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Full-length Article

The translocator protein gene is associated with symptom severity and cerebral pain processing in fibromyalgia

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ABSTRACT

The translocator protein (TSPO) is upregulated during glia activation in chronic pain patients. TSPO constitutes the rate-limiting step in neurosteroid synthesis, thus modulating synaptic transmission. Related serotonergic mechanisms influence if pro- or anti-nociceptive neurosteroids are produced. This study investigated the effects of a functional genetic polymorphism regulating the binding affinity to the TSPO, thus affecting symptom severity and cerebral pain processing in fibromyalgia patients. Gene-to-gene interactions with a functional polymorphism of the serotonin transporter gene were assessed. Fibromyalgia patients (n = 126) were genotyped regarding the polymorphisms of the TSPO (*rs6971*) and the serotonin transporter (*5-HTTLPR/rs25531*). Functional magnetic resonance imaging (n = 24) was used to study brain activation during individually calibrated pressure pain. Compared to mixed/low TSPO affinity binders, the high TSPO affinity binders rated more severe pain ($p = 0.016$) and fibromyalgia symptoms ($p = 0.02$). A significant interaction was found between the TSPO and the serotonin transporter polymorphisms regarding pain severity ($p < 0.0001$). Functional connectivity analyses revealed that the TSPO high affinity binding group had more pronounced pain-evoked functional connectivity in the right frontoparietal network, between the dorsolateral prefrontal area and the parietal cortex. In conclusion, fibromyalgia patients with the TSPO high affinity binding genotype reported a higher pain intensity and more severe fibromyalgia symptoms compared to mixed/low affinity binders, and this was modulated by interaction with the serotonin transporter gene. To our knowledge this is the first evidence of functional genetic polymorphisms affecting pain severity in FM and our findings are in line with proposed glia-related mechanisms. Furthermore, the functional magnetic resonance findings indicated an effect of translocator protein on the affective-motivational components of pain perception.

1. Introduction

Activated glial cells have been reported in animal models of chronic pain (Milligan and Watkins, 2009; Watkins and Maier, 2005). In humans, glia activation can be studied *in vivo*, using positron emission tomography with ligands for the peripheral benzodiazepine receptor, more frequently referred to as the translocator protein (TSPO). Small amounts of TSPO are expressed by glia in the healthy human brain (Rupprecht et al., 2010), but the expression is up-regulated during glia activation (Nothdurfter et al.,

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2012; Pinna et al., 2006). In clinical conditions, altered expression of TSPO has been reported in patients with various psychiatric (Bloomfield et al., 2016; Pozzo et al., 2012; Setiawan et al., 2015) and neurological (Girard et al., 2011; Zürcher et al., 2015) disorders that are linked to glia activation. Recently, increased thalamic TSPO binding was reported in chronic low back pain patients compared to healthy controls, thus linking glia cell activation to chronic pain in humans (Loggia et al., 2015).

Despite the fact that TSPO is an evolutionary well conserved protein, it's exact biological roles are yet to be determined (Gatliff and Campanella, 2016). TSPO is a mitochondrial membrane protein, important for the regulation of steroid hormone production and was believed to be necesasary for survival. This view was recently challanged by studies showing that TSPO is not necessary for steroid production (Banati et al., 2014; Morohaku et al., 2014) and by demonstrating an overtly normal phenotype of TSPO knockout mice, with the exeption of reduced mitochondrial ATP production in microglia (Banati et al., 2014). The authors speculated that TSPO-mediated changes in ATP production might exert indirect regulatory effects on the energy-dependent steroid biogenesis, particularly under stress challenges, thus influencing the course of inflammatory brain pathology (Banati et al., 2014). However, whereas knockout studies have yielded inconsistent results most likely due to differences in methodology, strains and compensatory mechanisms (Gatliff and Campanella, 2016), the evidence supporting an important role of TSPO in cholesterol metabolism and steroidogenesis is abundant (Gatliff and Campanella, 2016).

Previous studies have shown that by controlling the rate-limiting step in neurosteroid synthesis, TSPO has a large impact on neurosteroids (Costa et al., 2012; Pozzo et al., 2012). Neurosteroids act as potent modulators of synaptic transmission by exerting facilitatory or inhibitory effects on GABA-A receptors, thus affecting mood, cognition and pain (Aouad et al., 2009; Nothdurfter et al., 2012; Pozzo et al., 2012). Depending on their action on the GABA-A receptor subunits, neurosteroids can have analgesic (positive modulators) or hyperalgesic (negative modulators) effects (Scarf and Kassiou, 2011; Svensson et al., 2013). Serotonergic tone may influence which types of neurosteroids are synthesised, with low tone favouring negative modulators (Pinna et al., 2006; Schüle et al., 2011). Thus, whereas TSPO binding affinity regulates the rate of neurosteroid production, serotonergic tone influences if positive or negative neurosteroid modulators are synthesised.

The binding affinity to the human TSPO receptor is genetically determined by a functional polymorphism in the TSPO gene (*rs6971*) (Guo et al., 2013; Mizrahi et al., 2013; Owen et al., 2012; Venneti et al., 2013). This single-nucleotide polymorphism (SNP) substitutes the amino acid alanine 147 into threonine (Ala147Thr) in the C-terminal transmembrane domain containing the cholesterol recognition amino acid consensus sequence (Costa et al., 2009a). The SNP has been shown to affect neurosteroid production (Costa et al., 2009a) and has been associated with psychiatric diagnosis such as panic disorder (Nakamura et al., 2006), adult separation anxiety (Costa et al., 2009b), and bipolar disease (Colasanti et al., 2013).

