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Genetic variation in *P2RX7* and pain tolerance

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Abstract

P2X7 is a nonselective cation channel activated by extracellular ATP. P2X7 activation contributes to the proinflammatory response to injury or bacterial invasion and mediates apoptosis. Recently, P2X7 function has been linked to chronic inflammatory and neuropathic pain. P2X7 may contribute to pain modulation both by effects on peripheral tissue injury underlying clinical pain states, and through alterations in central nervous system processing, as suggested by animal models. To further test its role in pain sensitivity, we examined whether variation within the *P2RX7* gene, which encodes the P2X7 receptor, was associated with experimentally induced pain in human patients. Experimental pain was assessed in Tromsø 6, a longitudinal and cross-sectional population-based study (N = 3016), and the BrePainGen cohort, consisting of patients who underwent breast cancer surgery (N = 831). For both cohorts, experimental pain intensity and tolerance were assessed with the cold-pressor test. In addition, multisite chronic pain was assessed in Tromsø 6 and pain intensity 1 week after surgery was assessed in BrePainGen. We tested whether the single-nucleotide polymorphism rs7958311, previously implicated in clinical pain, was associated with experimental and clinical pain phenotypes. In addition, we examined effects of single-nucleotide polymorphisms rs208294 and rs208296, for which previous results have been equivocal. Rs7958311 was associated with experimental pain intensity in the meta-analysis of both cohorts. Significant associations were also found for multisite pain and postoperative pain. Our results strengthen the existing evidence and suggest that P2X7 and genetic variation in the *P2RX7*-gene may be involved in the modulation of human pain sensitivity.

Keywords: P2X7, Polymorphism, SNP, Experimental pain, Cold-pressor test

1. Introduction

P2X7 is a purinoceptor and nonselective cation channel activated by extracellular ATP. Activation increases permeability of small cations such as Ca^{2+} or K^+, but with prolonged activation, P2X7 can switch to pore function, allowing passage of larger molecules. This leads to activation of intracellular cascades and causes several functional consequences including maturation and release of excitatory and inflammatory mediators.[2,15,19,20,29,34,37,38,58] Signaling molecules accumulate in the extracellular space and the cerebrospinal fluid and induce gliogenic long-term potentiation of the nociceptive system at C-fiber synapses.[38] P2X7 activation and consequent cellular events contribute to the proinflammatory response to injury and mediate apoptosis.[30] It has been implicated in physiological and pathological conditions, including bone tissue homeostasis and remodeling,[26,36] inflammation,[3,11,17] oncogenesis,[17,51] neurodegenerative and neuropsychiatric diseases,[8] and in chronic inflammatory and neuropathic pain.[13]

P2X7 is expressed in peripheral and central nervous systems and the immune system, which mediate and modulate pain (gene atlas, http://biogps.org). P2X7 has been shown to be upregulated in both dorsal root ganglia and injured nerves,[13] and monocytes and lymphocytes[44] in patients with neuropathic pain. In animals, *p2rx7* disruption reduces hypersensitivity in neuropathic and inflammatory pain models.[13] Also, in inbred mouse strains, loss-of-function (LOF) variants are associated with reduced nociceptive sensitivity, a finding which has been attributed to impaired pore function.[54] Finally, P2X7 expression correlates with nociceptive hypersensitivity, whereas pharmacological blockade of P2X7 reduces allodynia and hyperalgesia in inflammatory and neuropathic pain models.[29,59]

Human *P2RX7* variants can change protein expression or functionality such as receptor trafficking,[61] ATP binding,[28] channel function or pore formation,[27,54] leading to LOF, and gain-of-function (GOF) phenotypes.[12,46,57] Several functional variants have been identified including GOF variants such as rs208294 (H155Y) and LOF variants such as rs7958311 (R270H). Human *P2RX7* variants have been associated with pain in

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osteoarthritis, painful diabetic neuropathy, and postoperative pain.[33,54,60] Although the involvement of P2X7 in neuroinflammatory processes which contribute to tissue pathology and clinical pain has been well shown, it is not known whether the *P2RX7* variants influence pain processing directly. Only 1 recent study has addressed pain sensitivity in a standardized setting. It showed association between *P2RX7* haplotypes and experimental pain sensitivity in a cohort of 100 patients.[33] However, the study failed to show which variants were responsible for the effect. Among the numerous studied *P2RX7* variants, the association of rs7958311 with clinical pain has been replicated and shown to be robust.[54] The remaining single-nucleotide polymorphisms (SNPs) assessed in the context of pain have mostly shown inconsistent results and/or did not survive multiple comparison corrections.

Our primary aim was to extend previous findings[54] and to characterize the effects of the *P2RX7* variant rs7958311 on experimental pain sensitivity in 2 independent cohorts. We hypothesized that, in line with the earlier findings,[54] LOF SNP rs7958311 (R270H) reduces experimental pain. Our secondary aim was to characterize the rs7958311 in additional clinical pain phenotypes—multisite chronic pain and postoperative pain. Finally, because the reported effects of SNPs rs208294 and rs208296 on pain have been inconsistent,[54,60] we included them in the analysis as exploratory targets.

2. Methods

2.1. Patients and study subjects

The study included a Norwegian and a Finnish cohort. An overview of the respective sample selections is given in Figure S1 (Supplementary Digital Material, available online at http://links.lww.com/PAIN/A547).

The Norwegian cohort was drawn from the longitudinal and cross-sectional population-based study of the population of the Tromsø municipality of Northern Norway, the Tromsø Study. In the sixth wave of the study (Tromsø 6), conducted in 2007 to 2008, 19,762 residents aged 30 years or older were invited, of whom 12,984 participated in the study. Genotyping was performed on all subjects aged 40 to 52 years, leaving a sample of 3462. After the removal of 377 first and second degree relatives and 18 cases with failed genotyping, 3067 subjects remained. Of these, complete experimental pain data were available for 3016 subjects who were included in statistical analysis.

