH* genes respectively. Put more simply, those participants with a genetic variant in one of these three genes tend to experience larger placebo-induced pain reduction [29; 31]. We demonstrated that adding a two-way interaction and even three-way interaction improved the prediction of placebo effects only marginally rather the gene-to-gene interact with and placebo procedure to predict placebo responsiveness. Patterns of responsiveness were observed in those carrying the *COMT* met/val allele independently of the other allele combinations for *OPRM1* rs179997 and *FAAH* rs324420. Additionally, individuals carrying *OPRM1* AA together with *FAAH* Pro/Pro and those with *COMT* met/met along with *FAAH* Pro/Pro showed significant placebo hypoalgesia (see Fig. 3). These findings might suggest that the endogenous mu-opioid system with a larger activation in response to pain in the met/val allele carriers [65] as well as the synergism between endogenous mu-opioid system and cannabinoids [47] may play the most relevant role in driving hypoalgesic responses in distinct contexts (e.g. different placebo manipulations).

We tested the main effect of *OPRM1 rs1799971* that was significantly linked to placebo reduction induced by verbal suggestion and learning. This is not totally surprising given that *OPRM1* rs1799971polymorphism has been associated with pain modulation in dysmenorrhea women [59] as well as hypnotizability traits [51]. The role of the *COMT* rs4680 in placebo effects is less consistent with both positive findings detecting an association [3; 24] as well as negative findings [30]. Given that we used a restrictive level of significance, we found a trend towards a main effect of *COMT* rs4680 in placebo hypoalgesia (p=0.002) that is in line with previous finding for main effect of *COMT* rs4680 on placebo hypoalgesia (p=0.035, [30] and p=0.008 [62], respectively). Molecular (but not behavioral, average VAS changes [47]) findings provided evidence of μ-opioid-mediated placebo and related changes in brain regions associated with pain, reward-motivated learning and memory processing are modulated by specific genetic variant in the *FAAH* rs324420 gene [47]. These associations between variants in genes encoding related functions further emphasize the need for considering gene-to-gene interaction. Our results expanded upon a recent published article that illustrated a significant *two-way* interaction between *OPRM1* rs1799971 and *COMT* rs4680 in association the learned placebo analgesia [4].

One of the goals of a genetic approach to placebo research is to identify genes with specific DNA variations that increase or decrease one's susceptibility to respond to treatments and placebo procedures. Previous research based on one SNP reported that *OPRM1* rs1799971 AA homozygotes compared with G carriers, showed greater mu-opioid and dopamine release in the NAc. Also, AA carriers showed lower scores in the NEO-depression and NEO-vulnerability facets of the NEO-Neuroticism domain [46]. For *COMT* rs4680 those with the met/met had a trend towards greater placebo hypoalgesic effects [62] and in the case of IBS patients [30] they benefitted most from the supportive doctor-patient relationship. Pro/Pro *FAAH* rs324420 carriers larger molecular changes and improved mood [47]. The one study with the *OPRM1* rs1799971 and *COMT* rs4680 interactions indicated that participants with *OPRM1* A/A carriers combined with *COMT* met/met and val/met reported significant pain relief after given placebo, whereas those with other combinations of the *OPRM1* rs1799971 and *COMT* rs4680 genotypes displayed no significant placebo effect [4]. Adding to previous findings [4], we found that *COMT* rs4680 val/val mutation, which was considered being not response to placebo manipulation, showed significant hypoalgesia if carrying *OPRM1* AA together with *FAAH* Pro/Pro or *OPRM1* G together with *FAAH* Thr allele combinations. The same team reported that *COMT* rs4680 genotype has an impact on fear of medical pain with Met-allele carriers reporting higher fear of medical pain compared with the Val-allele but observed no effect of the *COMT* rs4680 genotype on placebo analgesia [25].

We used permutation tests to generate responders and nonresponses and estimate the probability to predict placebo effects. The area under the curve values indicated that the single SNPs predicted placebo effects (*OPRM1* rs1799971, AUC =0.661; *COMT* rs4680, AUC = 0.665; *FAAH* rs324420, AUC=0.661). However, the *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 with placebo procedure as factors offered the best predictive value for estimating placebo responders (AUC =0.773) over the two-way interactions (*OPRM1* by *COMT* =0.666; and *OPRM1* by *FAAH* =0.661) and the three-way *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 interaction (AUC=0.667).