The amount of serotonin available in the synaptic cleft is genetically regulated by a common, functional polymorphism, the Long Promoter Repeat (*5-HTTLPR*) of the serotonin transporter (5-HTT) gene (*SCL6A4*) (Lesch et al., 1994). The human promoter region of the gene *SLC6A4* coding for the 5-HTT harbors a 43 base-pair (bp) insertion/deletion referred to as the 5-HTT linked polymorphic region (*5-HTTLPR*). This polymorphism consists of a long (L) allele and a short (S) allele, the latter coupled to reduced gene-expression (Lesch et al., 1994). In addition, the promoter region of the *SLC6A4* gene also harbors the single-nucleotide polymorphism (SNP) *rs25531* which includes an A to G substitution (Wendland et al., 2006). The *rs25531* has been shown to further alter the degree of 5-HTT gene expression. The minor G-allele is nearly always in phase with the L-allele of the 5-HTTLPR and has been shown to reduce transcriptional efficacy to the level of the S-allele (Caspi et al., 2010). When studied jointly, as in the present study, the mini-haplotypes constructed from *5-HTTLPR* and *rs25531* are usually referred to as 'tri-allelic' *5-HTTLPR* whereas analysis of only the L/S alleles are termed the 'biallelic' assay. Thus, the tri-allelic *5-HTTLPR* permits the functional division of individuals into high- (LA/LA), intermediate- (LA/LG, SA/LA) or low- (SA/SA, SA/LG) expressors of the 5-HTT (Caspi et al., 2010). This polymorphism affects endogenous pain modulation (Lindstedt et al., 2011) and has been associated with fibromyalgia (FM) (Ablin and Buskila, 2015; Arnold et al., 2013).

FM is characterized by chronic widespread pain and a generalized hypersensitivity to sensory stimuli, often in combination with fatigue, disturbed sleep and psychological distress. FM patients are characterized by pain hypersensitivity (Kosek et al., 1996) and an inability to activate endogenous pain inhibitory mechanisms (Kosek and Hansson, 1997; Lannersten and Kosek, 2010), which has been supported by neuroimaging studies showing augmented and abberrant cerebral pain processing (Gracely et al., 2002; Jensen et al., 2009, 2010, 2012, 2013). Furthermore, glia activation has been suggested in FM patients based on findings of elevated cerebrospinal fluid (CSF) concentrations of interleukin-8 (IL-8), compared to controls and patients with rheumatoid arthritis (Kadetoff et al., 2012; Kosek et al., 2015). The rodent equivalent of IL-8 (CXCL1) is co-localized with TSPO in glia cells (Liu et al., 2016). Furthermore, TSPO agonists regulate the expression of CXCL1 and it's receptor, thus affecting glia to neuron signalling and central sensitisation (Liu et al., 2016). Therefore, the elevated CSF concentrations of IL-8 in FM patients suggest that TSPO associated mechanisms may be involved in the pathophysiology of FM.

In the present study the influence of the functional polymorphism of the TSPO gene on FM symptoms and cerebral pain processing was investigated. We hypothesized that if pain in FM is associated with glia cell activation and TSPO/IL-8 related mechanisms, then genetically inferred differences in TSPO binding affinity would affect FM symptoms. Furthermore, an interaction between the TSPO and the 5-HTT functional polymorphisms would be expected.

2. Materials and methods

2.1. Subjects

Subjects were recruited to a multi-center experimental study (ClinicalTrials.gov identification number: NCT01226784) by newspaper advertisement, where FM patients were randomized to physical exercise or relaxation therapy (Larsson et al., 2015). Only baseline data were used in the current study. Out of 402 patients screened by telephone, 177 were assesed for eligibility at medical examination and 126 completed baseline examination and genotyping and were used for this analysis (Gothenburg n = 38, Linköping n = 41, Stockholm n = 47). The average age was 51 years, range 22–64 years. Inclusion criteria for FM patients were: female, age 20–65 years, and meeting the ACR-1990 classification criteria for FM (Wolfe et al., 1990). The patient characteristics are presented in Table 1. All patients were caucasian. A subgroup (the exercising part of Stockholm cohort) also performed functional magnetic resonance imaging (fMRI), to assess pain-evoked cerebral activations (n = 24, age 25–64 years).

Exclusion criteria were: high blood pressure (>160/90 mmHg), osteoarthritis in hip or knee, other severe somatic or psychiatric

Table 1
Characteristics of the participants.

N = 126	Average	Range
Age (years)	51.4	22–64
FM duration (years)	10.7	0.5–35
SF-36 bodily pain	34.0	0–74
FIQ total (%)	60.8	16.7–95.3
HAD-A	7.9	0–21
HAD-D	6.7	0–18
PPTs mean (kPa)	183	39–525

SF-36 BP = short form 36 bodily pain score, FIQ = fibromyalgia impact questionnaire, HAD-A = Hospital anxiety and depression scale, anxiety score, HAD-D = Hospital anxiety and depression scale, depression score, PPTs = pressure pain thresholds.

disorders, other primary causes of pain than FM, high consumption of alcohol (Audit >6 for women according to the Audit version used in Sweden), participation in a rehabilitation program within the past year, regular resistance exercise training or relaxation exercise training twice a week or more, inability to understand or speak Swedish, and inability to refrain from analgesics, NSAID or hypnotics for 48 h prior to study assessments. All patients had a physical exam to ensure that inclusion criteria were met, and that no exclusion criteria were present.

The study was approved by the Regional ethics committee in Stockholm (2010/1121-31/3). Written and oral information was given to all participants and written consent was obtained from all participants. The study followed the guidelines of the Declaration of Helsinki.

2.2. Procedures

During the first visit (V1), subjects completed standardized questionnaires regarding pain severity (short form (SF-36) bodily pain) (Contopoulos-Ioannidis et al., 2009; Hawker et al., 2011), FM impact (fibromyalgia impact questionnaire (FIQ)) (Bennett et al., 2005; Hedin et al., 1995) and depression and anxiety (Hospital Depression and Anxiety Scale (HADS)) (Bjellanda et al., 2002).

SF-36 consists of eight scaled scores, which are the weighted sums of the questions in their section, ranging from zero (maximal severity) to 100 (no severity). SF-36 bodily pain (SF-36 BP) is one subscale of SF-36 and was chosen since it is a validated instrument to assess pain severity and its interference with working activities, including housework, over a longer period of time (4 weeks) (Hawker et al., 2011). Severity of pain was rated on a scale from 1 to 6, ranging from none to very severe, and degree of interference was rated on a 1–6 scale, ranging from not at all to extreme interference. SF-36 BP does not segregate between different dimensions of the painful experience, e.g. pain intensity and pain unpleasantness. The SF-36 BP subscale is often used in patients with FM, and the different subscales have been used to differentiate FM from other painful conditions, but also used as an outcome measure in randomized controlled trials (Wallace and Clauw, 2005).