The Finnish cohort (BrePainGen) consisted of 1000 patients who underwent breast cancer surgery. Of the 1536 eligible consecutive patients with unilateral nonmetastasized breast cancer enrolled for surgical treatment at the Unit for Breast Surgery at the Women's Hospital, Helsinki University Hospital, between August 2006 and December 2010, 1149 patients were invited to participate in the study. Thousand patients aged 18 to 75 years agreed to participate and provided a written informed consent. Nine hundred ninety-six patients were genotyped. Experimental pain data were assessed in 900 patients of whom 831 remained after the genotype quality control. The specific criteria for exclusion were metastasized cancer, immediate breast reconstruction during surgery, and contraindications due to the anesthesia protocol (for detailed cohort description, see Ref. 35).

2.2. Ethics

2.2.1. BrePainGen

Coordinating ethics committee (136/E0/2006) and the ethics committee of the Department of Surgery (Dnro 148/E6/05) of the Hospital District of Helsinki and Uusimaa (HUS) approved the research protocol. A research nurse or a physician acquired a written informed consent from each subject participating in the study.

2.2.2. Tromsø 6

The study protocol was approved by the Data Inspectorate of Norway and the Regional Committee of Medical and Health Research Ethics, South-East Norway (2010/722). The Tromsø Study complies with the guidelines of the Declaration of Helsinki, and each participant provided a written informed consent before participation.

2.3. Pain assessment

2.3.1. Experimental pain

For both cohorts, experimental pain was assessed with the cold-pressor test providing estimates of cold pain intensity (CPI) and tolerance. In the cold-pressor test, pain was induced by immersion of the hand and wrist into circulating cold (3°C) water. The intensity of pain (CPI) was assessed with a numeric rating scale (NRS, 0-10) ranging from "No pain" (0) to "Most intense pain imaginable" (10). In BrePainGen, the cold-pressor task was performed before surgery and CPI was recorded every 15 seconds until withdrawal or until the cutoff time (90 seconds) was reached. In Tromsø 6, CPI was assessed at 4 seconds and every 9 seconds thereafter until withdrawal or until the cutoff time of 106 seconds was reached (eg, 12 times). Cold withdrawal times (CWTs) were recorded.

2.3.2. Clinical pain

To assess the clinical relevance of *P2RX7* variants, SNPs which showed association with experimental pain in the meta-analysis were assessed in clinical pain phenotypes in both the BrePainGen and Tromsø cohorts. In the BrePainGen cohort, postoperative pain was assessed using NRS (0-10). Pain in the breast, arm, or axilla on the operated site was assessed twice daily for 7 days after surgery. For each day, the most intense pain occurring at either of these sites was determined and the resulting 7 ratings were used as a phenotype in linear mixed-model analysis with time and genotype as independent variables. In the Tromsø 6 cohort, chronic multisite pain was assessed by questionnaire. Multisite pain was defined as pain with intensity of at least 4/10 on NRS, lasting at least for 3 months, and occurring daily at 4 or more body sites out of a total of 15. Participants fulfilling the phenotype criteria were considered as cases and the rest of the cohort as controls. Genotypes were compared using logistic regression.

2.4. Genetic analysis and genotypes

Three SNPs in the *P2RX7* gene were assessed in both cohorts: rs7958311 (R270H; primary aim), and rs208294 (H155Y) and rs208296 (exploratory targets).

2.4.1. BrePainGen

DNA was extracted from peripheral blood using the Autopure LS automated DNA purification instrument (Gentra Systems, Inc, Minneapolis, MN). Genotype data were produced at the Wellcome Trust Sanger Institute (Hinxton, United Kingdom) using the Human OmniExpress Illumina BeadChip (Illumina, Inc, San

Diego, CA). Genotyping was performed blinded to phenotypic information. Of the 996 samples sent for genotyping, 47 failed because of low quality of the DNA or other technical issues. The remaining 949 samples were subjected to quality control procedures consisting of several per-individual and per-marker steps. In the per-individual quality control, 19 subjects were excluded because of unexpected relatedness (first and second degree relatives, identity-by-descent [IBD] >0.2; n = 5) or heterozygosity (n = 14). In addition, after the creation and assessment of multidimensional scaling plots, 4 subjects were identified as outliers based on dimensions 9 and 10. In the per-marker quality control, the SNPs were filtered based on minor allele frequency (MAF > 0.005), Hardy–Weinberg equilibrium (HWE $P > 1 \times 10^{-6}$), and success rate (0.97). After the quality control, 926 individuals remained and their mean genotyping success rate was 0.997. Of these patients, 831 had complete pain data and were included in the statistical analysis.

2.4.2. Tromsø 6

In the Tromsø Study, variants were genotyped using Infinium HumanCoreExome BeadChip platform. Several quality control steps were taken to ensure genotype quality. For all genotyped variants, MAF and HWE were assessed together with genotyping call rate and IBD from genome-wide association data (see Ref. 24 for details). Individuals with a high proportion of missing genotypes (>5%) and related individuals (first and second degree relatives, IBD >0.2) were excluded from the data set. All included markers had a high success rate (>95%) and sufficient MAF (>1%). For rs208296, genotypes were imputed using 1000 Genomes Project imputation reference panel for mixed population (Phase I, 3rd version, March 2012)[23] and IMPUTE2 software.[31]

2.5. Statistical analysis

The primary outcome variables were CWT and the area under the time—CPI curve (CPI AUC%). Cold pain intensity values that were missing after CWT were recoded as 10 (maximum). Individual missing values before CWT were estimated by the average of adjacent data points, weighted by the distance in time to the data point being estimated. For this purpose, a dummy data point at time point 0 second with the value of NRS = 0 was created in cases where the first rating was missing. Area under the curve (AUC) was determined using the trapezoidal rule for the whole duration of the cold-pressor test (BrePainGen, 0-90 seconds; Tromsø, 0-106 seconds) and expressed as percentage from the maximal possible AUC (AUC%).