Overall, we demonstrated that the combined *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 approach provides an advantage in predicting those who respond to placebo as compared to the single SNP or the two-way SNP interaction and psychological procedure controlling for age is critical to improve predictability accuracy.

Caution is also warranted when translating this knowledge to a chronic pain patient population. For instance, a few recent studies have indicated that specific genotype or combination of genotypes (such as *COMT* haplotypes) could become a useful tool in identifying those patients who are likely to benefit from a specific treatment and predict clinical outcomes (e.g. pain reduction, improved quality of life, patient satisfaction) in IBS and Temporomandibular Disorder patients [20; 30; 32]. It is plausible to think that reward mechanisms and the dopaminergic system assume a distinct value in chronic pain as compared to healthy controls when we study placebo hypoalgesia. Additionally, there are some limitations to be considered. The difficulty in balancing type I error with type II error is a key issue in association studies of placebo analgesia with laboratory well-controlled small sample size. Herein, we conducted a replication study of previous published SNPs that

the function has been reported in relationship to placebo hypoalgesia [63; 30; 48; 47; 4]. Although larger replication studies including a second cohort are needed, the approach we proposed here, can serve as a model for future genetic epistasis-based studies. Despite the small and diverse sample size, this study helps not only replicate previous published studies and also expand our knowledge about the possibility that multiple systems and therefore genetic variants, might play a role in placebo responsiveness [13].

Research on genes that influence placebo hypoalgesia is nascent but the potential to inform and guide more effective, personalized and, mechanistic-based therapeutics, is promising. The use of specific combination of SNPs within *OPRM1* rs1799971, *COMT* rs4680 and *FAAH* rs324420 genes along with the careful evaluation of the procedure used to induce placebo effects is inexpensive and such information may be integrated into clinical practice for assessing the individual's propensity to benefit from the endogenous modulatory systems and placebo responsiveness. Moreover, SNP-related information may help separate drug and placebo effects [58] in larger randomized clinical trials with the potential to predict individual responses to treatments (and placebos). Future research needs to combine genetic influence with encounter factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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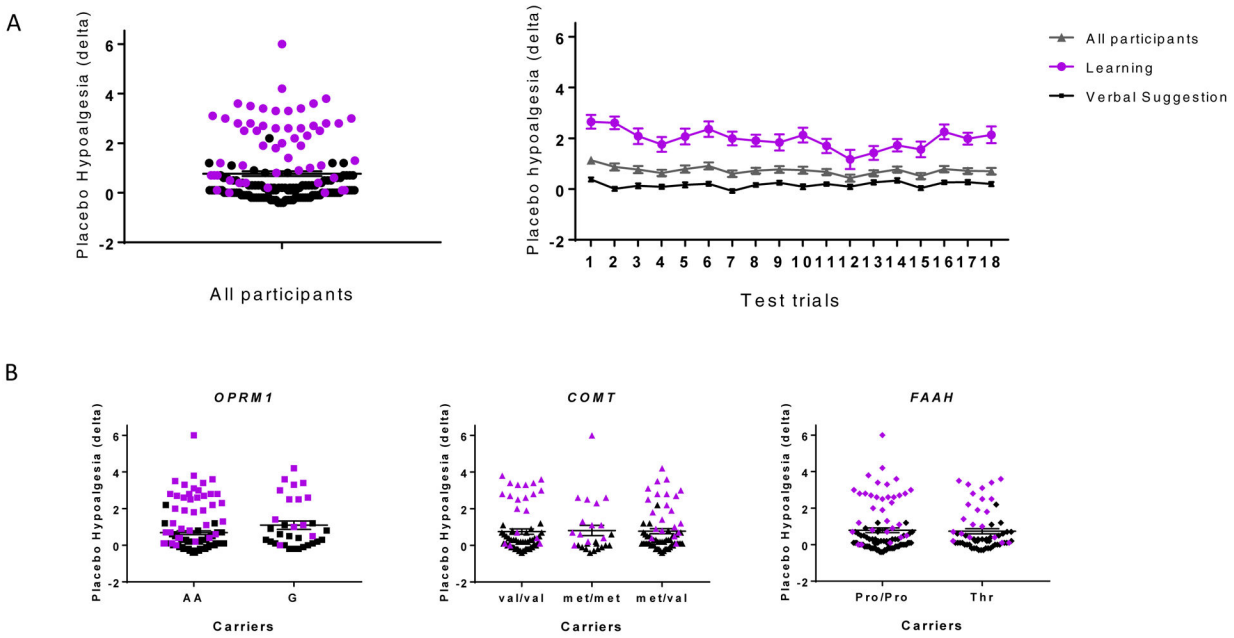