The FIQ is a disease-specific questionnaire consisting of 20 items assessing symptoms and disability common to FM. The total score ranges from 0 to 100, where a higher score indicates a lower health status (Bennett et al., 2005; Hedin et al., 1995). HADS is one of few psychometric questionnaries that has specifically been developed for non-psychiatric patients and it consists of two subscales, anxiety and depression, each ranging from zero (no anxiety/depression) to 21 (maximal anxiety/depression) (Bjellanda et al., 2002). Pressure pain thresholds (PPTs) were assessed using a pressure algometer (se below). Saliva was collected for genotyping using Oragene kits (OG-500). The Stockholm cohort returned for a second visit (V2), for individual calibration of experimental pain to be used in the MRI scanner, followed by an fMRI scan the next day (V3).

2.3. Genotyping

2.3.1. TSPO (Rs6971)

Genotyping was performed using TaqMan SNP genotyping assays and ABI 7900 HT instrument (Applied Biosystems (ABI), Foster City, CA). Polymerase chain reactions (PCR), with a total volume of 5 μl, were performed in 384-well plates containing 2.5 μl Universal Master Mix (UMM) and 5 ng dried-down genomic DNA per well. The PCR amplification protocol includes two holds, 50 °C for 2 min and denaturation at 95 °C for 10 min, followed by 45 cycles at 92 °C for 15 s and 60 °C for 1 min.

2.3.2. Tri-allelic 5-HTTLPR

For the biallelic *5-HTTLPR*, two fragments, 487 bp (short) and 530 bp (long), were amplified by PCR. Each PCR reaction contained 50 ng DNA, 0.2 mM deoxynucleocide triphosphate (dNTP), 0.4 μM of primer 17P-3F (5′-ggcgttgccgctctgaatgc-3′), 0.4 μM primer 17P-3R (5′-gagggactgagctggacaaccac-3′), 0.05 U/μl Quiagen HotStar®Polymerase, 1 M Q-solution and finally 1× buffer. Samples were amplified on Biorade Tetrade (BIORAD, Hercules, CA, USA) with an initial denaturation for 10 min at 95 °C followed by 33 cycles consisting of denaturation for 30 s at 95 °C, annealing for 30 s at 57 °C and elongation for 5 min at 72 °C and finally followed by another elongation step for 5 min at 72 °C. 8 μl of the PCR reactions were separated for 2 h at 100 V by gel-electrophoresis in TBE-buffer on a 2.5% Agarose gel containing GelRed® and visualized using ultraviolet light (UV).

In order to determine the *rs25531*, 10 μl of the PCR product were then digested with 0.1 μl MSP1 (New England Biolabs, Ipswich, MA, USA) and 1 μl buffer per sample for 12 h at 37 °C. The MSP1 restriction enzyme breaks the 5′-C/CGG′ sequence which gives rise to fragments of different length from which the triallelic *5-HTTLPR* genotype can be determined. LA results in 342 bp, 127 bp and 62 bp; SA results in 298 bp, 127 bp, and 62 bp; LG results in 173 bp, 166 bp, 127 and 62 bp and finally SG results in 166 bp, 130 bp,127 bp and 62 bp. The fragments were run on an 4% Agarose gel (3% normal Agarose and 1% low melting Agarose) containing GelRed® initially for 15 min at 70 V followed by 2 more hours at 100 V. The gels were then visualized with UV light. We were unable determine the triallelic *5-HTTLPR* genotype for one subject.

2.4. Psychophysical testing

Pressure pain thresholds (PPTs) were assessed in all subjects in order to get a semi-objective quantification of pain sensitivity. PPTs were assessed using a pressure algometer (Somedic Sales AB, Hörby, Sweden); a pistol-shaped apparatus with a 1 cm^2 hard rubber probe that is held at 90 degree angle against the body and then pressed with a steady rate of increased pressure (approximately 30 kPa/s) until the patient's pain threshold is reached, and the corresponding pressure is recorded (Kosek et al., 1993). PPTs were assessed bilaterally at four different sites, i.e., m. trapezius; elbows (lateral epicondyle), m. quadriceps femoris and knees (at the medial fat pad proximal to the joint line), with one assessment per anatomical site. The average PPT for all body sites (PPT_{mean}) was calculated for each subject and used for analysis.

2.5. Individual calibration of evoked pain during fMRI

An individual calibration of pressure pain stimuli to be used during fMRI was performed in the Stockholm cohort. Pressure stimuli were applied to the left thumbnail for 2.5 s with 30 s inter-

vals using an automated, pneumatic, computer controlled stimulator with a plastic piston that applies pressure via a 1 cm^2 hard rubber probe (Jensen et al., 2009). In order to avoid sensitisation, the calibration was performed the day before scannning. Each subject was calibrated for subjective pain ratings by receiving one ascending series of pressure stimuli and one randomized series. During the ascending series the pressure stimuli were presented in steps of 50 kPa of increased pressure, starting at 50 kPa. The pain threshold, i.e., pressure giving rise to the first VAS rating >0 mm and stimulation maximum, i.e., the pressure eliciting the first rating exceeding 60 mm on a 0–100 mm visual analogue scale anchored by "no pain" and "worst imaginable pain" was determined. These values were then used to compute the magnitude of five different pressure intensities evenly distributed within the range of each patient's threshold and maximum. During the randomized series, a total of 15 stimuli, three of each intensity, were delivered in a randomized order, and the pain intensity was rated on VAS following each stimulus (Jensen et al., 2009). A polynomial regression function was used to determine each individual's calibrated pain rating of 50 mm on the VAS, derived from the 15 randomized ratings (Jensen et al., 2009). The amount of pressure required to evoke pain at VAS 50 mm in each individual is referred to as P50 throughout this article.