Associations between genetic variants and pain phenotypes were assessed using SNPTEST (v2.3.0; https://mathgen.stats.ox.ac.uk/genetics_software/snptest/snptest.html), PLINK (v1.07),[50] SPSS (IBM Corp Released 2013, IBM SPSS Statistics for Windows, Version 22.0; IBM Corp, Armonk, NY), R software, and ProbABEL (v0.4.4).[4] Cold withdrawal times were analyzed with Cox proportional hazard models (Cox regression). Cold withdrawal time was used as a time factor and individuals completing the full time (BrePainGen, 90 seconds; Tromsø, 106 seconds) were treated as censored cases. Cold pain intensity AUC% was analyzed using linear regression. Additive genetic models were applied for both linear and Cox regressions. The additive genetic model presumes that in the studied phenotype, genotype effect is *r* for heterozygous variant carriers who have only 1 copy of the minor allele, 2*r* for homozygous carriers (who have 2 copies), and 0 for noncarriers.[42] Including heterozygous variant carriers in the analysis increases the number of subjects contributing to the genetic effect (AA + AG, n = 1088 in total) and decreases the risk of false-positive findings. For Tromsø 6, analysis was performed for each sex separately. In the follow-up analysis, significant associations were verified by 2-way analysis of variance where time and genotype were entered as independent factors and CPI as the dependent variable. The effect of sex was assessed in the Tromsø data set in the full sample. In the figures, pain intensity results are presented as mean ± SEM of n observations. Cold withdrawal time is shown as a Kaplan–Meier plot with the cumulative proportion of cold withdrawal plotted on the y-axis and time on the x-axis.

In all tests, empirical $P < 0.05$ was used as a limit of statistical significance. In the results, empirical *P* values are reported in the text and in the tables, together with corresponding β values (linear regression) or hazard ratios (HRs; Cox regression). The β values and HRs reflect the magnitude and direction of the genotype effect in each analysis, with β values <0 and HRs <1.0 indicating that minor allele carriers report less pain (CPI) and tolerate the test longer (CWT) than noncarriers. A meta-analysis of females from both cohorts was performed using the fixed effect model.

2.5.1. Clinical pain

The intensity of pain during the first postoperative week (BrePainGen) was analysed using linear mixed model analysis with time and genotype as independent factors. The Dunnett test was used for post hoc comparisons. In the Tromsø 6 cohort, chronic multisite pain was analyzed using logistic regression (additive model).

3. Results

3.1. Descriptive

The demographic characteristics of the study subjects are shown in **Table 1** and Table S1 (Supplementary Digital Material, http://links.lww.com/PAIN/A547). The mean age of the subjects was lower in the Tromsø cohort which consisted of both sexes, whereas in the BrePainGen cohort, patients were older and females only. The performance of the 2 cohorts in the cold pressure test is shown in **Figure 1**.

Minor allele frequencies, observed heterozygosity, and genotype counts of the studied SNPs are presented in **Table 2**. The SNPs did not deviate from the HWE and minor allele frequencies of genotyped variants varied 0.23 to 0.42, thus fulfilling the predetermined MAF criterion (MAF > 0.01) For the imputed SNP (rs208296, Tromsø data set), imputation quality was excellent in both sexes (infoscore: 0.908 and 0.905 for males and females, respectively). Linkage disequilibrium (LD) patterns of the study subjects are shown in **Table 3**.

Table 1
Demographics.

	Tromsø (m)	Tromsø (f)	BrePainGen (f)
n	1387	1629	831
Age (yr)	44.7 ± 3.9	44.6 ± 3.9	57.0 ± 9.3
Weight (kg)	87.3 ± 13.6	71.7 ± 13.7	69.4 ± 4.3
BMI (kg/m^2)	27.2 ± 3.8	26.2 ± 4.8	25.4 ± 4.3
Chronic pain	28.5%	37.4%	24%

Data are summarized as mean ± SD.
BMI, body mass index; f, female; m, male.

