BRAIN
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Genome-wide analysis identifies impaired axonogenesis in chronic overlapping pain conditions

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Chronic pain is often present at more than one anatomical location, leading to chronic overlapping pain conditions. Whether chronic overlapping pain conditions represent a distinct pathophysiology from the occurrence of pain at only one site is unknown. Using genome-wide approaches, we compared genetic determinants of chronic single-site versus multisite pain in the UK Biobank. We found that different genetic signals underlie chronic single-site and multisite pain with much stronger genetic contributions for the latter. Among 23 loci associated with multisite pain, nine loci replicated in the HUNT cohort, with the DCC netrin 1 receptor (*DCC*) as the top gene. Functional genomics identified axonogenesis in brain tissues as the major contributing pathway to chronic multisite pain. Finally, multimodal structural brain imaging analysis showed that *DCC* is most strongly expressed in subcortical limbic regions and is associated with alterations in the uncinate fasciculus microstructure, suggesting that DCC-dependent axonogenesis may contribute to chronic overlapping pain conditions via corticolimbic circuits.

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Abbreviations: COPC = chronic overlapping pain conditions; GO = Gene Ontology; GWAS = genome-wide association studies; LDSC = LD score regression; OD = orientation dispersion; PRS = polygenic risk score; SNP = single nucleotide polymorphisms; UF = uncinate fasciculus

Introduction

Chronic pain is a common and complex disease with a prevalence of 10–50% worldwide and is associated with substantial costs to affected individuals and society at large.[1–3] The clinical assessment of most chronic pain conditions relies on self-report of symptoms associated with a specific anatomical location. However, at least one-third of chronic pain patients diagnosed with one pain condition often simultaneously exhibit symptoms of another.[4,5] Epidemiological studies have examined the overlap between different bodily distribution of pain and suggested that they may share a common underlying aetiology.[5] In these pain conditions, recently referred to as nociplastic, altered network architecture of functional brain connectivity seems to contribute to central sensitization and co-occurring symptoms include fatigue, mood and cognitive problems, sleep disturbances and multisensory hypersensitivity.[6] The most common set of pain disorders that tend to overlap includes temporomandibular disorders, fibromyalgia, irritable bowel syndrome, vulvodynia, myalgic encephalomyelitis/chronic fatigue syndrome, headaches and chronic lower back pain. This manifestation of multiple chronic pain conditions that frequently occur together and are associated with similar risk factors are referred to as chronic overlapping pain conditions (COPC), and are now recognized by the National Institute for Health as a set of disorders that co-occur.[7] Although the pathophysiological processes that underlie most of these conditions are still poorly understood, COPC have been proposed to have common genetic, neurological and psychological vulnerabilities.

Twin studies have indicated that chronic pain conditions show a heritability between 16% and 50%.[8] Shared heritability between pelvic pain and facial pain and between widespread pain and abdominal pain have been reported.[9,10] Candidate gene studies have suggested that the same genetic variants are associated with multiple pain conditions, which implicated a possible shared genetic basis.[11] There remains a paucity of genetic findings based on genome-wide association studies (GWAS) in large cohorts that have systematically assessed multiple chronic pain conditions. To date, most genetic association studies of pain have featured small samples of a single pain condition, with a few exceptions for back pain and multisite pain.[12,13] It is still unknown whether the reports of COPC versus one specific chronic pain condition feature distinct pathophysiologies or are simply a manifestation of one another.

In this study, we employed genome-wide and brain structure analysis to understand the pathophysiology of COPC. Our first objective was to understand the genetic basis of chronic pain manifestation at one body site versus multiple body sites as a proxy for COPC. Our second objective was to uncover the molecular pathophysiology underlying COPC. Our final objective was to investigate whether CNS mechanisms are genetically related to COPC. Our goal was to uncover the shared genetic heritability between chronic pain conditions and to search for potential underlying biological pathways for COPC.

Materials and methods

Study cohort: UK Biobank

The UK Biobank is a large, prospective, multicentre study of the United Kingdom's population recruited between 2006 and 2010.[14,15] Participants were 40–69 years old and lived within 25 miles of a study recruitment centre. Chronic pain conditions were assessed for 502 599 individuals at the initial assessment visit (2006–10) using a touchscreen-based question: 'In the last month, have you experienced any of the following that interfered with your usual activities?' (Data field 6159). The participants had a choice between pain all over the body, back pain, facial pain, headaches, knee pain, stomach/abdominal pain, hip pain, neck/shoulder pain, none of the above and prefer not to answer. For each pain site selected, participants were asked if that pain lasted for more than 3 months (Data fields 2956: pain all over the body; 3404: neck/shoulder pain; 3414: hip pain; 3571: back pain; 3741: stomach/abdominal pain; 3773: knee pain; 3799: headaches; 4067: facial pain). Participants that answered pain all over the body could not indicate any other body site. Cases were defined as individuals self-reporting pain that interfered with their usual activities in the last month and/or that had lasted for more than 3 months. Participants that reported pain at 1 month and at 3 months at the same site were defined as having pain chronification. Controls were defined as the participants that answered 'none of the above' to data field 6159. Participants that answered 'prefer not to answer' and 'do not know' were excluded. Of the 502 599 individuals, 404 381 had phenotype and genotype data available and therefore were analysed in this paper. For the analysis of the distance between two sites, each reported pain site was assigned a number from the top (head = 1) to bottom (knee = 7) (Fig. 1A). Then, the absolute value of the difference between corresponding numbers was calculated. Widespread pain (=8) was excluded from this analysis. Comparison to previously published GWAS on pain phenotypes in the UK biobank is presented in Supplementary Table 1A.

Medication

Medication used was assessed using field 6154. Participants were asked 'Do you regularly take any of the following for pain relief, constipation and heartburn?' For the purpose of this study only pain medications were considered: aspirin, ibuprofen and paracetamol. ANOVA was used to assess the statistical difference between the groups.

Statistical analysis

Statistical analyses were done using SPSS IBM v 22.0. The prevalence of each chronic pain condition was assessed. The OR and 95% CI were calculated to quantify the degree of overlap between conditions. Next, we classified the study population in two groups. The first group included individuals that reported only one pain site that lasted for more than 3 months. The second group included individuals that reported more than one pain site that lasted for more than 3 months, including those who reported widespread pain. This second group was defined as cases reporting multisite pain as a proxy for COPC.

Genetic analysis

Out of the 404 381 participants that underwent genotyping and that have available phenotype information, we excluded participants that were not genetically confirmed as 'white British', that had sex aneuploidy, or that have a high (⩾2%) genotypic missingness rate. After quality control filters were applied, 340 547 participants were considered for analysis. We conducted eight GWAS, one for each pain site, using a logistic regression model to assess heritability and genetic correlations. Next, we also conducted a GWAS contrasting the report of one pain site ($n = 93\,964$) with a randomly selected half of participants that answered 'none of the above' to data field 6159 ($n = 81\,805$). We also conducted a GWAS for chronic multisite pain, with cases defined as individuals reporting more than one pain site ($n = 82\,812$) and controls as the rest of the randomly selected participants that answered 'none of the above' to data field 6159 ($n = 81\,966$). All genetic analyses were conducted using a logistic regression model with the following covariates: 40 principal components to account for population stratification, age, age^2, sex, genotyping array and dummy-coded recruitment sites. BOLT-LMM v.2.3 was used in all GWAS analyses, as it accounts for cryptic relatedness.[16] Autosomal analysis was restricted to variants with a minor allele frequency (MAF) >0.1%, info score >0.8, genotype hard call rate >0.95 and Hardy–Weinberg $P > 1 \times 10^{-12}$. A total of 8 239 177 autosomal makers with minor allele frequencies above 0.1% that passed quality controls were tested. Genome-wide statistical significance was established from Bonferroni's 5×10^{-8}. Heritability was estimated from single nucleotide polymorphisms (SNPs) under an additive model of inheritance using BOLT-REML and linkage disequilibrium score regression (LDSC).[16,17]

Genetic correlations were estimated for each pair of pain conditions using LDSC.[18] The outliers were defined when distances to distribution's means (μ) were greater than three sigmas (σ), $|Z - \mu| > 3\sigma$. Tissue-based partitioned heritability was evaluated using LDSC,[19,20] with the dataset from the Xavier laboratory.[21]

Gene-based analysis

Gene-based analysis was done using MAGMA. SNPs derived from the summary GWAS were mapped to 18 714 protein-coding genes. A threshold of genome-wide significance level was estimated at $P < 2.67 \times 10^{-6}$.