2.6. Neuroimaging assessments

Patients were placed in the bore of the magnet and asked to place their left thumb in the pressure-pain device. During fMRI scanning, two different pressures were used: P50, and a non-painful pressure corresponding to 50 kPa. All stimulations were randomly presented over the scanning time, preventing subjects from anticipating the onset time and event type. The time interval between stimuli was randomized with a mean stimulus onset asynchronicity (SOA) of 15 s (range 10–20 s). The total duration was approximately 16 min. No pain ratings were performed during the scan, and subjects were instructed to focus on the thumb pressures and not use any distraction or coping strategies.

Images were collected using a 3 T General Electric scanner. Multiple T2*-weighted single-shot gradient echo EPI sequences were used to acquire blood oxygen level dependent (BOLD) contrast images with the following parameters: repetition time: 3000 ms (35 slices acquired), echo time: 40 ms, flip angle: 90 degrees, field of view: 24 × 24 cm, 64 × 64 matrix, 4 mm slice thickness with 0.4 mm gap and sequential image acquisition order. In the scanner, cushions and headphones were used to reduce head movement and dampen scanner noise. The placement of a blank screen in front of the patient's field of view minimized visual distraction during scans. In addition to the functional scans, high-resolution T1-weighted structural images were acquired in coronal orientation for anatomical reference purposes and screening for cerebral anomalies. Parameters were: Spoiled Gradient Recalled 3D sequence, repetition time: 24 ms, echo time: 6 ms, flip angle 35 degrees with a voxel size of 0.9 × 1.5 × 0.9 mm^3.

2.7. Statistics

First, the effects of the TSPO polymorphism on FM symptoms were analysed by a Multivariate analysis of variance with SF-36 bodily pain, FIQ total score, HAD-depression, HAD-anxiety and PPT_{mean} as dependent variables, TSPO as fixed factor and age as covariate. The same procedure was used to analyse the effects of the 5-HTT gene polymorphisms on FM symptoms. For dependent variables where TSPO had a significant effect, the interaction between TSPO and 5-HTT polymorphisms was analysed by a univariate analysis of variance with the particular factor as dependent variable and TSPO and 5-HTT as fixed factors. In order to exclude that antidepressive medication influenced our results, we also performed separate analyses for patients on (n = 53, 42%) and off antidepressants (n = 73, 58%), respectively. Post hoc analysis was performed using Students' independent sample *t*-test. The statistical analysis was performed using IBM SPSS Statistics version 22. The data are presented as mean ± standard deviation (SD) if not otherwise stated. For all non-fMRI analyses, $p < 0.05$ was considerd significant.

2.8. Functional magnetic resonance imaging

Pre-processing and analyses of imaging data were performed using the Statistical Parametric Mapping 8 (SPM8) software (http://www.fil.ion.ucl.ac.uk/spm/) and Matlab (Mathworks). All functional brain volumes were realigned to the first volume, spatially normalized to a standard Echo Planar Imaging template and finally smoothed using a 8 mm full-width at half-maximum isotropic Gaussian kernel. Data analysis was performed using the general linear model (GLM) and modelling of the two different conditions ('painful pressure' and 'non-painful pressure'). A file containing the movement parameters for each individual (6 directions) were obtained from the realignment step and saved for inclusion in the model. A design matrix was prepared for each subject and included regressors for the two conditions. To assess pain-specific cerebral activity, brain activation during non-painful pressures was individually subtracted from activity during the calibrated painful pressures.

In the overall analysis of pain-evoked cerebral activity across genotype groups, an initial image threshold of $p < 0.001$, uncorrected for multiple comparisons, was used together with a cluster threshold of $p < 0.05$ Family-Wise Error (FWE) corrected. In all other analyses, including the psychophysiological interaction (PPI)-analysis, we adopted an initial image threshold of $p < 0.005$ and 20 contiguous voxels uncorrected for multiple comparisons and a cluster threshold of $p < 0.05$ FWE-corrected. Anatomical locations were expressed in Montreal Neurological Institute (MNI) stereotactic atlas coordinates (x, y, z) (Mazziotta et al., 1995).

A PPI analysis was performed based on "seeds"; i.e. anatomical locations from which pain-evoked functional connectivity was to be calculated. A seed coordinate in the dorsolateral Prefrontal Cortex (dlPFC) was defined by a coordinate from the univariate analysis, and chosen based on evidence for dlPFC pain regulatory functions, together with a seed in the Periaqueductal Grey (PAG) as it is a key region for descending pain regulation. For each subject, voxel-wise PPI effects were estimated, and statistical parametric maps (SPM's) were produced for the PPI term. The resulting contrast images were used in a second level PPI group analysis, comparing the PPI contrast images between TSPO high affinity binder genotype (TSPO HAB) and the pooled TSPO mixed/low affinity binders (TSPO MLAB) in a two-sample *t*-test.

3. Results

3.1. Genetics

3.1.1. Effects of the functional polymorphism of TSPO and tri-allelic 5-HTTLPR on FM symptoms

Sixty FM patients had the TSPO high affinity binder (HAB) genotype, and 66 were genetically inferred mixed (n = 52) or low affinity binders (n = 14). The data from mixed and low affinity binders was pooled and is referred to as mixed/low affinity binders (MLAB). Higher pain severity ratings ($p = 0.016$) and higher FIQ scores ($p = 0.02$) were seen in the genetically inferred TSPO HAB compared to MLAB (Table 2). No statistically significant group differences were seen regarding depression or anxiety ratings or

Table 2
Impact of genetically inferred TSPO binding affinity and 5-HTT expression on FM symptoms (means and standard deviations).