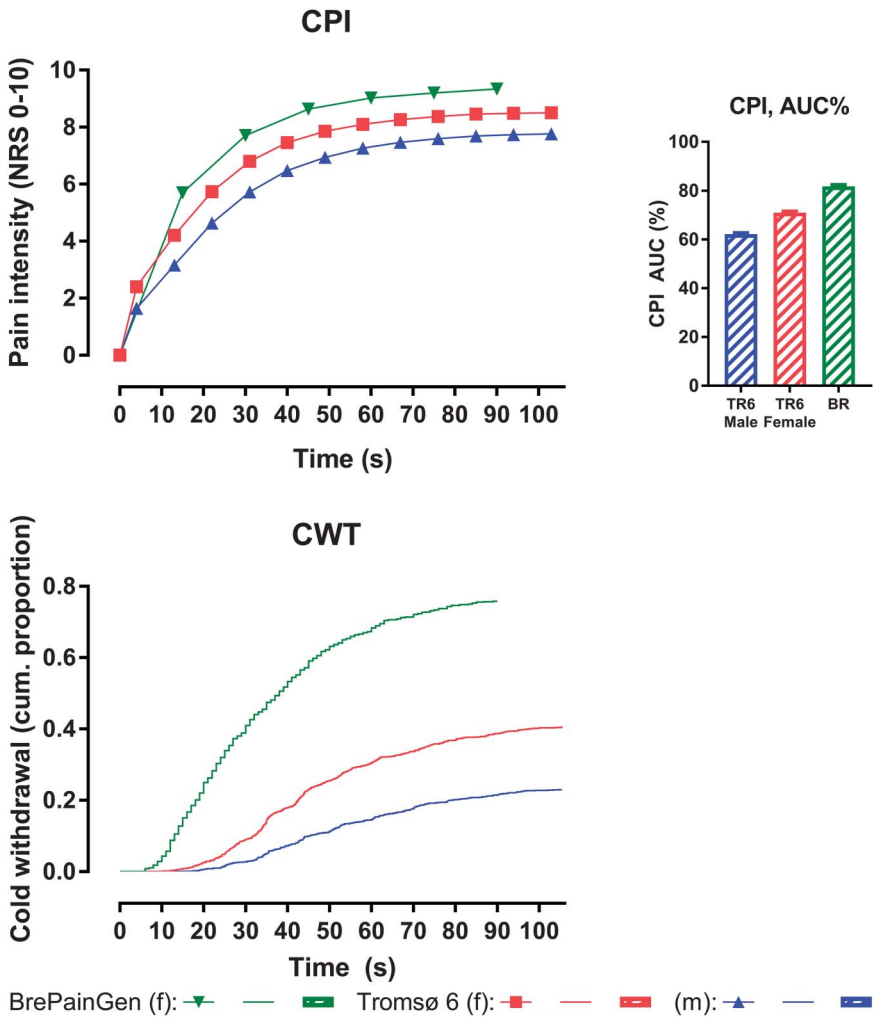

Figure 1. Pain intensity and tolerance in the cold-pressor task in the Tromsø 6 and BrePainGen cohorts. Upper left panel shows the time vs cold pain intensity (CPI) curve during the cold-pressor task. Cold pain intensity (±SEM) is plotted on the y-axis and time on the x-axis. Area under the time-CPI curve (CPI AUC% + SEM) is shown on the upper right panel. Cold withdrawal times (CWTs) are shown in the lower panel as a Kaplan–Meier plot with the cumulative proportion of cold withdrawal plotted on y-axis and time on x-axis. BR, BrePainGen; f, female; m, male; NRS, numerical rating scale; TR6, Tromsø 6.

3.2. Effects of rs7958311 on cold pain intensity and cold withdrawal time

3.2.1. Cold pain intensity: area under the time-pain intensity curve (%)

In the *BrePainGen* cohort, SNP rs7958311 showed significant association with CPI and reduced the area under the time-pain intensity curve (linear regression, additive model, $P = 0.01$; **Table 4** and **Fig. 2**). Although the association did not reach the level of statistical significance in *Tromsø6* (linear regression, additive model; $P > 0.05$ for all comparisons; **Table 4** and **Fig. 2**), it was significant in the meta-analysis of the 2 cohorts (fixed effect model, $P = 0.006$; **Fig. 3A**). The minor allele of rs7958311 reduced cold pain and heterogeneity was not significant. Direction of the effect was the same as reported in the literature.[54]

Table 2

Genetic variants, minor allele frequencies, and observed heterozygosity in the BrePainGen and Tromsø data sets.

	SNP	Source	a	A	aa	aA	AA	OBS HET	MAF
Tromsø (m, n = 1387)	rs7958311	*g*	A	G	95	537	755	0.39	0.26
	rs208296	*i*	A	G	151.06	572.71	663.23	0.41	0.32
	rs208294	*g*	A	G	240	668	479	0.48	0.41
Tromsø (f, n = 1629)	rs7958311	*g*	A	G	120	618	891	0.38	0.26
	rs208296	*i*	A	G	171.74	697.81	759.45	0.43	0.32
	rs208294	*g*	A	G	298	763	568	0.47	0.42
BrePainGen (f, n = 831)	rs7958311	*g*	A	G	37	313	481	0.38	0.23
	rs208296	*g*	A	G	99	349	383	0.42	0.33
	rs208294	*g*	A	G	98	395	338	0.48	0.36

Assessed single-nucleotide polymorphisms (SNPs) are referred to as their reference SNP cluster IDs (rsids). a, minor allele; A, major allele; f, female; g, genotyped; i, imputed; m, male; MAF, minor allele frequency; OBS HET, observed heterozygosity.

Table 3

Linkage disequilibrium.

SNP 1	SNP 2	Tromsø 6		BrePainGen	
		D′	r^2	*D′*	r^2
rs208294	rs208296	0.731	0.175	0.715	0.138
rs208294	rs7958311	0.286	0.021	0.284	0.013
rs208296	rs7958311	0.665	0.317	0.578	0.207

Table shows pairwise linkage disequilibrium measures between the studied SNPs in Tromsø and BrePainGen data sets.
D′, D-prime; r^2, correlation coefficient; SNP, single-nucleotide polymorphism.

3.2.2. Follow-up analysis of individual pain ratings

In the follow-up analysis of rs7958311 and CPI, similar effects were observed in both the *BrePainGen* and *Tromsø6* data sets. There was a significant effect of genotype and time but no interaction, suggesting that the effect of genotype was consistent across time points (2-way analysis of variance, genotype effect $P < 0.0001$ and time effect $P < 0.0001$ for both data sets; **Fig. 2**). In the post hoc comparisons (the Dunnett multiple comparison test), homozygous minor allele carriers (AA) differed from the main allele homozygotes (GG) (the Dunnett multiple comparison test, GG vs AA, adjusted $P = 0.0001$ for both data sets). There were no differences between heterozygotes and main allele homozygotes (GG vs AG, $P =$ ns), suggesting a possible nonadditive effect.