Figure 1.

Distribution of behavioral and genetic placebo hypoalgesic effects. Individual pain reduction delta scores (red- minus green trials) and time-courses are presented for the overall group verbal suggestion,and learning subgroups (A). Moreover, distribution of genotypes and placebo hypoanalgesic effects are shown (B) Data are presented as mean ± sem.

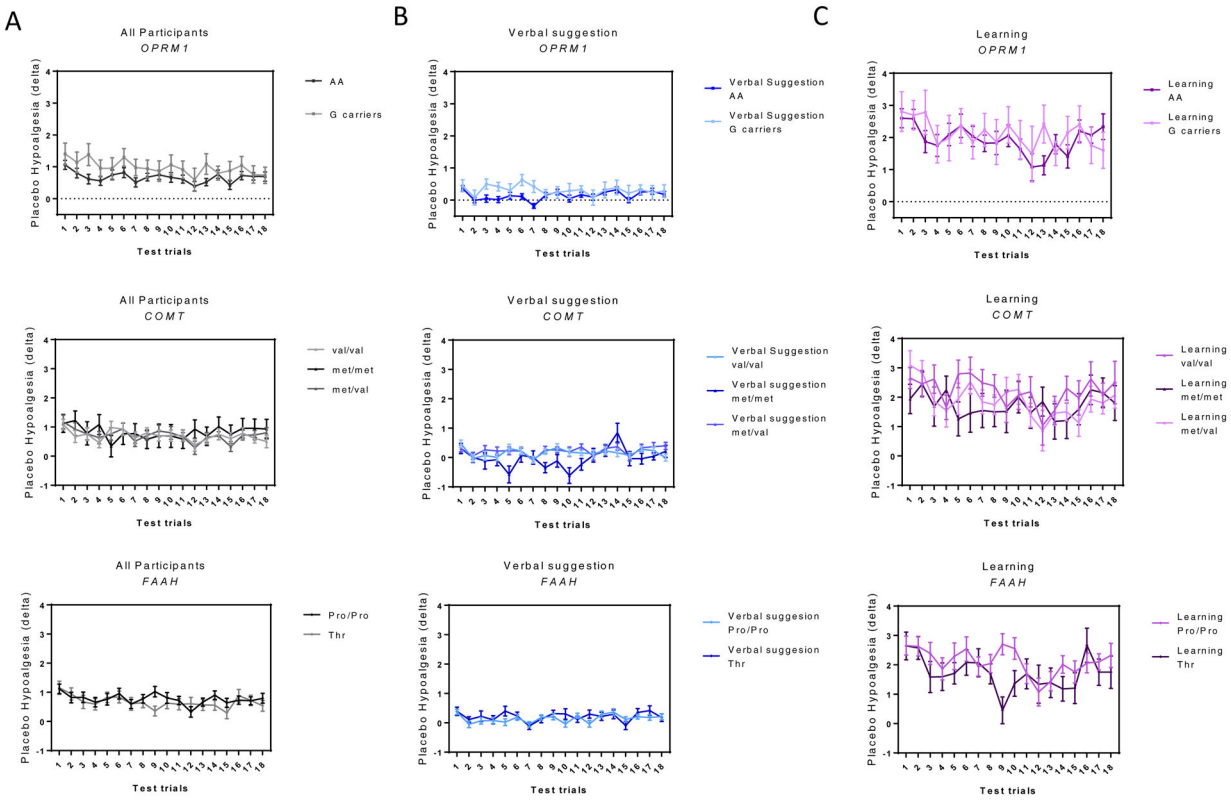

Figure 2.