	TSPO HAB (n = 60)	TSPO MLAB (n = 66)	Statistics p-value	5-HTT-low (n = 28)	5-HTT-inter (n = 64)	5-HTT-high (n = 33)	Statistics p-value
SF-36 BP	30.9 ± 11.6	36.9 ± 14.8	p = 0.016	31.0 ± 11.9	34.2 ± 14.3	35.9 ± 13.8	NS
FIQ total (%)	64.3 ± 15.7	57.6 ± 15.6	P = 0.02	61.1 ± 13.4	59.6 ± 17.3	62.3 ± 15.4	NS
HAD-A	8.1 ± 4.3	7.7 ± 4.6	NS	8.3 ± 4.1	7.8 ± 4.5	7.7 ± 4.8	NS
HAD-D	6.9 ± 3.6	6.5 ± 3.7	NS	6.6 ± 3.2	6.7 ± 4.0	6.6 ± 3.2	NS
PPTs (kPa)	178.0 ± 82.4	187.0 ± 77.2	NS	196 ± 76	177 ± 71	184 ± 99	NS

TSPO = Translocator protein, HAB = high affinity binders. MLAB = mixed/low affinity binders, 5-HTT = serotonin transporter, SF-36 BP = short form 36 bodily pain score (lower score indicates higher pain severity), FIQ = fibromyalgia impact questionnaire, HAD-A = Hospital anxiety and depression scale, anxiety score, HAD-D = Hospital anxiety and depression scale, depression score, PPTs = pressure pain thresholds. P-values refer to group differences between TSPO HAB/MLAB and 5-HTT low/intermediate and high expressing, respectively. There was an inverse correlation between SF-36 BP and FIQ ($r = -0.599$, $p < 0.001$).

PPT_{mean} between TSPO HAB and MLAB (Table 2). Furthermore, we found no statistically significant effects of the 5-HTT tri-allelic polymorphism on SF36 bodily pain, FIQ, HADS or PPT_{mean} (Table 2).

A separate analysis was peformed of FM patients on antidepressants (selective serotonin re-uptake inhibitors n = 22, tricyclic antidepressants n = 15, serotonin-noradrenalin re-uptake inhibitors n = 11, and combinations of these n = 5) and those not taking antidepressants. There were 24 (45%) MLAB and 29 (55%) HAB taking anti-depressant medication, to compare with 42 (58%) MLAB and 31 HAB (42%) among FM patients who were not on antidepressants. We could reproduce our overall findings in the subgroup without antidepressants, e.g., TSPO HAB had more severe pain (SF-36 BP m = 31.7) than TSPO MLAB (SF-36 BP = 38.5)(p = 0.036). However, this was not seen in the subgroup taking antidepressants (HAB: SF36-BP m = 30.3, MLAB SF-36BP = 33.5) (p = 0.36).

3.1.2. Effects of gene-to-gene interactions on FM symptoms

There was a statistically significant interaction between the TSPO and tri-allelic 5-HTT polymorphism regarding pain severity ($df = 2$, $F = 8.32$, $p < 0.0001$), and this was true also when patients on antidepressants ($df = 2$, $F = 4.33$, $p = 0.019$) and patients not taking antidepressants ($df = 2$, $F = 3.26$, $p = 0.045$) were analysed separately. The TSPO × tri-allelic 5-HTT interaction did not reach statistical significance regarding FIQ ($df = 2$, $F = 2.81$, $p = 0.064$), but was in the same direction. Compared to TSPO MLAB, higher pain severity was reported by TSPO HAB who had genetically inferred high (n = 33, p = 0.012), or mixed (n = 64, p = 0.001) 5-HTT expression. However, the opposite was true for the genetically inferred 5-HTT low expressing subjects, i.e., lower pain severity ratings were reported by the TSPO HAB compared to MLAB (n = 28, p = 0.015) (Fig. 1). Within the TSPO MLAB group, *higher*

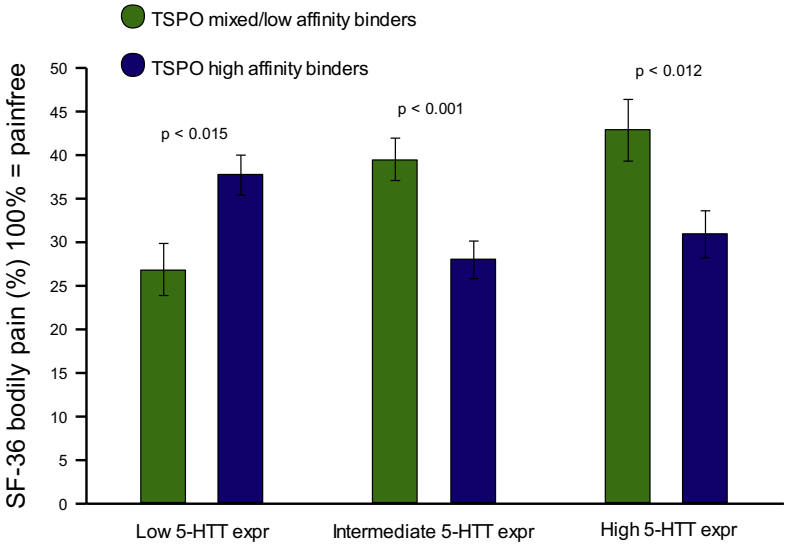

Fig. 1. Bodily pain scores across TSPO and 5-HTT genotypes. Average SF-36 bodily pain (SF-36 BP) (means ± SE) in FM patients (n = 126). There was a statistically significant gene-to-gene interaction between the TSPO and the 5-HTT gene regarding pain severity ($p < 0.0001$). In genetically inferred 5-HTT low expressing FM patients, lower pain severity (higher SF-36 BP) was reported in TSPO HAB compared to MLAB (n = 28, p = 0.015). On the contrary, higher pain severity (lower SF-36 BP) was reported in 5-HTT intermediate (n = 64, p = 0.001) or high (n = 33, p = 0.012) expressing FM patients who were TSPO HAB compared to MLAB.

Table 3
Main effects when pooling TSPO HAB and MLAB during painful stimulation (painful pressure – non-painful pressure).