3.2.3. Cold withdrawal time

Association between CWT and SNP rs7958311 was not significant in either of the cohorts (Cox regression, additive model; $P > 0.05$ for all comparisons; **Table 4** and **Fig. 2**). In the meta-analysis of both cohorts, the signal was strengthened, but failed to reach the level of statistical significance (fixed effect model, $P = 0.059$; **Fig. 3B**). Kaplan–Meier curves seemed to indicate that minor allele homozygotes differ from those of main allele carriers, suggesting a possible nonadditive effect (**Figs. 2G–I**).

3.2.4. Sex effect

In the Tromsø data set, the effect of sex and gene-by-sex interactions were tested using PLINK software. There was a significant effect of sex when AUC% was analyzed in the whole sample ($P = 6.66E\text{-}33$, $\beta = -4.521$; **Fig. 1**) and the subsequent analyses were performed for each sex separately. Gene-by-sex interactions were not significant ($P > 0.05$).

3.3. Effects of rs7958311 on clinical pain

3.3.1. BrePainGen

In the BrePainGen cohort, associations between rs7958311 variants and postoperative pain during the first postoperative week were assessed. There was a significant effect of genotype ($P = 0.0046$) and time ($P < 0.0001$) but no interaction ($P > 0.05$). The post hoc analysis showed that the effect was driven by minor allele homozygotes (AA) who reported lower pain intensity compared with the main allele homozygotes (GG) (the Dunnett test for multiple comparisons, AA vs GG: $P = 0.027$). Heterozygous carriers did not differ from main allele homozygotes (the Dunnett test for multiple comparisons, AG vs GG: $P = 0.98$) (**Fig. 4A**).

3.3.2. Tromsø 6

In the Tromsø 6 data set, associations between rs7958311 variants and chronic multisite pain were assessed. Minor allele carriers were less likely to have multisite pain and MAF was lower in multisite pain patients (21%) compared with the controls (27%) ($P = 0.047$, $\beta = -0.27 \pm 0.14$; odds ratio = 0.75, confidence interval 95 = 0.57-0.99) (**Fig. 4B**).

3.4. Exploratory targets: effects of single-nucleotide polymorphisms rs208296 and rs208294

Single-nucleotide polymorphism rs208294 was not associated with CPI AUC% or CWT in the BrePainGen, Tromsø 6, or meta-analysis ($P > 0.05$ for all comparisons; Supplementary Digital Material, Figures S3 and S4, available online at http://links.lww.com/PAIN/A547). In the BrePainGen cohort, SNP rs208296 was associated with cold-pressor phenotypes reducing CPI AUC% and prolonging CWT (CPI AUC%, linear regression, additive model, $P = 0.02$; CWT, Cox regression, additive model; $P = 0.04$; Supplementary Digital Material, Table S2 and Figure S2, available online at http://links.lww.com/PAIN/A547). These associations did not replicate in the Tromsø 6 data set or in the meta-analysis ($P > 0.05$ for all comparisons; Supplementary Digital Material, Table S2, Figures S2 and S4, available online at http://links.lww.com/PAIN/A547).

4. Discussion

We found that carriers of minor allele of *P2RX7* LOF SNP rs7958311 (R270H) report less pain during the cold-pressor test, in line with our initial hypothesis and previous findings.[54] Furthermore, we found similar effects for 2 clinical phenotypes, multisite chronic pain, and postoperative pain.

Table 4

Single-nucleotide polymorphism rs7958311 and pain in the cold-pressor task in the BrePainGen and Tromsø data sets.

	CWT		CPI, AUC%	
	HR (95% CI)	*P*	β (β SE)	*P*
Tromsø (m, n = 1387)	0.98 (0.82-1.17)	0.84	−0.01 (0.88)	0.99
Tromsø (f, n = 1629)	0.92 (0.81-1.10)	0.16	−1.3 (0.81)	0.11
BrePainGen (f, n = 831)	0.81 (0.6-1.08)	0.15	−3.01 (1.18)	0.01
Meta-analysis (ff, n = 2460)	0.895 (0.77-1.02)	0.059	−1.83 (0.55)	0.006

Cold pain was assessed using the cold-pressor test. Cold withdrawal time was used as an indicator of pain tolerance and CPI was assessed using CPI AUC%. Cold withdrawal time was analyzed using Cox proportional hazards model and CPI AUC% with linear regression. An additive genetic model was assumed in the CWT and CPI AUC% analyses. Meta-analysis of females from both cohorts was performed using the fixed effect model.
CI, confidence interval; CPI, cold pain intensity; CPI AUC%, area under the time-CPI curve; CWT, cold withdrawal time; HR, hazard ratio.

4.1. Effect of rs7958311

The direction of the effect was the same as reported earlier.[54] Although minor allele carriers reported reduced pain intensity during the cold-pressor test, differences in pain tolerance were not significant. This could indicate that rs7958311 or P2X7-function is more important for pain intensity than tolerance. On the other hand, in Tromsø 6, the majority of participants reached the cutoff time, whereas differences between the genotypes were assessed mainly based on the most sensitive fraction of the study subjects who discontinued the test. As rs7958311 minor allele plays a protective role in pain, this can also explain the lack of effect in CWT. There were also clear sex differences in the Tromsø 6 data set. Although no gene-by-sex interactions were found, this may be due to the lack of statistical power because genotype differences were only observed in females. The magnitude of effect was in line with effect sizes reported in literature for clinical pain.[54] In fact, the effect was even more pronounced in our study (meta-analysis of CPI AUC %, $\beta = -1.83$) compared with the post-mastectomy pain cohort described by Sorge et al.,[54] ($\beta = -1.19$) especially considering the differences between used scales (NRS 0-10 vs NRS 0-20). Nevertheless, the effect sizes observed in our study were modest, possibly because rs7958311 only produces partial LOF, and P2X7 can be replaced by other P2X-family subunits, such as P2X4, resulting in functional compensation. Finally, pain is a multigenic trait and the effects of *P2RX7* may be limited by redundancies in the pain network.[63] To confirm the clinical relevance of the rs7958311 effects, we assessed its effects in clinical pain phenotypes. Clinical pain states correlate with experimental pain outcomes, although variation is usually larger, requiring a stronger signal or bigger sample sizes.[25,35,48,52,55,62] Also, the pain modulatory system may play an important role in clinical pain because of longer duration of stimuli and the involvement of tissue damage and psychological influences. Clinical phenotypes, however, provide a more holistic and clinically relevant picture of the effects and their clinical significance. The effects of rs7958311 were similar in clinical and experimental pain phenotypes, which confirms their clinical relevance.

