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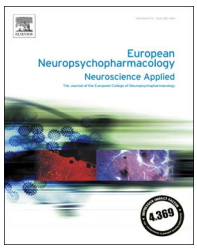

Raloxifene increases prefrontal activity during emotional inhibition in schizophrenia based on estrogen receptor genotype

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Abstract
People with schizophrenia show decreased prefrontal cortex (PFC) activity during emotional response inhibition, a cognitive process sensitive to hormonal influences. Raloxifene, a selective estrogen receptor modulator, binds estrogen receptor alpha (ESR-α), improves memory, attention and normalizes cortical and hippocampal activity during learning and emotional face recognition in schizophrenia. Here, we tested the extent to which raloxifene restores neuronal activity during emotional response inhibition in schizophrenia. Since genetic variation in estrogen receptor alpha (ESR-1) determines cortical ESR-α production and correlates with cognition, we also predicted that genetic ESR-1 variation would differentially relate to increased cortical activity by raloxifene administration. Thirty people with schizophrenia participated in a thirteen-week randomized, double-blind, placebo-controlled, cross-over adjunctive treatment trial of raloxifene administered at 120 mg/day. Effects of raloxifene on brain activation were assessed based on ESR-1 genotype using functional magnetic resonance imaging during emotional word inhibition. Raloxifene increased PFC activity during inhibition of response to negative words and the raloxifene related increased PFC activity was greater in patients homozygous for ESR-1 rs9340799 AA relative to G carriers. Comparison to 23 healthy controls demonstrated that PFC activity of people with schizophrenia receiving raloxifene was more similar to controls than to their own brain activity during placebo. Estrogen receptor modulation by raloxifene restores PFC activity during emotional response inhibition in schizophrenia and ESR-1 genotype predicts degree of increased neural

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activity in response to raloxifene. While these preliminary results require replication, they suggest the potential for personalized pharmacotherapy using ESR-1 and estrogen receptor targeting compounds in schizophrenia.

1. Introduction

Cognitive deficits, particularly impaired executive control processes related to response inhibition and working memory, are core symptoms of schizophrenia (Ravizza et al., 2010; Weickert et al., 2000), have been linked to impaired prefrontal cortex (PFC) function (Barch et al., 2001) and worse prognosis (Green et al., 2000). Decreased PFC activity has been routinely reported in people with schizophrenia relative to healthy controls during response inhibition and working memory (Glahn et al., 2005; Vercammen et al., 2012). Since antipsychotics are unable to restore cognition or dysfunctional cortical brain activity in people with schizophrenia, there is an urgent need to identify treatments that can restore the underlying neural processes and improve attention and memory which are related to functional capacity.

In light of the need for novel cognitive treatments in schizophrenia, we have recently reported that the selective estrogen receptor modulator (SERM), raloxifene, improved attention and memory in men and women with schizophrenia (Weickert et al., 2015). While these results are encouraging, the neural mechanisms underlying raloxifene based improvement are only beginning to be identified since different cognitive processes are often dependent upon specific brain regions to various degrees. Thus, we have shown that adjunctive raloxifene administration can improve brain activity in two distinct brain regions (hippocampus and inferior frontal gyrus) on the basis of distinct cognitive tests: one involving implicit learning and one involving emotional face recognition (Ji et al., 2016; Kindler et al., 2015). However, we do not know the extent to which adjunctive raloxifene will restore PFC neural activity during emotional word inhibition in schizophrenia.

There is abundant evidence for emotional processing impairments in schizophrenia, with deficits during basic emotion recognition, emotional memory, the experience of affective states (Schneider et al., 1995), and the recognition and interpretation of negative emotional content (Kohler et al., 2003). The emotional go/no-go word inhibition test is considered to be a complex emotional task that requires the recognition, processing and inhibition of responses to written words with negative valence which assesses the emotion-cognition interaction and robustly activates the PFC (Vercammen et al., 2012) in healthy participants. Our lab and others have shown that people with schizophrenia have impaired accuracy and blunted PFC activity during response inhibition (Ravizza et al., 2010; Vercammen et al., 2012; Weisbrod et al., 2000). Furthermore, PFC activity during response inhibition to emotional words is sensitive to sex hormones, as activity correlates with circulating estrogen levels in healthy females (Amin et al., 2006) and with circulating testosterone in men with schizophrenia (Vercammen et al., 2013). This suggests that sex hormones, both estrogen and testosterone (which can be converted to estrogen), may play a role in modulating cortical activity during evaluating and directing responses to emotive stimuli and suggests that the emotional go/no go test is an ideal cognitive task to test the neural response of a novel sex steroid receptor treatment.