Genome-wide meta-analysis

In order to identify shared and unique genetic loci between single and multisite chronic pain summary GWAS datasets, a meta-analysis was performed using GWAMA that was adapted from the sex-specific analysis described previously.[22,23] The code was adapted to replace the 'sex-differentiated' option where we assigned 'males' as single-site pain and 'females' as multisite pain.[22] The results of GWAMA will show unique and pleiotropic loci.

Functional mapping and annotation

We used the online platform of FUMA v.1.3.4 to obtain comprehensive annotation information from GWAS summary data.[24] Gene-based tests were obtained using MAGMA.[25] Pathway analyses were conducted with MAGMA within Gene Ontology's (GO) biological processes.[26] Reduction and visualization of GO pathways was done using reviGO.[27]

Replication study cohort—HUNT

Participants in the HUNT Study

The Nord-Trøndelag Health Study (HUNT) is an ongoing population-based cohort study from the county of Nord-Trøndelag in Norway.[28,29] All inhabitants aged 20 years or older were invited to participate in the HUNT1 survey (1984–86), the HUNT2 survey (1995–97) and the HUNT3 survey (2006–2008). Participation rates in HUNT1, HUNT2 and HUNT3 were 89.4% ($n = 77\,212$), 69.5% ($n = 65\,237$) and 54.1% ($n = 50\,807$), respectively.[29] Taken together, the study included more than 120 000 different individuals from Nord-Trøndelag County. For the present study, we included participants from HUNT2 and HUNT3. All participants have provided questionnaire, interview and measurement data, which can be found at the HUNT databank (https://hunt-db.medisin.ntnu.no/hunt-db, last accessed June 2019). In addition, about 80 000 participants have provided biological samples for storage at the HUNT biobank (https://www.ntnu.edu/hunt/hunt-biobank, last accessed June 2019).

Phenotype definition in HUNT

The pain questionnaires in HUNT2 and HUNT3 have been described in detail previously.[30] In brief, participants who answered 'yes' to the screening question 'Have you during the last year continuously for at least 3 months had pain and/or stiffness in muscles and joints?' were requested to indicate the site of the pain, with the possibility to select one or more sites among the following: neck, shoulders, elbows, wrist/hands, upper back, low back, hips, knees and/or ankles/feet. Cases with chronic multisite pain were defined as those reporting pain at two or more sites. Controls were defined as those who answered 'no' to the screening question on chronic pain. If an individual had participated in both HUNT2 and HUNT3, information from HUNT2 was used. This resulted in a total of 25 747 cases with multisite pain and 35 753 controls without chronic pain.

Genotyping, quality control and imputation

In total, DNA from 71 860 HUNT samples was genotyped using one of three different Illumina HumanCoreExome arrays (HumanCoreExome12 v1.0, HumanCoreExome12 v1.1 and UM HUNT Biobank v1.0). Samples that failed to reach a 99% call rate, had contamination > 2.5% as estimated with BAF Regress,[31] large chromosomal copy number variants, lower call rate of a technical duplicate pair and twins, gonosomal constellations other than XX and XY, or whose inferred sex contradicted the reported gender were excluded. Samples that passed quality control were analysed in a second round of genotype calling following the Genome Studio quality control protocol described elsewhere.[32] Genomic position, strand orientation and the reference allele of genotyped variants were determined by aligning their probe sequences against the human genome (Genome Reference Consortium Human genome build 37 and revised Cambridge Reference Sequence of the human mitochondrial DNA; http://genome.ucsc.edu) using BLAT.[33] Variants were excluded if their probe sequences could not be perfectly mapped, cluster separation was < 0.3,

Gentrain score < 0.15, showed deviations from Hardy–Weinberg equilibrium in unrelated samples of European ancestry with *P*-value < 0.0001), had a call rate < 99%, or another assay with higher call rate genotyped the same variant. Ancestry of all samples was inferred by projecting all genotyped samples into the space of the principal components of the Human Genome Diversity Project reference panel (938 unrelated individuals; downloaded from http://csg.sph.umich.edu/chaolong/LASER/),[34,35] using PLINK.[36] Recent European ancestry was defined as samples that fell into an ellipsoid spanning exclusively European population of the Human Genome Diversity Project panel. The different arrays were harmonized by reducing to a set of overlapping variants and excluding variants that showed frequency differences > 15% between datasets, or that were monomorphic in one and had MAF > 1% in another dataset. The resulting genotype data were phased using Eagle2 v2.3.[37]

Imputation was performed on the 69 715 samples of recent European ancestry using Minimac3[38] (v2.0.1, http://genome.sph.umich.edu/wiki/Minimac3) with default settings (2.5 Mb reference based chunking with 500 kb windows) and a customized Haplotype Reference Consortium release 1.1 (HRC v1.1) for autosomal variants and HRC v1.1 for chromosome X variants.[39] The customized reference panel represented the merged panel of two reciprocally imputed reference panels: (i) 2201 low-coverage whole-genome sequences (WGS) samples from the HUNT study; and (ii) HRC v1.1 with 1023 HUNT WGS samples removed before merging. We excluded imputed variants with $R^2 < 0.3$ or minor allele count <3.

Association testing

We used the Scalable and Accurate Implementation of GEneralized mixed model, which uses a generalized mixed model to account for sample relatedness and cryptic population structure.[40] We ran a mixed logistic regression model, including sex, age, genotyping batch and the first four principal components as covariates. The principal components were calculated by projecting all samples into the space of the principal components of unrelated HUNT samples, using directly genotyped variants in PLINK v1.90.[36]

Ethics

The current study is approved by the Regional Committee for Medical and Health Research Ethics (ref. 2015/573).

Allen Brain Atlas

Human gene expression data for visualization of *DCC* expression in the brain were obtained from the Allen Human Brain Atlas (http://human.brain-map.org). A detailed description of this dataset can be found elsewhere.[41] The Neurosynth platform (https://neurosynth.org/) was used extract heat map of normalized expression of *DCC* across the cerebral cortex and subcortical regions. Visualization of the extracted heat map was done using either Brain Net Viewer[42] or MRICron (https://www.nitrc.org/projects/mricron).