4.2. Mechanisms and site of action

The main effects of the rs7958311 genotype were seen in pain intensity. Cold pain intensity AUC% is affected by both the primary afferent drive, the role of which may be the most prominent at the beginning of the task, and pain-modulatory mechanisms which are more likely to activate at a later stage. The lack of time-interaction in the post hoc test suggests that the genotype effect was already present at the beginning of the test. P2X7 expression in the nervous and immune systems, involved in modulation of pain (gene atlas, http://biogps.org), and upregulation in nerves and immune cells in patients with neuropathic pain suggest at least 3 potential sites of action: peripheral immune cells, central immune cells, and neurons.[13,44]

P2X7 induces maturation and release of proinflammatory mediators from spinal and peripheral immune cells,[1,6,18,40,53] in a manner modulated by genetic polymorphisms.[56] As increased proinflammatory mediator levels and inflammation have been linked to both clinical and experimental pain,[5,44,52] their attenuation provides a plausible and explanatorily sufficient mechanism for rs7958311.

As P2RX7 is expressed in several brain areas and is associated with neuropsychiatric phenotypes, supraspinal mechanisms should also be considered.[7,43,45] Modulation of gene expression can mediate SNP effects and reveal potential site of action. In previously published in vitro assays, both rs208294 and rs7958311 have shown effects consistent with and potentially contributing to their functional profiles (GOF and LOF, respectively).[57,60] These effects were dependent on other co-occurring variants. The GTEx database showed eQTL associations between rs7958311 and P2RX7 expression in human tissues, most significant for the brain (hippocampus, $P = 0.015$, $\beta = -0.17$), although the effect was not sufficient to survive multiple comparison corrections. Interestingly, rs7958311 decreased the expression of P2RX4 in whole blood ($P = 1.4e\text{-}7$, $\beta = -0.23$) and fibroblasts ($P = 0.000059$, $\beta = -0.17$) and these effects remained significant after accounting for multiple comparisons (https://gtexportal.org). Thus, rs7958311 is involved in the regulation of P2RX4 expression as a cis-eQTL. An association was detected because of the close proximity of *P2RX4* and *P2RX7* genes which are structurally related, have overlapping functions, form heteromers, and interact functionally.[16] Although the mechanisms and physiological relevance of regulation of P2RX4 expression by rs7958311 remain to be revealed, they are likely to emphasize direct P2RX7-mediated effects: as P2RX4 compensates for the lack of P2X7 function, its downregulation by rs7958311 could provide cumulative or synergistic effect.

4.3. Limitations of the study

Despite several advantages (clearly defined phenotypes, large and homogenous cohorts, and 2 independent samples), our study also has limitations that can impact the effect of rs7958311. Cold withdrawal time data were right-censored, particularly in the Tromsø 6 data set, resulting in loss of statistical power. The additive genetic model of regression was applied, which might not be the most sensitive or powerful approach.[41,42,47] Homozygous rs7958311 minor allele carriers differed from the main allele homozygotes in the follow-up CPI analysis and based on the shape of Kaplan–Meier CWT curves. Heterozygous carriers, although representing a larger group and having more weight in the statistical analysis, did not differ, suggesting that both minor allele copies are required for the effect and thus a recessive model could be a better fit with the data. Although our sample size is quite large for the experimental pain field, it is still modest compared with larger genome-wide association studies. Increasing the sample size could increase statistical power.

4.4. Limiting factors

Several other factors can limit the observed effects of rs7958311. As P2X7 is involved in pathogen defense and other important processes, the mutations resulting in total LOF phenotype are likely to be rare and compensated by co-occurring GOF variants. At least 16 nonsynonymous *P2RX7* variants with MAF $> 1\%$ have been identified and can be considered as a mechanism for genetic compensation. On the other hand, other functional genetic variants in LD with rs7958311 can also contribute to its effect. Although rs7958311 modulates P2X7 function,[54] the design of our study does not allow us to exclude the possible involvement of other variants from the list of potential mechanisms. From the genetic perspective, the scope of our study was limited to 3 SNPs and considering the presence of other functional variants could increase the effect size or identify variants responsible for the effect. Although we did not see an independent effect for the GOF SNP rs208294 and the effect of rs208296 did not survive in the meta-analysis, it is possible that rs208294 and rs208296 could modulate the effect of rs7958311