Evidence ranging from clinical behavioral data to molecular neuropathology suggests that estrogen signaling may be altered in the brains of people with schizophrenia. The onset of schizophrenia often occurs during late adolescence (Lieberman et al., 2001), there are sex differences in age of onset, symptoms of psychosis (Shtasel et al., 1992), and course of schizophrenia (Gur et al., 1996) and low estrogen/testosterone levels in females/males with schizophrenia have been related to worse negative symptoms and more impaired cognition (Ko et al., 2006; Moore et al., 2013). Estrogen is a potent regulator of both emotional responses (Amin et al., 2005) and PFC activity (Berman et al., 1997), and estrogen therapy improves recovery from acute psychotic symptoms and reduces positive symptoms and general anxiety in females with schizophrenia (Kulkarni et al., 2014). SERMs activate estrogen receptors in brain and bone but do not stimulate estrogen receptors in breast or uterine tissue; therefore, reducing the risk of some adverse events (Lonard and Smith, 2002). Raloxifene, a SERM approved for use in postmenopausal women for osteoporosis, has been shown to be effective in enhancing brain activity and maintaining cognition in older adults (Goekoop et al., 2006; Yaffe et al., 2005). Raloxifene administration can also reduce negative symptom severity (Usall et al., 2015), has beneficial effects on memory and executive function in postmenopausal women with schizophrenia (Huerta-Ramos et al., 2014), and improves attention and memory in younger men and women with schizophrenia (Weickert et al., 2015) supporting the need for a greater understanding of the mechanism by which raloxifene improves cognition in schizophrenia. Furthermore, we found approximately 40% of men and women with schizophrenia showed cognitive improvement with raloxifene administration (Weickert et al., 2015), suggesting that there is heterogeneity in the response to raloxifene treatment. Thus, identifying biomarkers that predict the neuronal response to raloxifene would be of clinical value.

The neuronal response to pharmacological treatment in humans can vary depending on functional genotypes for the target receptor. The estrogen receptor-α gene (ESR1), located on chromosome 6q25.1, undergoes complex transcriptional regulation and alternative splicing (Hirata et al., 2003). While the ESR1 gene contains many variants, two DNA changes of primary interest are two ESR1 single nucleotide polymorphisms (SNPs) in intron 1: rs9340799 (A/G) and rs2234693 (T/C) which are in strong linkage disequilibrium (Becherini et al., 2000) and were related to

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cognitive performance in independent studies (Ryan et al., 2013; Yaffe et al., 2009, 2002). The G and C alleles of rs9340799 and rs2234693, respectively, are considered risk alleles for cognitive deficits and schizophrenia (Sundermann et al., 2010; Weickert et al., 2008). Genetic variation within intron 1 is associated with reduced ESR-α mRNA levels in the PFC of men and women with schizophrenia (Perlman et al., 2005; Weickert et al., 2008). Both ESR1 intron 1 SNPs (rs9340799 and rs2234693) are functional and regulate the level of gene transcription (Maruyama et al., 2000).

Our first aim was to determine the extent to which adjunctive raloxifene treatment at 120 mg daily alters neuronal activity during emotional response inhibition in men and women with schizophrenia. Our hypothesis was that administration of the SERM raloxifene would improve emotional response inhibition and increase PFC neural activity in both men and women with schizophrenia. Additionally, here, we report results of the first "pharmaconeuroimaging genomics" study of ESR1 to determine the extent to which the effect of adjunctive raloxifene therapy on brain activity varies depending on ESR1 genotype. Specifically, we predicted the G and C alleles of ESR1 rs9340799 and rs2234693, respectively would be associated with less PFC activity and decreased performance in people with schizophrenia administered adjunctive raloxifene during emotional response inhibition.

2. Experimental procedures

2.1. Overall organization of this study

There were three separate and main comparisons in the present study. First, raloxifene and placebo was administered to men and women with schizophrenia using a randomized, double-blind, placebo-controlled, cross-over design to determine the extent to which raloxifene influenced brain activity during emotional inhibition in schizophrenia. A secondary analysis in that trial assessing the effects of raloxifene on brain activity was performed in people with schizophrenia on the basis of their ESR-1 genotype. Finally, in a separate series of analyses, people with schizophrenia receiving raloxifene and the same people with schizophrenia receiving placebo were compared to a group of healthy adults (not receiving medication) to determine the extent to which raloxifene administration to people with schizophrenia can normalize brain activity in schizophrenia during emotional inhibition.

2.2. Participants

Thirty chronically ill adult patients with schizophrenia or schizoaffective disorder (18–55 years of age) were recruited for participation in the study. These patients were a subset of patients from our larger trial in which the CONSORT diagram was provided (Weickert et al., 2015). See Figure 1 for a flow diagram describing this neuroimaging study. See Table 1a for the demographics and clinical characteristics of the patients on basis of treatment order. All patients met the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria for schizophrenia or schizoaffective disorder on the basis of the Structured Clinical Interview for DSM-IV Axis 1 disorders (SCID) (First, 2007) which was administered by a trained psychiatrist or psychologist.