Single SNP-placebo associations. Single SNP-placebo association for *OPRM1* rs1799971, *COMT* rs4680, and *FAAH* rs324420 are shown for the overall group(A), verbal suggestion (B) and learning (C). Data are presented as time-courses with test trial-by-trial mean ± sem.

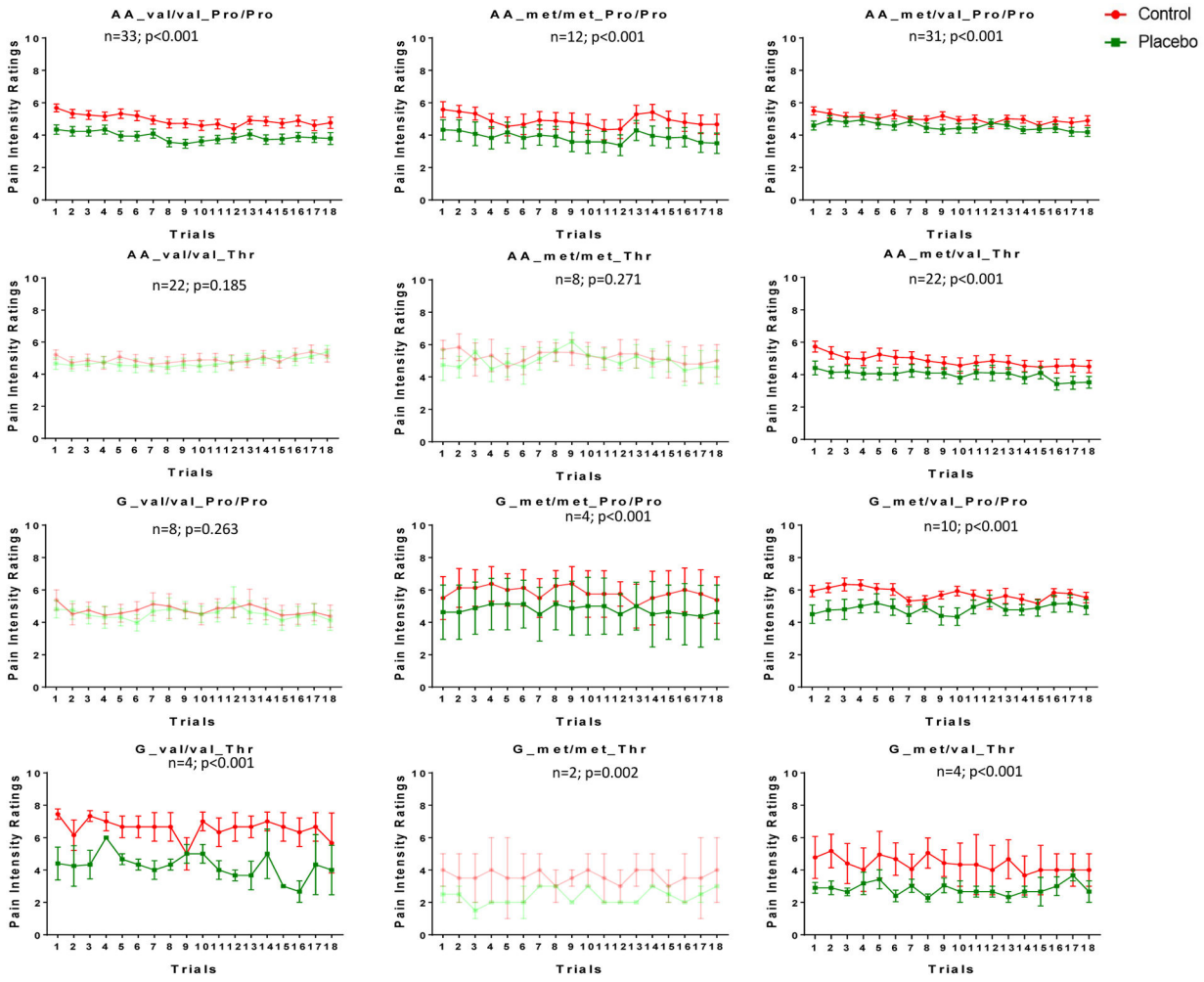

Figure 3.