Brain imaging in the UK Biobank

Brain imaging occurred on a subset of subjects at a subsequent brain imaging visit. Inclusion into the pain groups therefore necessitated that subjects met the same chronic pain report on both the initial baseline visit and brain imaging visit. This resulted in 3985 subjects with no pain, 593 subjects with one-site pain and 800 subjects with multisite pain. Based on previous literature showing involvement of corticolimbic networks connecting the prefrontal cortex with limbic structures (striatum, amygdala and hippocampus) in chronic pain, and based on results from the Allen Brain Atlas showing clear expression of *DCC* in limbic structures, we decided to restrict our analyses to the uncinate fasciculus (UF), which is the only corticolimbic tract readily provided as an imaging derived phenotype (IDP) in the UK Biobank.[43,44]

Diffusion data were acquired using a spin-echo echo-planar imaging sequence with two *b*-values (b = 1000 and 2000 s/mm^2) at 2-mm spatial resolution. The diffusion-weighted volumes were acquired with 100 distinct diffusion-encoding directions with multiband acceleration factor of 3. The field of view was 104 × 104 mm, imaging matrix 52 × 52, 72 slices with slice thickness 2 mm, giving 2 mm isotropic voxels. Additional details about the sequence of acquisitions and extraction of IDPs in the UK Biobank can be obtained here: https://biobank.ctsu.ox.ac.uk/crystal/refer.cgi?id=1977. Briefly, the data were first corrected for eddy currents and head motion using the Eddy tool. Second, the tracts were derived using probabilistic tractography analysis (BEDPOSTx/PROBTRACKx). The automatic mapping of the 27 major white matter tracts was conducted in standard space of each participant using start/stop region of interest masks (implemented using the AutoPtx plugin for FSL). Maps of fractional anisotropy, mean diffusivity, intracellular volume fraction, isotropic volume fraction and orientation dispersion (OD) were registered with the AutoPtx tract masks, allowing the calculation of the averaged value for each parameter across all voxels pertaining to each tract of interest. Here, we specifically focused on the angular variation in neurite orientation (OD) in the UF.

The OD of neurites can range from highly parallel (coherently oriented white matter structures, such as the corpus callosum) to highly dispersed (grey matter structures characterized by sprawling dendritic processes in all directions).

Polygenic risk scores

Polygenic risk scores (PRSs) were generated using PRSice v.2.3.3,[45] using as a base summary GWAS results derived from the single-site and the multisite GWAS by excluding participants with imaging results. PRSet was used to generate PRSs for the axonogenesis pathway (GO: 0007409) and the *DCC* gene with 100 kb on each side. SNPs were clumped using the maximum haplotype frequency estimates and permutation was performed 10 000 times to generate an empirical *P*-value and to prevent type 1 errors. A regression model that included sex, age, scan site and head scales were used as covariates in a model where each participant's PRS was the dependent variable. A PRS was generated for a series of *P*-value thresholds (5×10^{-8}, 1×10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 0.04, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 1) in the summary GWAS were to determine the association between pain-related genetic variants and left and right OD of the UF. The best-fit *P*-value threshold was used in the analysis.

Data availability

GWAS summary results and code are available upon request.

Results

Prevalence of chronic pain sites

In the UK Biobank, 294 627 participants (60%) reported pain that interfered with their usual activities in the past month. Participants were given the choice among eight pain sites, with the possibility to report more than one site (Fig. 1A): head, facial, neck/shoulder, back, stomach/abdominal, hip, knee and 'all over the body'. The highest prevalence reported was for back (26%) and neck/shoulder (23%) pains. These participants were then asked if

their pain lasted for more than 3 months. Participants who answered 'yes' for pain that lasted for more than 3 months were classified as having chronic pain. Participants reported chronic pain for at least one site at 72%. The highest prevalence of chronic pain was reported for back (18%), knee (17%) and neck (16%) pains. Headache (9%), hip (9%) and abdominal (5%) pains showed less than 10% prevalence. Pain all over the body (1%) and facial pain (1%) displayed the lowest prevalence. Participants that reported pain in the last month and for more than 3 months at the same site were defined as having pain chronification. Pain all over the body, knee and hip pains showed the highest rates of chronification (81%, 78% and 77%, respectively; Supplementary Table 1B).

Next, we created two distinct groups to represent participants who reported only one chronic pain site and those who reported pain at two or more pain sites, which include participants with pain all over the body. We defined participants who reported more than one pain site for more than 3 months as participants with multisite pain as a proxy for COPC. One-third (34.1%) of participants with chronic pain reported multisite pain and 38% reported single-site pain. Around 28% of participants did not report any chronic pain site (Supplementary Fig. 1). In participants with multisite pain, the highest OR for pain at two sites was for facial pain and headache [OR (95% CI) = 10.7 (10.1–11.5)], followed by back and hip pain [(OR (95% CI) = 5.9 (5.8–6.1)] (Fig. 1B and Supplementary Table 1C). Pain all over the body was excluded from this analysis because participants who indicated pain all over the body did not have the option to report any other pain site. Participants who reported multisite pain were more likely to be older, female, have higher body mass index and have lower socioeconomic status. They were also more likely to report more cancer and non-cancer illnesses and to consume more paracetamol and ibuprofen, but not aspirin. In terms of mental health status, participants with multisite pain reported higher neuroticism scores and a higher number of and more severe depressive episodes (Table 1).

Genetic correlation of chronic pain sites

Most chronic pain sites were found to be genetically correlated (Fig. 1B and Supplementary Table 1D). The largest genetic correlation was observed between facial and abdominal pain ($r_g = 1.04$, $P = 1.8 \times 10^{-10}$), followed by pain all over the body and abdominal pain ($r_g = 0.99$, $P = 8.2 \times 10^{-8}$). Headaches presented the smallest genetic correlations with any other chronic pain sites (r_g between 0.37 and 0.54). In a latent causal variable analysis to infer causality, we detected evidence for genetically causal effect of facial pain on hip pain. We also detected a genetic causal effect of headache on back, knee and neck/shoulder pains (Supplementary Table 1E).

Pain site pairs that are physically close displayed stronger correlations (Fig. 1B). Close physical proximity between two pain sites yields an increased chance of their being reported together (% variance explained: $r^2 = 54\%$, $P = 1.4 \times 10^{-4}$; Fig. 1C). Also, increased genetic correlation is observed with close physical proximity ($r^2 = 15\%$, $P = 4.9 \times 10^{-2}$; Fig. 1D). Genetic and epidemiological variables (pain sites) were also observed to be correlated ($r^2 = 16\%$, $P = 4.7 \times 10^{-2}$; Fig. 1E). Similar epidemiological correlations have been shown before and it has been proposed that the observed anatomical selectivity is a consequence of neurosensory and/or affective processes that differentially amplify pain according to its location, rather than a presentation of referred pain.[9,46,47]

Heritability of chronic pain sites

For each chronic pain site, we calculated the heritability derived from genome-wide association (h^2_g), defined as the proportion of phenotypic variance explained by common SNPs under an additive model of inheritance. Between 1% and 10% of the heritability can be explained for each pain site (Fig. 1F and Supplementary Table 1F). The highest heritability was identified for back pain ($h^2_g = 10.0\%$, $P = 7 \times 10^{-106}$) while the lowest was for facial pain ($h^2_g = 1.4\%$, $P = 1 \times 10^{-5}$).

Genome-wide associations of chronic overlapping pain conditions

Next, we performed a comparative GWAS analysis for the report of chronic single-site pain with the report of chronic multisite pain. In a total sample of 340 547 participants, we conducted a GWAS contrasting the report of one pain site ($n = 93\,964$) with a randomly selected half of participants who reported no pain at any site ($n = 81\,805$). We also conducted a GWAS contrasting the report of multisite pain ($n = 82\,812$) with non-overlapping controls as the rest of the randomly selected participants who reported no pain at any site ($n = 81\,966$).

We then computed the percentage of variance explained by genetic and by environmental factors for the report of single-site versus multisite pain. We found a substantial contribution of environmental factors for both the report of single-site (93.2%; SEM 0.4%) and multisite (80.9%; SEM 0.4%) pain. However, we found a significant difference ($P < 2.2 \times 10^{-16}$) for genetic factors between the report of single-site pain (6.9%; SEM 0.4%) and the report of multisite pain (19.1%; SEM 0.4), with a much greater genetic contribution in chronic multisite pain (Fig. 1F). Importantly, the heritability for multisite pain was twice higher than heritability for any individual pain site.