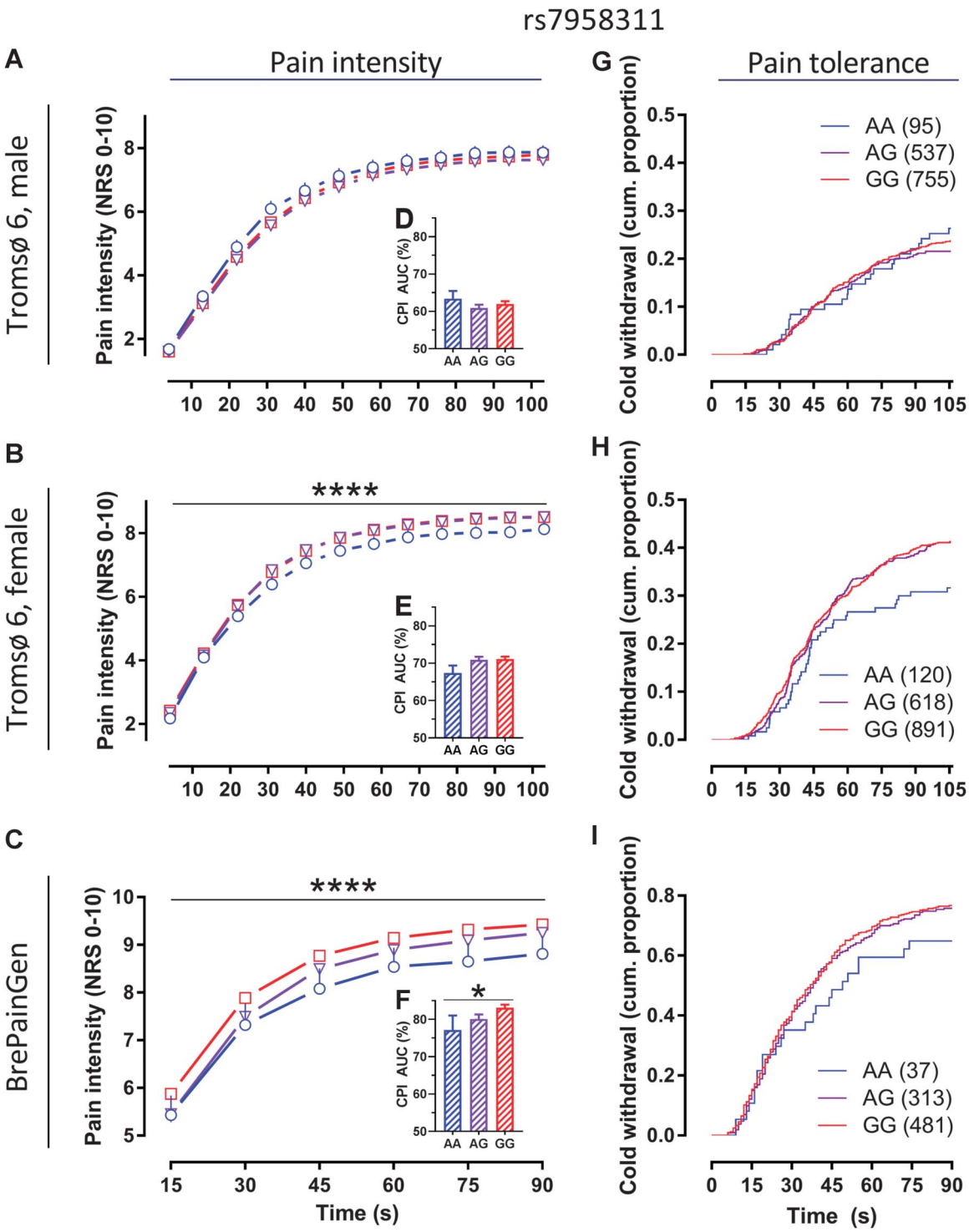

Figure 2. Human *P2RX7* variants and cold-pressor pain in the Tromsø and BrePainGen data sets: SNP rs7958311. Experimental pain was assessed using the cold-pressor task. Cold pain intensity (CPI) was assessed using the numeric rating scale (NRS 0-10). Cold pain intensity values missing after CWT were recoded as 10 (maximum). Time-CPI curves of the different genotypes are shown (A–C) and CPI (±SEM) is plotted on y-axis and time on x-axis. Areas under the time-CPI curves (AUC%) were calculated by trapezoidal rule and are shown as genotype mean ± SEM (D–F). Withdrawal time (CWT; G–I) was used as an indicator of pain tolerance. Cold withdrawal time was analyzed using an additive genetic model and Cox proportional hazards model. The duration of the cold-pressor task is plotted on x-axis. The y-axis shows the cumulative proportion of cold withdrawal. Numbers in parentheses indicate n for each genotype group. Asterisks indicate the statistical significance of the genotype effects (*$P < 0.05$, linear regression, additive model; ****$P < 0.0001$, effect of genotype, 2-way ANOVA). ANOVA, analysis of variance; CWT, cold withdrawal time.

or other *P2RX7* variants. *P2RX7* function is modulated by other genes involved in the same biological pathway, such as enzymes synthesizing or eliminating receptor ligands, and receptors sharing similar functions. The P2X4 receptor is related to P2X7, also expressed in microglia, capable to form pores, and potentiated by lipopolysaccharide.[9] ENTPD inactivates ATP, limiting its effects on P2X7. Interestingly, ENTPD is a negative regulator of P2X7 effects on cellular level[39] and its genetic