Anatomical region	Cluster size	x	y	z	Peak T-value	P-value cluster
L. insula	1214	-44	10	-6	7.88	p < 0.0001
R. primary sensory cortex	7742	44	-36	66	7.20	p < 0.0001
L. cerebellum	3360	-28	-52	-32	7.05	p < 0.0001
R. dlPFC	305	38	44	28	5.54	p < 0.05
R. temporal/secondary sensory cortex	725	-52	-36	16	5.48	p < 0.001
L. dlPFC	444	-32	42	28	5.48	p < 0.01

Coordinates (x, y, z) correspond to the anatomical space as defined by the MNI standard brain atlas (Mazziotta et al., 1995). Results are reported at an initial threshold setting of p < 0.001, uncorrected for multiple comparisons; cluster corrected for multiple comparisons, FWE p < 0.05. Laterality (Left/Right) for anatomical regions are indicated with L/R. dlPFC = dorsolateral prefrontal cortex, TSPO = Translocator protein, HAB = high affinity binders. MLAB = mixed/low affinity binders.

pain severity was reported by FM patients with the low expressing 5-HTT genotype, compared to mixed (p = 0.003) and high (p = 0.002) expressing, respectively. Paradoxically, within the TSPO HAB group, the 5-HTT low expressing genotype was associated with *lower* pain severity, compared to the intermediate (p = 0.015) and high (p = 0.106) expressing 5-HTT genotypes.

3.2. Neuroimaging results

3.2.1. Main effect of painful stimulation on brain activity

The fMRI subgroup can be considered representative of the whole study cohort as no statistically significant differences were found between this subgroup and the rest of the cohort regarding age, SF-36 BP, FIQ, HAD or PPTs. There was no statistically significant difference in the calibrated thumb pressure (P50) between TSPO HAB (286 ± 172 kPa) and MLAB patients (233 ± 110 kPa). In order to validate the pressure pain neuroimaging paradigm, we calculated the main effect of the painful pressure minus the non-painful pressure using a one-sample *t*-test across all FM patients (TSPO HAB and MLAB pooled, n = 24). As expected, we found activation in areas traditionally associated with pain processing, such as the insula, the primary (S1) and secondary (S2) somatosensory cortex and the cerebellum (Table 3).

3.2.2. Effects of TSPO HAB and MLAB on brain activity

There were no brain regions where the HAB group had greater brain activations than MLAB. Conversely, the MLAB group had greater activity in several brain areas (Table 4), including the dorsolateral prefrontal cortex (dlPFC) (MNI peak coordinate x = 40, y = 44, z = 28). The similarities and differences in brain activations between TSPO HAB and MLAB are shown in Fig. 2.

3.2.3. Psychophysiological interaction (PPI) Connectivity

3.2.3.1. Dorsolateral prefrontal cortex (dlPFC). A PPI-analysis of pain-evoked functional connectivity revealed significant positive connectivity between the right dlPFC and the right parietal cortex (MNI peak coordinate: x = 50, y = −46, z = 58) in TSPO HAB patients (n = 11), compared to MLAB (n = 13). In addition, TSPO HAB had

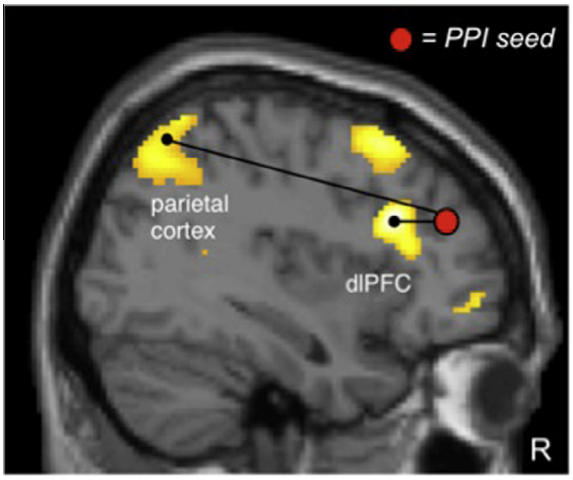

Fig. 3. TSPO genotype differences in dlPFC functional connectivity. Functional connectivity between the dlPFC and the rest of the brain was estimated using psychophysiological interaction analyses (PPI). TSPO HAB patients (n = 11) displayed higher pain-evoked connectivity than MLAB patients (n = 13) between the dlPFC seed-region and another anatomical location in the dlPFC (p < 0.001 FWE, corrected for multiple comparisons), as well as the right parietal cortex (p < 0.005, FWE corrected for multiple comparisons).

Table 4
Anatomical regions where brain activity is greater for TSPO MLAB than HAB during painful stimulation (painful pressure - non-painful pressure).

Anatomical region	Cluster size	x	y	z	Peak T-value	P-value peak voxel
L. superior temporal gyrus	445	−42	−22	−6	4.59	p < 0.001
R. primary sensory cortex	48	44	−38	66	4.34	p < 0.001
R. primary sensory cortex	34	18	−32	80	4.32	p < 0.001
R. secondary sensory cortex/temporal	27	68	−38	18	4.17	p < 0.001
R. dACC	127	10	−12	52	3.77	p < 0.001
R. primary sensory cortex	32	58	−22	52	3.71	p < 0.001
R. dACC	95	14	4	40	3.44	p < 0.001
L. suppl. motor area	22	−14	−8	76	3.41	p < 0.001
R. insula	57	38	−26	18	3.34	p < 0.001
R. insula	65	40	−14	2	3.34	p < 0.001
R. dlPFC	56	40	44	28	3.31	p < 0.001
L. precentral gyrus	25	−48	−4	20	3.15	p < 0.005
L. insula	21	−42	6	−4	3.14	p < 0.005

Results are exploratory as they are reported at a liberal statistical threshold setting of p < 0.005, uncorrected for multiple comparisons. Coordinates (x, y, z) correspond to the anatomical space as defined by the MNI standard brain atlas (Mazziotta et al., 1995). Laterality (Left/Right) for anatomical regions are indicated with L/R. dACC = dorsal anterior cingulate cortex, dlPFC = dorsolateral prefrontal cortex, TSPO = Translocator protein, HAB = high affinity binders, MLAB = mixed/low affinity binders.