In the case-control association study, where cases were defined as participants reporting chronic single-site pain ($n = 93\,964$), and controls being participants not reporting any pain site ($n = 81\,805$), there were no individual loci that passed the threshold of genome-wide significance (Fig. 2A and Supplementary Table 2A). The genomic inflation factor lambda was 1.07, but the LDSC regression intercept value was 1.015, suggesting a polygenic signal rather than inflation from unaccounted population stratification (Supplementary Fig. 2A). A gene-level association analysis in MAGMA testing for 18 220 genes showed that 11 genes passed multiple testing (Bonferroni threshold $P < 2.7 \times 10^{-6}$; Supplementary Table 2B). Importantly, all previous GWAS that reported genome-wide significant hits in UK Biobank (Supplementary Table 1A) were concentrated on a particular chronic pain condition. Thus, a subject would be included in the analysis if a subject reports the pain site of interest regardless of the subject's other chronic pain site report. In our study, we tested genetic variants underlying report of a single pain site GWAS. In line with its low heritability, this analysis did not identify any genome-wide significant SNPs, pointing to low genetic contribution.

In the case-control GWAS, where cases were defined as participants reporting chronic multisite pain ($n = 82\,812$) and controls being participants not reporting any pain site ($n = 81\,966$), there were 896 SNPs spanning 23 loci that passed the genome-wide threshold (Fig. 2B and Supplementary Fig. 3 and Table 2C). The genomic inflation factor lambda was 1.20, but the LDSC regression intercept value was 1.017, suggesting again, a contribution of LD structure of associated loci rather than inflation from unaccounted-for population stratification (Supplementary Fig. 2B). A gene-level analysis showed that 97 genes passed multiple testing ($P = 2.7 \times 10^{-6}$). The two top associations were with genes involved in neuronal connectivity in model animals: *DCC*,[48] encoding the DCC receptor for netrin1 ($P = 7.4 \times 10^{-19}$), and *SDK1*,[49] encoding the sidekick cell adhesion molecule 1 ($P = 5.4 \times 10^{-18}$; Supplementary Table 2D). Due to the known contribution of depression in the report of multisite chronic pain, as well the importance of DCC in depression, we repeated the gene-level analysis using depression as

Table 1 Demographic and phenotypic characteristics of study population

	Controls	One-site	Multisite	P-value
Number of participants (*n*)	163 771	93 964	82 812	
Females (%)	52.4%	54.2%	60.7%	<0.0001
Age (mean)	56.78	56.67	56.98	<0.0001
BMI (mean)	26.70	27.67	28.66	<0.0001
Current smoking status (%)	8.8%	10.8%	13.6%	<0.0001
Townsend deprivation index (mean)	–1.60	–1.32	–0.80	<0.0001
Number of self-reported cancers (mean)	0.09	0.09	0.1	<0.0001
Number of self-reported non-cancer illnesses (mean)	1.44	1.94	2.83	<0.0001
Medication for pain relief (%)				
Paracetamol	12.7%	30.6%	49.5%	<0.0001
Ibuprofen	8.8%	22.5%	29.5%	<0.0001
Aspirin	14.3%	17.3%	21.3%	<0.0001
Depressed mood last 2 weeks (%)				
Severe days	12.9%	18.9%	25.6%	<0.0001
More than half the days	1.6%	3.0%	5.5%	<0.0001
Nearly every day	0.9%	1.7%	4.4%	<0.0001
Number of depression episodes (mean)	2.44	2.78	3.21	<0.0001
Neuroticism score (mean)	3.35	4.32	5.41	<0.0001

Categorical data were compared using a chi-square test while quantitative data using a *t*-test. The overall *P*-value is an ANOVA between the three groups.

a covariate. We found that at the gene-level, the top two genes, *SDK1* and *DCC* were still genome-wide significant with *P*-values of 7.9×10^{-18} and 9.6×10^{-17}, respectively (Supplementary Table 3). Because both GWAS were equally powered, the differences observed at both the SNP and the gene-level analyses might partially account for the differences in heritability estimates, establishing distinct genetic backgrounds.

Genome-wide meta-analysis

In order to identify loci that were specific to individual pain states (i.e. single-site and multisite pain) and pleiotropic loci that contribute to both states, we performed two meta-analyses using GWAMA.[23] The first meta-analysis aimed to identify loci that are distinct for each of the GWAS (Fig. 2C). Of the 18 066 genes tested, 41 genes passed the threshold for multiple testing (Supplementary Table 4A). The top two genes shown in the meta-analysis are *DCC* and *SDK1*, which are also the top two genes in chronic multisite pain. The second meta-analysis aimed to identify loci that are pleiotropic between the report of single-site pain and multisite pain by running a classical fixed-effect meta-analysis between the two GWAS (Fig. 2D). There are 36 genes that passed the threshold for multiple testing, with the top two genes being *BBX* and *PABPC4* (Supplementary Table 4B). Overall, we found that there are both distinct and common genetic loci underlying chronic single-site pain and chronic multisite pain.

Tissue-expression based functional analyses

Next, we performed partitioned heritability analyses by means of a stratified LDSC regression[19,20] to examine whether the observed heritability was enriched in any tissue, regulatory region or functional category.[21] Analyses in a wide range of tissues and cell types were done for both the report of single-site pain and multisite pain.[21] Partitioned heritability analysis for single-site pain did not show any enrichment in any of the tested tissues at a 10% FDR (Fig. 3A—top panel and Supplementary Table 5A). The analysis of a wide range of tissues and cell types for chronic multisite pain yielded significant results exclusively in the CNS, but not in other tissue types like adipose, blood or immune, and connective or musculoskeletal, nor in the PNS (Fig. 3A—bottom panel and Supplementary Table 5B). We found an exclusive significant enrichment in most brain tissues (Fig. 3B). Finally, in order to quantify whether the enrichment was exclusive to multisite pain, we correlated the heritability estimates in brain-specific tissues. We found no evidence for tissue-based congruency between the two heritability estimates, which suggests distinct tissue heritability (Fig. 3C). Tissue-expression based analysis concluded that heritability for chronic multisite pain, and not chronic single-site pain, is exclusively enriched in the CNS.

Pathway-based functional analyses

We next performed pathway-based enrichment analyses from SNPs in gene sets using GO's[26] biological processes for both chronic single-site pain and multisite pain. For the report of chronic single-site pain, there was no enrichment in any pathway at FDR 10% in GO biological process (Supplementary Table 6A). For the report of chronic multisite pain, a total of 60 pathways were significant at the FDR 10% level in GO biological process, with most pathways involved in neural development, including *DCC* and *SDK1* as leading-edge genes (Supplementary Table 6B). We then used reviGO[27] to reduce redundancy and extricate meaningful information regarding biological processes. The top reviGO class of pathway identified regulation of nervous system development that encompasses pathways involving neurogenesis, axonal development and post-synaptic specialization (Supplementary Table 6C). Here, similar to single variant analysis, we repeated the pathway-level analysis using depression as a covariate to account for its potential confounding effect. We found that axonogenesis and axonal development were still present as top pathways with *P*-values of 3.8×10^{-4} and 1.3×10^{-4}, respectively (Supplementary Table 3). Taken altogether, our pathway analysis results were in line with tissue-expression based functional analysis, suggesting that pathways acting in the CNS in general and associated with neural development in particular contribute to the pathophysiology of chronic multisite pain. Moreover, pathway analysis further supported a strong genetic basis for chronic multisite pain but not for chronic single-site pain.