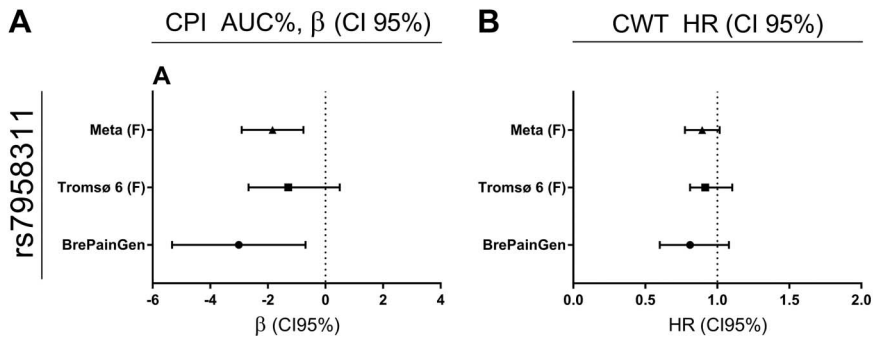

Figure 3. Meta-analysis of *P2RX7* rs7958311 variants and pain in the cold-pressor task in the BrePainGen and Tromsø data sets. Experimental cold pain was assessed using the cold-pressor task. Cold withdrawal time (CWT) was analyzed using an additive genetic model and Cox proportional hazards model (Cox regression). Pain intensity (CPI) was assessed using numeric rating scale (NRS 0-10). Area under the time-CPI curve (CPI AUC%) was calculated as an indicator of pain intensity and analyzed using an additive genetic model and linear regression. The figure shows associations between CPI AUC% (A) and CWT (B) and SNP rs7958311 in the BrePainGen and Tromsø 6 cohorts as well as in the meta-analysis (fixed effect model, females). CI, confidence interval; CPI, cold pain intensity; HR, hazard ratio.

variation has been linked to diabetic neuropathy and inflammatory bowel disease.[21,22] Genetic factors are not the only determinants of protein function. The *P2RX7* gene contains binding sites for several regulatory factors, providing potential for interactions and modulation (miRTarBase).[14,32] Because of alternatively spliced isoforms (n ≥ 9; UniProtKB and Swiss-Prot database)[10] with different exon structures, SNPs can be present in different molecular contexts or be lacking in some spliced variants. Finally, P2X7 is regulated by posttranslational processing. ADP-ribosylation is necessary for channel activation and gating, palmitoylation for localization to cell surface, and phosphorylation for regulation of the activation state.

4.5. Rationale and clinical significance

The P2X7 receptor is of clinical importance, and several pharmaceutical companies have launched drug-discovery campaigns and claimed patents for drugs modulating P2X7 receptors.[49] Identification of clinically important and relevant genetic variants may help to stratify patients and identify groups with higher risk and groups who are more likely to benefit from treatment. In addition, it provides insights into different functional aspects of the receptor and their clinical relevance. The effects of rs7958311 on clinical pain phenotypes confirm their clinical relevance. As the effects occur in 2 distinct clinical paradigms, these effects may be broad and pertain to pain more generally, rather than in condition-specific manner. This was further supported by rs7958311 effects in experimental pain, which suggest that its effects are not restricted to pain elicited by tissue injury. Further studies are required to assess whether these differences in clinical pain we see for rs7958311 would translate into different treatment requirements.

5. Conclusions

Our results support the previous findings suggesting the involvement of P2X7 and genetic variation in the *P2RX7*-gene,

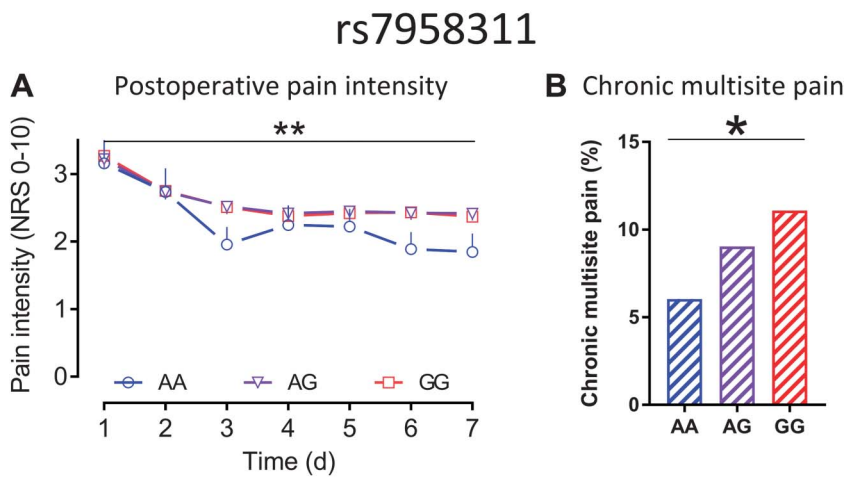

Figure 4. Human *P2RX7* variant SNP rs7958311 reduces the intensity and incidence of clinical pain. Postoperative pain intensity was assessed in the BrePainGen cohort using the numeric rating scale (NRS 0-10) (A). Worst pain occurring at any site was recorded daily for 7 days after breast cancer surgery and used as the phenotype. Pain intensity was analysed using 2-way analysis of variances with time and genotype as independent variables (genotypes: AA, n = 45; AG, n = 351, GG, n = 527). In the figure, pain intensity is shown as genotype mean ± SEM. Asterisks indicate the effect of genotype (*$P < 0.05$, logistic regression; **$P < 0.005$, 2-way ANOVA). Chronic multisite pain was assessed in the Tromsø 6 cohort (B). Multisite pain was defined as pain with intensity of at least 4 on the numeric rating scale (NRS) which lasted at least for 3 months occurring daily at 4 or more body sites out of a total of 15. Patients fulfilling the phenotype criteria were considered as cases and the rest of the cohort as controls. Genotypes were compared using logistic regression (additive model; genotypes: AA, n = 116; AG, n = 620, GG, n = 902). Prevalence of chronic multisite pain in different genotypes is shown in panel (B). ANOVA, analysis of variance.

particularly SNP rs7958311, in the modulation of human pain sensitivity. Our results suggest that the effects of *P2RX7* variants may be generalized across different experimental and clinical pain phenotypes. The magnitude of the effect, however, was modest and more studies are needed to confirm and elucidate the clinical impact of *P2RX7* variation on clinically relevant outcomes and analgesic requirements.

Conflict of interest statement

The authors have no conflict of interest to declare.

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Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A547.

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