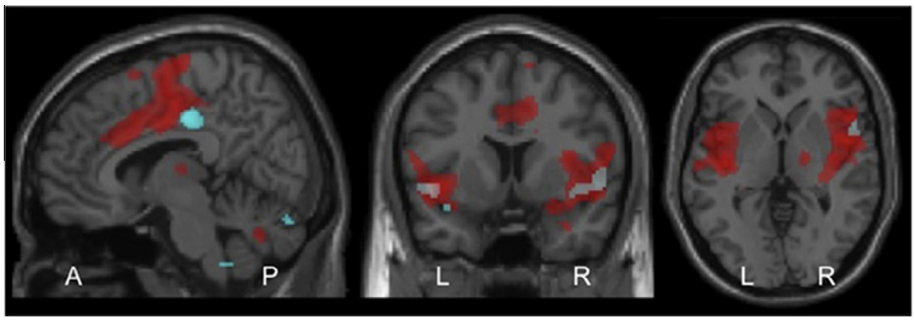

Fig. 2. Similarities and differences in brain activations between TSPO MLAB and HAB. Colors represent brain activity during painful pressure minus non-painful pressure in MLAB (red) and HAB (cyan) patients. Regions with overlapping activity are grey. Anterior and posterior orientation is denoted A/P. Left and Right is indicated with L/R. Results are thresholded at a statistical level of p < 0.005, uncorrected for multiple comparisons, with 20 contiguous voxels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significantly higher dlPFC connectivity within the right prefrontal area (MNI peak coordinate: x = 38, y = 22, z = 26), compared to TSPO MLAB. There were no regions where TSPO MLAB had higher dlPFC connectivity compared to HAB (See Fig. 3).

3.2.3.2. Periaqueductal grey (PAG). As the PAG is a key brain region for pain inhibition, a PPI analysis from a seed in the PAG was performed. There was no significant difference in PAG brain connectivity between the two TSPO genotype groups HAB/MLAB.

4. Discussion

Female FM patients with genetically inferred TSPO HAB reported more severe pain and more disability, compared to MLAB. In addition, we found a strong interaction between the TSPO and 5-HTT polymorphisms regarding pain severity. While the combination of genetically inferred TSPO HAB and high or intermediate 5-HTT expression was associated with higher pain intensities, the opposite was true for 5-HTT low expressing individuals. To our knowledge, this is the first evidence of a genetic functional polymorphism affecting pain severity in FM patients. Furthermore, cerebral activation patterns during evoked pressure pain differed between TSPO HAB and MLAB patients. A functional connectivity analysis revealed that TSPO HAB was associated with higher pain related functional connectivity, between the dlPFC and the parietal cortex. These structures constitute the frontoparietal network and are likely to be of major importance for vigilance as well as the anticipation and appraisal of pain (Kong et al., 2013; Wager and Atlas, 2015). In sum, we combine genetics and fuctional imaging to suggest that TSPO-related mechanisms affect FM severity, possibly by influencing the anticipatory and affective-motivational components of cerebral pain processing.

4.1. The influence of the TSPO functional polymorphism on FM symptoms

TSPO is widely expressed throughout the body and plays an important role in integrating hormon- and redox-sensivite pathways. It exerts regulatory effects on processes such as steroidogenesis, hormone synthesis, modulation of immune/inflammatory processes and energy metabolism, particularly during oxidative stress (Gatliff and Campanella, 2016). Our finding that FM symptom severity was associated with the functional polymorphism of TSPO (*rs6971*) can therefore hypothetically be explained by peripheral as well as central mechanisms. As peripheral abberations such as mitochondrial dysfunction with oxidative stress and low grade inflammation have been reported in FM patients (Cordero et al., 2010, 2013; Sánchez-Domínguez et al., 2015) peripheral TSPO effects could be of relevance. However, our finding that the TSPO polymorphism was associated with differences in cerebral pain related functional connectivity would suggest the involvement also of central mechanisms, such as glia cell activation. The latter is consistent with the report of elevated CSF concentrations of IL-8 in FM patients (Kadetoff et al., 2012, Kosek et al., 2015), a chemokine which is co-expressed with TSPO in glia cells and regulated by TSPO (Liu et al., 2016).

Previous studies have documented increased glia expression of TSPO in animal models of inflammatory (Hernstadt et al., 2009) and neuropathic (Liu et al., 2016; Wei et al., 2013) pain. In these pain models, TSPO agonists have analgesic and anti-hyperalgesic effects, mediated by steroid/neurosteroid synthesis (Liu et al., 2016). Similar results were found in a human positron emission tomography study where the comparison between ten patient-control pairs indicated an inverse correlation between TSPO binding in the thalamus and pain severity in chronic low back pain patients (Loggia et al., 2015). Therefore, our result showing more severe pain and higher impact of FM symptoms in TSPO HAB than MLAB may at first seem contra-intuitive. Yet, it is important to note that TSPO effects may vary considerably with neurochemical context and with time. Thus, there is evidence that the duration of the painful condition affects the physiological effects of TSPO agonists (Liu et al., 2016). Whereas TSPO may have analgesic effects and promote recovery in the earlier stages when pain is still localized, this may not apply once chronic widespread pain has developed. Furthermore, given that the pain modulatory effects of TSPO are mediated by neurosteroids synthesis (Liu et al., 2016), the net effect will depend on the ratios of positive and negative GABA-A receptor modulators, respectively. FM patients have elevated CSF concentrations of substance P (Russell et al., 1994; Vaeroy et al., 1988), which dose-dependently inhibit the synthesis of analgesic neurosteroids (Patte-Mensah et al., 2014). Finally, serotonergic tone influences neurosteroid synthesis, with low tone favouring negative modulators (Pinna et al., 2006; Schüle et al., 2011) and FM patients have lower CSF concentrations of serotonin metabolites (Legangneux et al., 2001; Russell et al., 1992). The importance of the serotonergic mechanisms for TSPO effects are further supported by the significant interaction between the TSPO and *5-HTTLPR* polymorphisms regarding pain severity in FM.

4.2. The role of the 5-HTTLPR functional polymorphism in FM

The role of genetics has been extensively studied in FM (Ablin and Buskila, 2015). In a genome-wide association study (GWAS) the chromosomal region of 5-HTT was linked to FM in one study (Arnold et al., 2013), but not confirmed in a subsequent study (Docampo et al., 2014). It is important to note that the LPR as such, is a DNA repeat that varies in length to produce a polymorphism. This type of variation is not detected per se in GWAS studies, such that one would have to rely on SNPs in high linkage disequilibrium to detect a signal, a fact that may reduce statistical power. The *5-HTTLPR* (*rs25531*) has been reported in higher frequencies in FM patients compared to controls (Offenbaecher et al., 1999; Cohen et al., 2002), and was associated with psychological distress (Offenbaecher et al., 1999). However, in the present study, no effects of the triallelic *5-HTTLPR* polymorphism on FM symptoms were found when studied in isolation.