Replication of genome-wide loci in an independent cohort

Next, we attempted to replicate the genome-wide significant SNPs in the independent HUNT cohort. Due to the absence of genome-

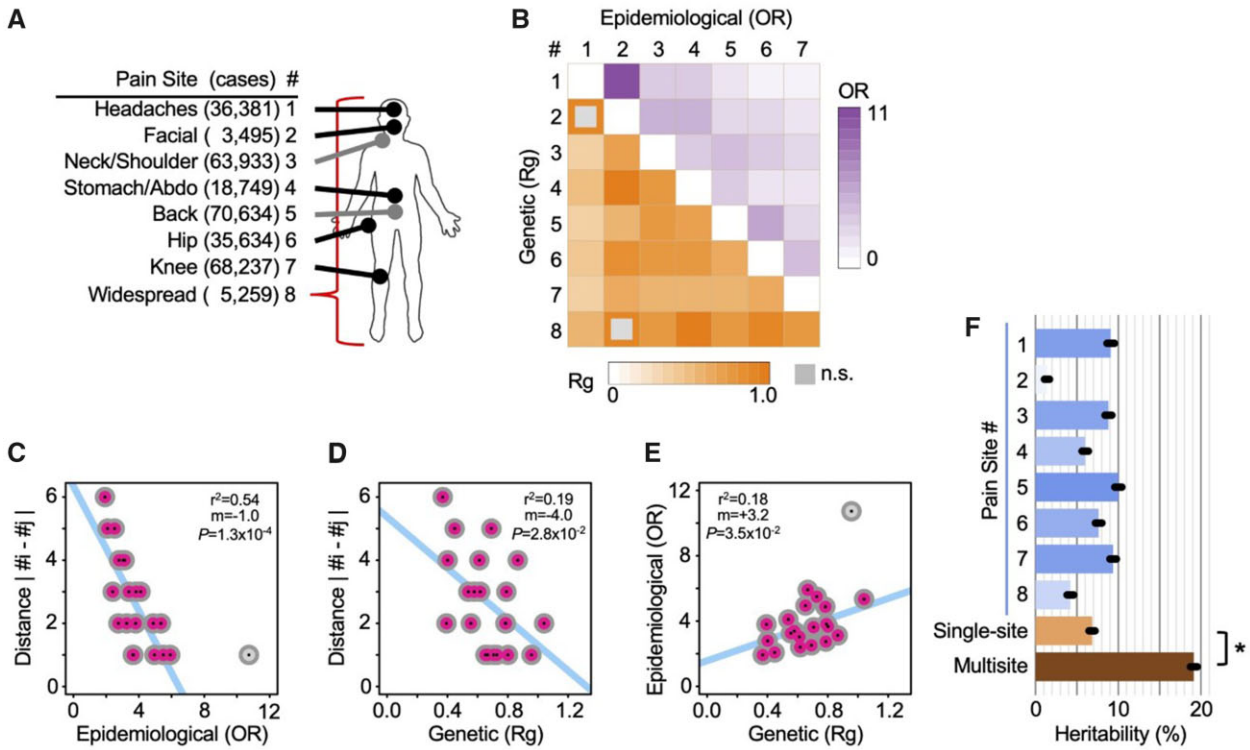

Figure 1 Pain sites characteristics and correlations in UK Biobank. (**A**) Pain sites mapped to the human body. Black dots indicate the sites in the front of the body, while grey dots indicate the sites in the back of the body. Number of cases at each site shown in parenthesis. Human body image from clipart-library.com. (**B**) Epidemiological and genetic correlations between pain sites. Heat map showing correlations for co-occurrence of pain sites. Correlations at the epidemiological odds ratios (OR) are shown in purple hues, while genetic odds ratios (Rg) are shown in orange hues. Grey cells indicate statistical non-significance after Bonferroni correction for the number of same-coloured cells. (**C**) Scatterplot showing correlation between epidemiological OR and body map distance. The body map distance between sites #i and #j is |#i–#j|, where #i and #j are defined in **A**. Each dot is a pair of pain sites out of a total of 21. Also shown are per cent variance explained (r^2), slope of regression (m), and associated *P*-value (*P*). The grey circle defines an outlier. (**D**) Scatterplot showing correlation between genetic Rg and body map distance. (**E**) Scatterplot showing correlation between genetic Rg and epidemiological OR. The grey circle defines an outlier. (**F**) Narrow-sense heritability estimates for each pain site (blue), for chronic single-site pain (orange) and for chronic multisite pain (brown). 95% CIs are shown in black. The difference in heritability is highly significant ($^{***}P < 2.2 \times 10^{-16}$).

wide significant SNPs in the chronic single-site pain GWAS, we only replicated the chronic multisite pain variants. We attempted the replication of the lead SNP in each of the loci and for SNPs that are in medium ($r^2 >= 0.5$) and high LD ($r^2 >= 0.8$) with it in the HUNT cohort. Of the 23 loci, nine reached nominal significance at $P \leqslant 0.05$, of which four reached statistical significance at $P \leqslant 0.002$ (corrected for 23 tests; Supplementary Table 7A). The following four loci passed the threshold for multiple testing: locus 4, with lead SNP rs11709734, located on chromosome 3 in the inositol hexakisphosphatase kinase 1 (*IP6K1*) gene; locus 8, with lead SNP rs34595097, located on chromosome 4 in the mastermind like transcriptional coactivator 3 (*MAML3*) gene; locus 11, with lead SNP rs12672683, located on chromosome 7 in the forkhead box P2 (*FOXP2*) gene; finally, locus 20, with lead SNP rs8099145, located on chromosome 18 in the *DCC* gene, showed the most robust replication ($P = 2.0 \times 10^{-4}$). Similar to the discovery cohort, here we also adjusted for depression and found that the association holds (rs9807752; uncorrected for depression $P = 4.6 \times 10^{-6}$; corrected for depression $P = 2.4 \times 10^{-6}$).

Next, we attempted to replicate the 97 genes associated with chronic multisite pain in the UK Biobank within the HUNT cohort. The threshold for replication was corrected for 97 tests and set at $P = 5.6 \times 10^{-4}$. Of the 97 genes, 11 genes successfully replicated. The most striking association is with the *DCC* gene with a *P*-value of 2.6×10^{-8}, reaching genome-wide statistical significance (Supplementary Table 7B).

Finally, at the pathway level, we attempted to replicate the pathways that passed FDR 10% in the UK Biobank. The axonogenesis pathway (GO: 0007409) showed the lowest *P*-value in the HUNT cohort. This pathway represents mechanisms involved in *do novo* generation of axons, including the terminal branched region. This morphogenesis also includes the shape and form of the developing axon. The second pathway was axon development (GO: 0061564), which covers processes that involve axon regeneration or regrowth after loss or damage (Supplementary Table 7C).

In summary, the replication of our results in HUNT cohort provided further evidence that axonogenesis through the netrin receptor DCC is important in the pathophysiology of chronic multisite pain.

Functional validation for the role of *DCC* in the human brain

Chronic multisite pain-related heritability seems to be expressed in brain tissues with a significant role for the axonogenesis pathway through the *DCC* gene. We therefore attempted to localize where *DCC* is most strongly expressed using a fine-grained representation of genomic information across the human brain and identify the location of axonal structures using diffusion weighted imaging.