3-way SNP-placebo associations. The *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 interaction orchestrated distinct profiles of placebo responsiveness although their effect sizes were not significantly different. Statistically, significant placebo hypoalgesia was observed in participants characterized by specific combinations of genotypes (bold lines, AA_val/val_Pro/Pro, AA_met/met_Pro/Pro, AA_met/val_Pro/Pro, AA_met/val_Thr, G_met/met_Pro/Pro, G_met/val_Pro/Pro, G_val/val_Thr, and G_met/val_Thr). Conversely, no significant placebo effects were seen in other participants with AA_val/val_Thr, AA_met/met_Thr, G_val/val_Pro/Pro and G_met/met_Thr (transparent lines).

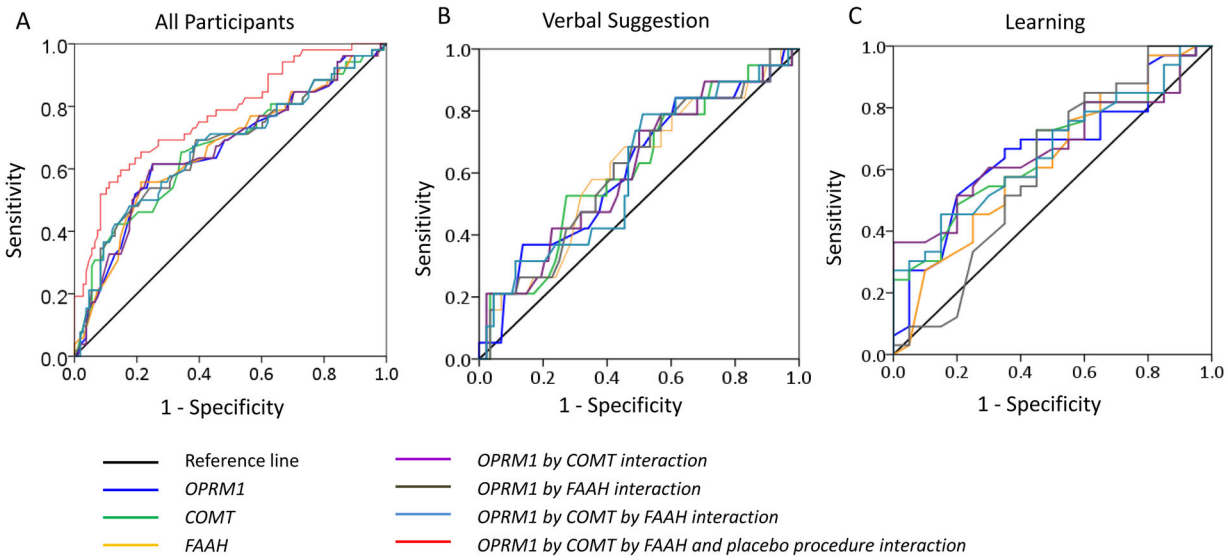

Figure 4. ROC curves.

Receiver Operating Characteristic (*ROC*) *curves are presented with* the true positive rate (*Sensitivity*) plotted in function of the false positive rate (1-*Specificity*) for the different cut-off points. Each point on the *ROC curves* represents the *sensitivity*/*specificity* pair corresponding to a particular decision threshold. The area under the ROC curve (AUC) is the measure of how well a parameter can distinguish predictors of placebo responder status. The ROC curves are presented for the overall group (A), verbal suggestion (B) and learning (C). Controlling for age, the *OPRM1* rs1799971 by *COMT* rs4680 by *FAAH* rs324420 by placebo procedure interaction offered the best predictive value for estimating placebo responders (placebo responder status based on the statistical cut-off as per individual permutation test).

Table 1.