4.3. Interactions between the TSPO and the 5-HTTLPR polymorphisms

We found a statistically significant interaction effect between the TSPO and *5-HTTLPR* polymorphisms regarding pain severity in FM. In the TSPO MLAB group, *higher* pain severity was reported by FM patients with the low expressing 5-HTT genotype, compared to intermediate and high expressing, respectively. Paradoxically, in the TSPO HAB group, the 5-HTT low expressing genotype was associated with *lower* pain severity. The reduced serotonin transport seen in the 5-HTT low expressing genotype has been reported to be comparable to inhibition of 5-HTT during treatment with selective serotonin re-uptake inhibitors (SSRIs) (Serretti et al., 2007). Since SSRIs promote the synthesis of neurosteroids with analgesic effects (Kawano et al., 2011; Pinna et al., 2006; Serretti et al., 2007), this mechanism could hypothetically explain why SSRIs can have beneficial effects on FM symptoms (Carville et al., 2008; Haüser et al., 2012). If, also the 5-HTT low expressing genotype favours the synthesis of neurosteroids with analgesic effects, then this would explain the reduced pain in TSPO HAB FM patients compared to MLAB seen in our study, since the impact would be more pronounced in TSPO HAB with an expected higher rate of neurosteroid synthesis.

4.4. Significance of the genetic findings and gene-to-gene interactions

To our knowledge this is the first report linking a functional genetic polymorphism to pain and symptom severity and demonstrating gene-to-gene interactions on symptom severity in FM. The finding that FM symptoms are associated with genetic variations of TSPO (*rs6971*) is consistent with the hypothesis of glia activation (Clauw, 2015; Kadetoff et al., 2012) and suggests that the ongoing drug development targeting TSPO associated neurosteroid mechanisms (Pinna et al., 2006) could be beneficial for treating FM. In addition, the significant interactions between TSPO and 5-HTT polymorphisms stress the importance to assess gene-to-gene interactions, which will hopefully result in more consistent, reproducible results. Furthermore, the results suggest that genotyping could become a valuable tool for patient stratification in treatment studies and form part of individualized medicine in clinical practice. For example, despite the fact that antidepressants play a major role in the treatment of pain in FM (Haüser et al., 2012), the analgesic response is typically very heterogenous (Jensen et al., 2014). Based on our results the most likely responders to SSRI/SNRI treatment regarding pain relief would be found among FM patients with the TSPO HAB genotype. The latter was supported by the lack of statictically significant difference in SF-36 BP between TSPO HAB and MLAB among patients taking antidepressants, as opposed to those who were not. This hypothesis should be tested in a large clinical trial.

4.5. Neuroimaging results related to the TSPO polymorphism

In line with the effects of the TSPO polymorphism on pain reports, there was a segregation of functional brain connectivity depending on TSPO genotype, when comparing differences in cerebral responses to pressure pain. Using a liberal statistical threshold (i.e. uncorrected for multiple comparisons), there were indications that MLAB patients displayed higher activation in nociceptive and pain modulatory areas, compared to HAB patients, including somatosensory cortices, dACC and dlPFC. Our univariate data indicate that the TSPO polymorphisms may influence cerebral processing of pain in FM patients, but need to be replicated in a larger cohort before more definite conclusions can be drawn.

Psychophysiological interaction (PPI), including anatomical seed coordinates in the dlPFC and PAG, was used to examine if these regions displayed differences in functional connectivity depending on TSPO genotype. We found significantly increased pain-related connectivity in the frontoparietal network in TSPO HAB individuals, compared to MLAB, using the dlPFC as seed region. The frontoparietal network includes the dlPFC and parietal cortex and has been implicated in expectancy-induced modulation of pain (Kong et al., 2013; Wager and Atlas, 2015). More specifically, it has been suggested that the frontoparietal network integrates information from the external environment with stored internal representations, and controls top-down attention during conflicting sensory processing (Kong et al., 2013).

Interestingly, the frontoparietal network (Wager et al., 2011) as well as dlPFC (Wager et al., 2004) have been implicated in placebo analgesia, supporting their role in endogenous pain regulation. However, the analgesic placebo effects involving activation of dlPFC were also reflected as increased connectivity between these structures and PAG (Wager and Atlas, 2015). The fact that PAG did not display any differences in pain-evoked connectivity between groups is in line with the lack of difference in pressure pain sensitivity between the TSPO HAB and MLAB groups. Thus, our results do not support the notion that the TSPO polymorphism modulates top-down regulation of nociceptive input, i.e., the more sensory-discriminative aspects of nociception. Rather, our findings suggest that TSPO may affect cognitive and affective-motivational aspects of pain, as TSPO HAB patients reported larger negative impact of pain, and more intense clinical symptoms.

4.6. Limitations

The study has several limitations. First, we assessed the genetically inferred binding affinity to TSPO as well as the genetically inferred expression of 5-HTT only. Also due to the limited number of patients examined by fMRI, the gene-to-gene interactions regarding cerebral pain processing could not be assessed. The data presented are baseline data from subjects enrolled in a clinical trial assessing the effects of exercise on FM symptoms, therefore we can not exclude a certain bias towards less disabled subjects, compared to FM patients typically seen at specialized pain clinics.

4.7. Conclusions

Female FM patients with genetically inferred TSPO HAB reported higher pain severity and more severe FM symptoms compared to MLAB. To our knowledge this is the first report of a functional genetic polymorphism affecting pain severity in FM. There was further a strong gene-to-gene interaction between the TSPO and the 5-HTTLPR polymorphisms, indicating the modulatory importance of serotonergic mechanisms. Finally, the TSPO polymorphism was associated with different pain related cerebral functional connectivity patterns, suggesting an effect of TSPO related mechanisms on the affective-motivational components of pain perception.

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Declaration of interest

None declared.

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