First, normalized *DCC* expression information was obtained from approximately 500 brain samples (per hemisphere) of six

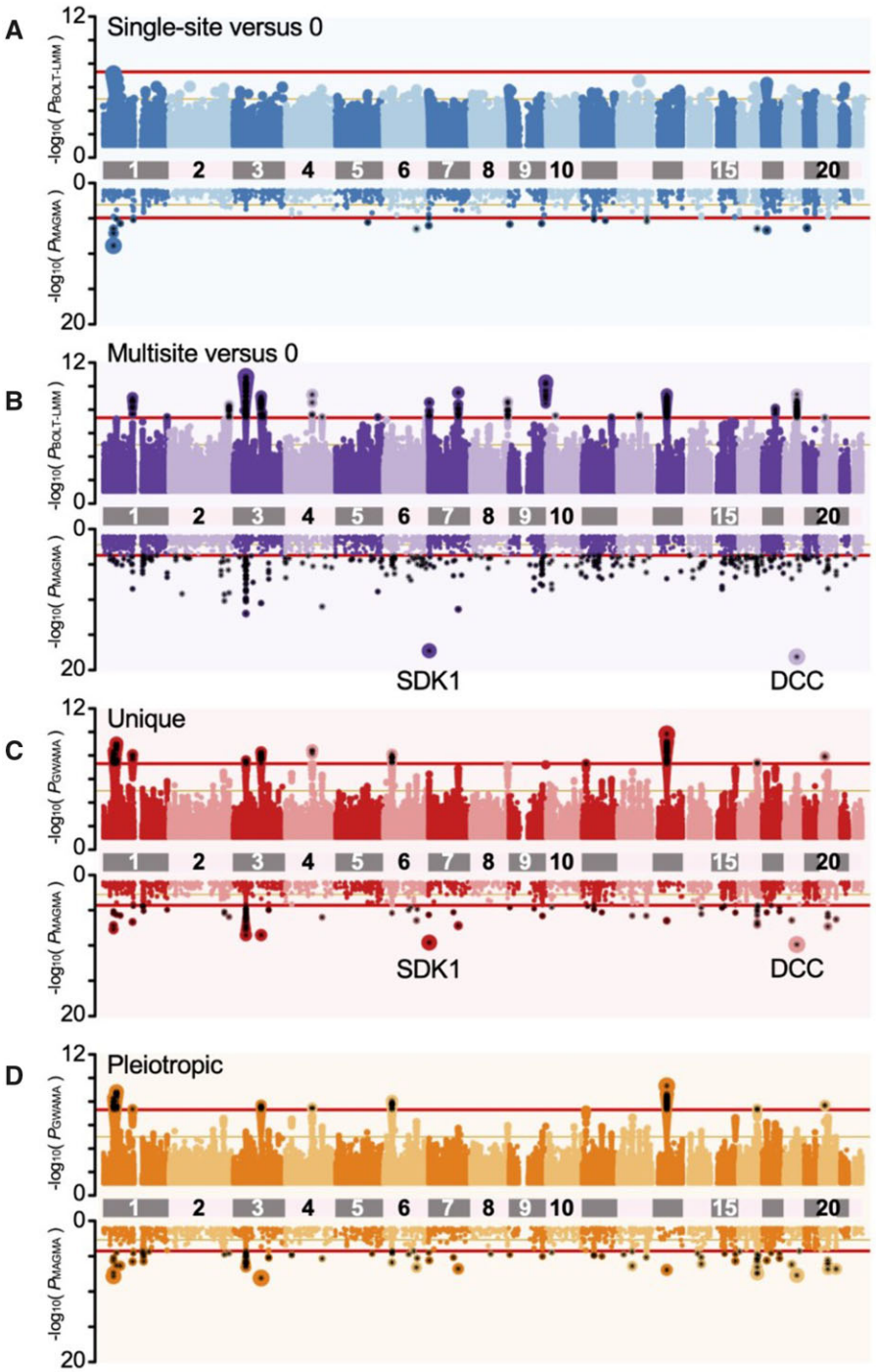

Figure 2 GWAS for single-site pain and multisite pain. Shown are Manhattan plots at the SNP-level (*top*) and at the gene-level (*bottom*). SNP *P*-values are obtained from BOLT or GWAMA, while gene *P*-values are obtained from MAGMA. Alternating dark and light colour hues used for odd and even chromosome numbers. Genome-wide significance highlighted by a horizontal red line at SNP-level is from Bonferroni's threshold of 5×10^{-8}, while the gene level is at FDR 1%. (**A**) Single- versus no chronic pain site. (**B**) Multisite- versus no chronic pain sites. (**C**) Unique loci derived from a meta-analysis in GWAMA. (**D**) Pleiotropic loci from a meta-analysis in GWAMA.

deceased human donors from the Allen Human Brain Atlas.[41] A heat map representing the normalized *DCC* expression across the donors was generated using the neurosynth platform. We observed that *DCC* is specifically expressed in subcortical limbic regions, such as the hippocampus, and basal ganglia (Fig. 4A and B), the corticolimbic system involved in motivation and affect regulation as well as the amplification and the chronification of pain.

Given our findings on the role of *DCC*-driven axonogenesis in chronic multisite pain and *DCC* expression in corticolimbic circuits, we next examined the associations between the microstructure of the UF, which connects the prefrontal cortex to limbic structures of the temporal lobe such as the amygdala and the hippocampus (Fig. 4C). The UF is also the main corticolimbic tract available as an IDP in the UK Biobank. Analyses of the UF were performed on 5378 participants that consistently reported no pain