Linear regression exploring the predicting factors on placebo hypoalgesia

	Mean (N)	SD (%)	Standard beta	t	p
Sex (M/F)	58/102	36.3%	0.03	0.48	0.635
Age	28.51	7.69	−0.18***	−3.04	**0.003**
Ancestry-informative markers[a]					
African	0.22	0.36	−0.04	−0.68	0.500
Asian	0.03	0.07	0.04	0.59	0.554
Fast East Asian	0.06	0.20	0.02	0.24	0.812
Oceania	0.01	0.02	0.07	1.19	0.236
America	0.02	0.06	−0.09	−1.53	0.130
Type of pain stimulation (Electricity vs. Heat)	144	90%	0.02	0.29	0.772
Placebo Procedure (Verbal suggestion vs. Learning)	107	66.9%	0.65***	7.49	**0.000**
Pain Sensitivity	94.52	66.88	−0.03	−0.38	0.702
Empathy	90.15	12.57	−0.01	−0.21	0.833

[a]Ancestry-informative markers (AIMs) Europe was excluded in the linear regression model to avoid multi-collinearity problem as AIMs Europe was highly correlated with AIMs African (r=−0.82, p<0.001).

Table 2.

Hardy–Weinberg equilibrium tests

Gene	Chromosome	SNP	Position	Genotype	n	HWE tests for each race[b]					
						White n=103		African American n=42		Asian n=13	
						MAF	p-value	MAF	p-value	MAF	p-value
OPRM1	6	rs1799971	154039662	AA	128	0.131	1.000	0.036	0.072	0.231	1.000
				AG/GG	32						
COMT	22	rs4680	19963748	Val/Val	67	0.442	0.541	0.214	1.000	0.308	1.000
				Met/Val	67						
				Met/Met	26						
FAAH	1	rs324420	46405089	Pro/Pro	98	0.199	0.730	0.298	1.000	0.115	1.000
				Pro/Thr, Thr/Thr	62						

[a] MAF = Minor Allele Frequency

[b] Two participants reported mixed race

Table 3.

Placebo Hypoalgesia within genotype combinations across placebo procedure.

Placebo Procedure	*OPRM1*	*COMT*	*FAAH*	Delta (red minus green)	SEM	p	95% CI	
							Lower Bound	Upper Bound
Verbal suggestion	AA	Val/Val	Pro/Pro	0.20	0.12	0.084	−0.03	0.43
			Thr carriers	0.05	0.13	0.705	−0.20	0.30
		Met/Met	Pro/Pro	−0.05	0.20	0.822	−0.45	0.35
			Thr carriers	−0.10	0.27	0.712	−0.63	0.43
		Met/Val	Pro/Pro	0.06	0.12	0.622	−0.17	0.28
			Thr carriers	0.43	0.14	0.002	0.16	0.69
	G carriers	Val/Val	Pro/Pro	0.22	0.19	0.240	−0.15	0.60
			Thr carriers	1.15	0.54	0.033	0.09	2.21
		Met/Met	Pro/Pro	0.53	0.54	0.330	−0.53	1.58
			Thr carriers	0.01	0.54	0.985	−1.05	1.07
		Met/Val	Pro/Pro	0.34	0.20	0.096	−0.06	0.74
			Thr carriers	0.21	0.54	0.695	−0.85	1.27
Learning	AA	Val/Val	Pro/Pro	2.57***	0.16	**0.000**	2.26	2.88
			Thr carriers	0.75	0.28	0.007	0.20	1.30
		Met/Met	Pro/Pro	2.56***	0.24	**0.000**	2.09	3.04
			Thr carriers	0.63	0.30	0.036	0.04	1.21
		Met/Val	Pro/Pro	1.51***	0.18	**0.000**	1.15	1.87
			Thr carriers	2.23***	0.23	**0.000**	1.77	2.68
	G carriers	Val/Val	Pro/Pro	-	-	-	-	-
			Thr carriers	2.91***	0.37	**0.000**	2.19	3.64
		Met/Met	Pro/Pro	1.22***	0.31	**0.000**	0.61	1.83
			Thr carriers	2.50***	0.54	**0.000**	1.44	3.56
		Met/Val	Pro/Pro	2.44***	0.33	**0.000**	1.80	3.08
			Thr carriers	2.13***	0.34	**0.000**	1.47	2.80