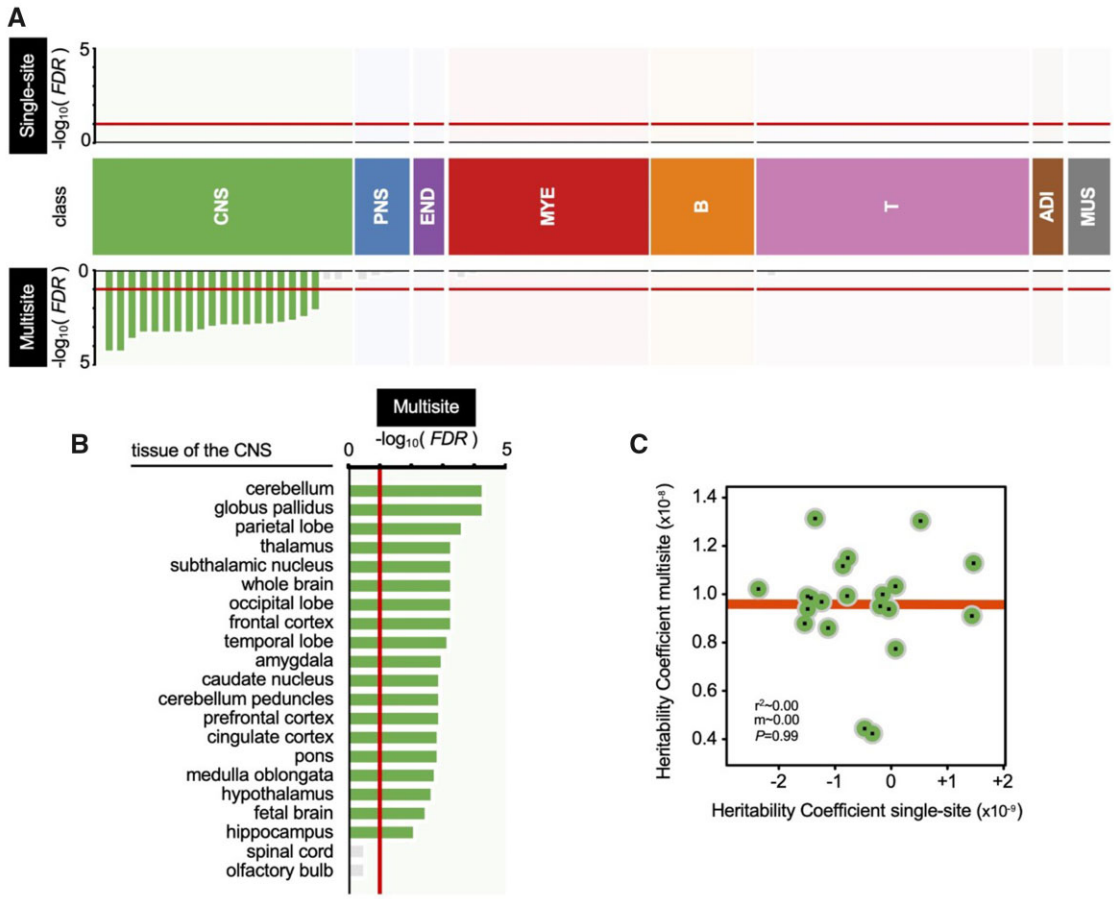

Figure 3 Partitioned heritability for single-site pain and multisite pain. (**A**) Seventy-eight tissues were grouped into eight tissue classes: CNS (green, $n = 21$), PNS (blue, $n = 4$), endocrine (END, purple, $n = 2$), myeloid (MYE, red, $n = 16$), B cells (B, orange, $n = 8$), T cells (T, purple, $n = 22$), adipose (ADI, brown, $n = 2$) and muscle (MUS, grey, $n = 3$). Shown for each tissue is –log10 of FDR-adjusted *P*-value for enrichment. Heritability estimated for single-site pain (*top*) and for multi-pain sites are shown (COPC; *bottom*). Statistical threshold of significance is highlighted at the FDR 10% level with horizontal red lines, while significant tissues are highlighted with coloured filled boxes. (**B**) Zoom into the CNS tissues for multisite pain. (**C**) Scatter plot of heritability coefficients in single-site pain versus multisite pain. Each dot is a tissue of the CNS. Orange line obtained from linear regression, with per cent variance explained (r^2), slope (m) and regression *P*-value (*P*) shown.

($n = 3985$), single-site pain ($n = 593$), or multisite pain ($n = 800$) on both the initial visit and the brain imaging visit (about 10 years apart). OD, a spatial organization metric that characterizes angular variation of neurites (dendrites and axons), was extracted as a metric with potential relevance to axon guidance for the left and the right UF and was compared between the groups. Our analysis revealed that participants with multisite pain showed significantly higher OD in UF compared to single-site pain and healthy controls (Fig. 4D), indicating that UF white matter tracts in patients with COPC are less structured.

In order to assess whether genetic variants in *DCC* and axonogenesis pathway contribute to the OD of the UF, we generated a PRS using summary statistics of single-site pain, multisite pain, the axonogenesis pathway and the *DCC* gene using the best PRS, i.e. that which explains the highest variance. Each of the four scores was used as dependent variables in a regression model with left and right OD of the UF as an independent variable (Supplementary Table 8). The score generated using *DCC* showed the highest significance for both brain sides OD of the UF. The PRS derived from the single-site GWAS at a *P*-value threshold of 5×10^{-8} explained 0.034–0.044% of the variability ($P = 1.0 \times 10^{-5}$; $P = 5.5 \times 10^{-4}$) for the left and right UF, respectively. PRS derived from the multisite pain GWAS at a *P*-value threshold of 4×10^{-2} explained 0.035% and 0.029% of the variability ($P = 4.8 \times 10^{-4}$; $P = 1.4 \times 10^{-3}$) for the left and right UF, respectively. PRS derived from the axonogenesis pathway at a *P*-value threshold of 5.5×10^{-2} explained 0.017% of the variability ($P = 1.6 \times 10^{-2}$) for both left and right UF, respectively. PRS derived from the *DCC* gene at a *P*-value threshold of 7×10^{-2} explained 0.05% of the variability ($P = 2.5 \times 10^{-5}$; $P = 1.3 \times 10^{-4}$) for the left and right UF, respectively (Fig. 4E). Overall, our results showed that the UF is an important structure associated with chronic pain and especially multisite pain at least through *DCC*, bridging for the first-time the genetic determinants of COPC with corticolimbic structures of the human brain.

Discussion

The propensity of chronic pain patients to report more than one location of chronic pain is often observed in clinical settings. Patients diagnosed with one chronic pain condition, such as fibromyalgia, temporomandibular disorder or headaches, have higher chances of presenting symptoms of other pain conditions.[4,5] Moreover, these patients also report comorbid symptoms such as sleep disturbances, depression and anxiety.[50–52] Whether COPC is a distinct pathophysiology from the occurrence of single-site chronic pain is unknown.[5]

Our analysis of the UK Biobank, one of the largest available datasets, confirmed the high degree of overlap between different

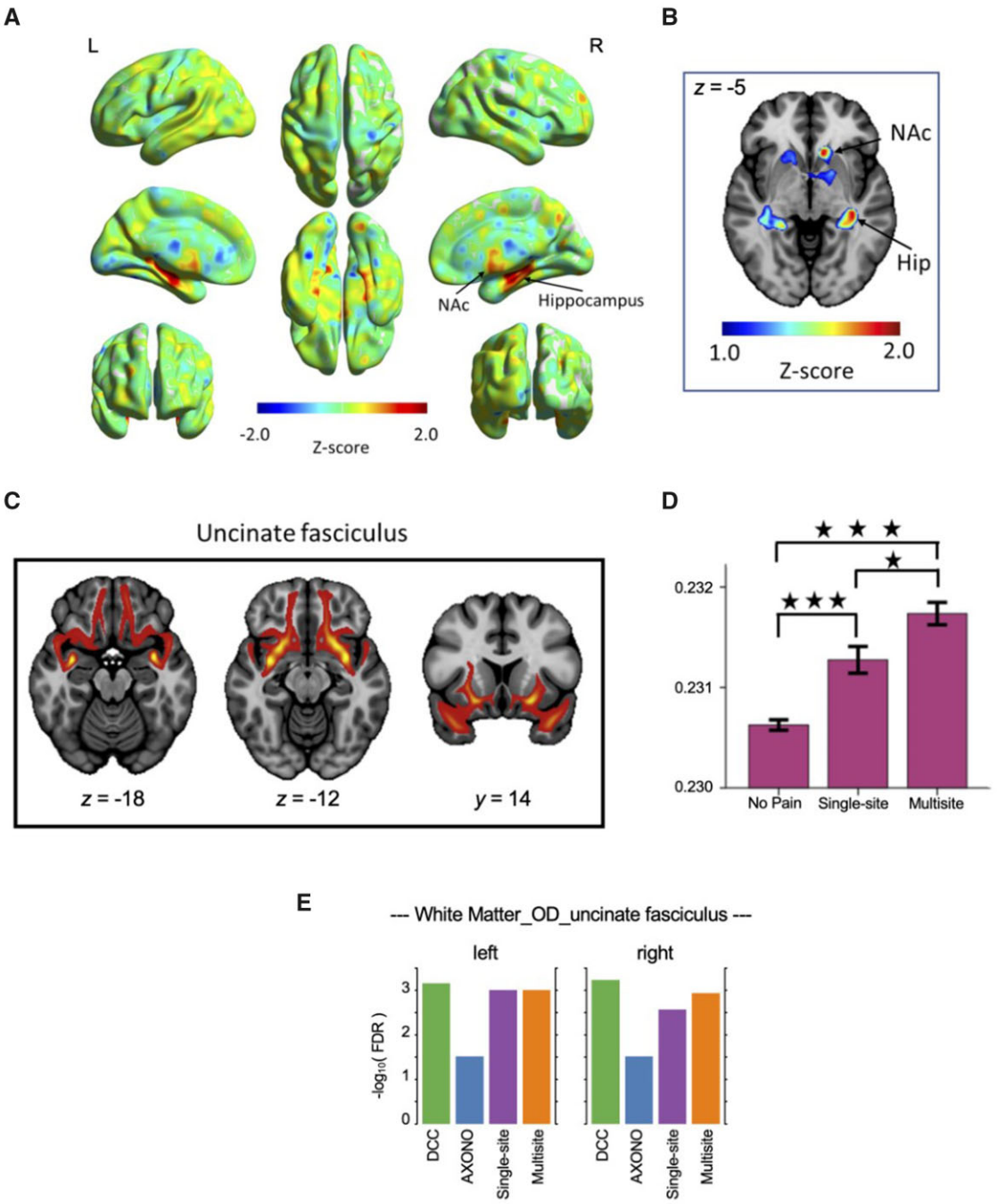

Figure 4 Functional validation for a role of *DCC* in the human brain. (**A**) Whole brain expression of *DCC* computed from the Allen Brain Atlas. (**B**) Zoom into the expression of *DCC* in the subcortical limbic regions. (**C**) Representation of the uncinate fasciculus (UF) white matter tract. (**D**) Bar plot of bilateral dispersion orientation (OD) of the UF in the no-pain controls, single-site pain, multisite pain states. The y-axis represents OD values for the UF. Bars represent standard error. $^{*}P < 0.05$; $^{***}P < 0.0001$. (**E**) PRS generated using PRSice from summary GWAS of single-site pain, multisite pain, axonogenesis pathway and *DCC*. Plotted is the –log10 *P*-value of the regression model using PRS with the score selected at the best fit *P*-value threshold.

chronic pain sites, with one-third of participants with chronic pain reporting multiple pain sites, another third reporting only one pain site and the remaining third reporting no pain. Our GWAS results showed that distinct genetic factors underlie the report of a single pain condition versus the report of COPC, with multisite pain having a much stronger genetic component than single-site pain. Furthermore, our study identified a genetic correlation between different chronic pain sites derived from genome-wide data. The strong genetic correlation between chronic pain sites and the causal latent analysis suggests that there is a specific pathway of vulnerability that underlies co-occurring pain conditions, confirming previous observations of twin studies.[9] Headaches, although also highly heritable, did not show genetic overlap with other chronic pain sites, which suggests a distinct pathophysiology. Indeed, previous GWASs of headaches and migraines have shown a strong cardiovascular component,[53] whereas in this paper we demonstrated a substantial involvement of CNS components in the genetic pathophysiology of COPCs.

Finally, we also confirmed the results of a previous twin study demonstrating a high genetic correlation between widespread pain and abdominal pain.[9] One limitation of this work lies in the unavailability of pain intensity data, and so the phenotypes considered might also have been 'more pain' (multisite) versus 'less pain' (single-site). Conclusions from the literature about the correlation between the number of pain sites and pain intensity was mixed.[54,55]

In the field of pain, the majority of existing genetic findings are derived from candidate gene approaches related to specific pain conditions.[11,56] Only recently have large genome-wide studies started to emerge from the UK Biobank for migraine, back pain, as well as multisite pain, where investigators found many of the SNPs that we uncovered as well (Supplementary Table 1A).[12,13,57] Here, we aimed to identify the genetic architecture and associated biological pathways of COPC rather than any specific SNP for a specific pain condition and discovered more than 900 variants associated with COPC. These genetic factors explain up to 20% of the variance for multisite pain, while the heritability for any individual pain site was lower, suggesting a much stronger genetic basis for COPC in comparison with single pain conditions. When we compared the genetic relationship between the report of chronic single-site pain and chronic multisite pain, we find both common and distinct loci. Contrary to the report of single-site pain, COPC is highly polygenic, with a large portion of its heritability conferred by common genetic variants. The loci that are specific to COPC are enriched in the CNS and are involved in mechanisms related to axonogenesis with a leading role for the *DCC* gene. While the previous studies have found an association between SNPs in *DCC* locus and pain among many others,[12,13] our approaches took single SNP associations results further and identified the central role of *DCC* in the genetics of COPC and uncovered corresponding functional role for netrin and its receptor in the human brain contributing to COPC pathophysiology. Importantly, we also replicated our human findings in another large and independent cohort.

Axon guidance is a process by which neuronal growth cones guide axon extension in the developing nervous system.[58] It involves molecular cues such as netrin 1, present in the environment of growth cones, signalling via dedicated receptors, such as DCC, expressed on the surface of growth cones.[48,59–62] Interestingly, changes in netrin 1-dependent peripheral nerve outgrowth have been reported in patients with chronic pain,[59,63] suggesting that netrin may continue to play an important role following nervous system assembly. The results of the present study further suggest that cerebral axonogenesis may contribute to COPC. First, heritability partitioning analyses clearly indicated that heritability for multisite pain was related to genes expressed in the brain. Second, brain imaging data from the Allen Brain Atlas and UK Biobank pointed towards corticolimbic circuits with the UF as a candidate structure for explaining the relationship between the *DCC* gene and COPC.

More specifically, *DCC* gene expression in the human brain appears to be relatively circumscribed within the basal ganglia and hippocampus. However, we should note that the rodent atlas also shows that *DCC* gene is prominently expressed in the hindbrain.[64] Therefore, we cannot unequivocally conclude that the UF is the only white matter tract explaining the relationship between *DCC* gene and COPC, although our results indicate that this may be a leading hypothesis in humans. Indeed, not only did the findings from the Allen Brain Atlas show prominent expression of *DCC* gene in limbic structures, but structural connectivity of the UF was also found to be related to both the *DCC* gene and to COPC. Increased OD values in the UF for multisite pain suggests that white matter tracts in the UF are less structured in patients exhibiting multisite pain. This finding seems to be highly consistent with the role of the UF in emotional regulation. The UF, which develops well into the fourth decade of life, connects the medial and lateral orbitofrontal cortex with limbic structures in the temporal lobe such as the amygdala and parahippocampal gyrus.[65] One of the main functions of the UF is to provide subcortical structures with contextual information about potential threats and reward available in the orbitofrontal cortex. As such, UF anatomy has been related to general deficits in the capacity to flexibly predict rewards and punishments, as well as to various neuropsychiatric disorders characterized by emotional dysregulation and poor impulse control, such as major depressive disorder, attention deficit/hyperactivity disorder and drug abuse.[65]

Interestingly, previous studies have shown that the *DCC* gene orchestrates the development of the prefrontal cortex during adolescence.[66] Moreover, GWASs of the UK Biobank have also associated the *DCC* gene with neuropsychiatric disorders characterized by mood instability such as major depressive disorder, post-traumatic stress disorder, bipolar disorder (BD), or attention deficit/hyperactivity disorder.[67,68]

Our findings add to these results by linking *DCC* with disorganization of the UF and multisite pain. Here, we showed that participants who report COPC have higher disorganization in axonal tracks versus participants that report only one pain site or healthy participants. This finding suggests that rewiring of the developing brain predispose to the development of chronic pain. A PRS analysis shed the light on a potential relationship between white matter tract organization in the brain and COPC and showed that variants belonging to *DCC* gene are important mediators of this relationship.

An exclusive involvement of the CNS in pathophysiology of COPC found in our study should be interpreted with caution. Our current results are limited by the broadness of the datasets we use. For instance, our partition heritability analyses did not identify expression from spinal cord, dorsal root ganglia, or peripheral nerves contributing to multisite pain. Yet, we are limited here in our analyses of the expression of adult tissues, when we know that *NTN1* and *DCC* are not expressed in the adult spinal cord but only during development. With the increasing broadness of the available expression datasets, new roles for *DCC* may be discovered in addition to that identified here: its crucial contribution to COPC through the wiring of the CNS, such as in the developing dorsal horn of the spinal cord.[69]

In conclusion, we identified a unique and distinct genetic basis for COPC that points to netrin-driven axonogenesis. Our results suggest that genetically determined *DCC*-dependent axonogenesis in the UF microstructure may contribute to COPC via corticolimbic circuits. CNS mechanisms, whether overlapping or distinct, have been suggested as a common neurobiological substrate that may underlie the development of COPC.[5,70] Here, we identified a genetic and structural basis of this CNS input. Thus, our results suggest a new direction in both fundamental research and therapeutics development.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

Appendix 1

Full details are available in the Supplementary material.

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