All patients were screened for exclusion criteria, which included a concurrent DSM-IV Axis I diagnosis other than schizophrenia or schizoaffective disorder, history of uncontrolled diabetes or cardiovascular disease including hypertension, recent alcohol/substance abuse (within the past 5 years), head injury with loss of consciousness, epileptic seizures, structural brain abnormalities, developmental disorders, mental retardation and/or central nervous system infection. A four subtest version of the Wechsler Adult Intelligence Scale, 3rd Edition (WAIS-III) (Wechsler, 1997) and the Wechsler Test of Adult Reading (WTAR) (Wechsler, 2001) were administered to all patients to obtain estimates of current IQ and

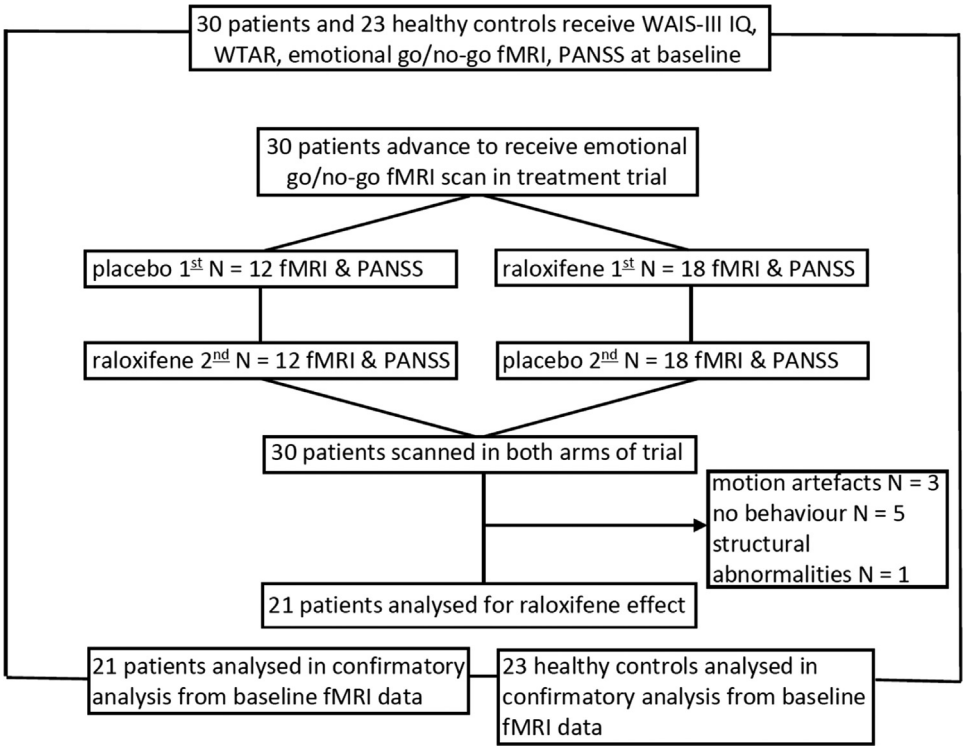

Figure 1 Flow diagram describing the neuroimaging study.

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Table 1a Demographic summary of patients in raloxifene treatment trial on basis of treatment order.

	Schizophrenia total $n=21$ baseline	placebo treatment first	raloxifene treatment first	df	t value	p
Age	35.5 (7.3)	—	—	—	—	—
Gender	13 m/ 8f	—	—	—	—	—
Education	13.6 (2.4)	—	—	—	—	—
WAIS-III IQ	95.5 (14.2)	—	—	—	—	—
WTAR	107.5 (8.4)	—	—	—	—	—
Age of Onset	22.8 (4.1)	—	—	—	—	—
Duration of illness	12.8 (7.7)	—	—	—	—	—
CPZ	479.6 (409.3)	484.0 (424.8)	448.3 (387.0)	20	−1.4	0.18
PANSS total	62.6 (19.9)	61.0 (16.7)	59.4 (16.4)	20	−1.1	0.37
PANSS pos	15.4 (4.9)	15.2 (5.5)	14.1 (4.8)	20	−2.3	0.09
PANSS neg	13.8 (6.6)	13.5 (5.6)	13.0 (5.0)	20	−0.9	0.37

Table 1b Demographic comparison of healthy controls and patients with schizophrenia.

	Healthy controls $n=23$	Schizophrenia total $n=21$	df	t value/ Chi-square	p
Age	33.4 (7.6)	35.5 (7.3)	42	−0.91	0.37
Gender	11m/12f	13m/ 8f	42	0.9	0.35
Education	15.7(1.8)	13.6 (2.4)	42	3.3	0.002*
WAIS-III IQ	114.2(15.4)	95.5 (14.2)	42	4.3	0.001*
WTAR	112.1(4.8)	107.5 (8.4)	42	2.3	0.028*

Notes: means with ± standard deviations in parentheses. CPZ = mean daily chlorpromazine equivalent dose. PANSS = Positive And Negative Syndrome Scale. WAIS-III = Wechsler Adult Intelligence Scale, 3rd Edition. WTAR = Wechsler Test of Adult Reading. Healthy controls were compared to schizophrenia patients at baseline. * $p < 0.05$ FDR corrected.

premorbid IQ. All patients were receiving antipsychotic medication (82% receiving second-generation antipsychotics) for at least 1 year prior to participation. Mean daily dose of antipsychotic medication for each person with schizophrenia was converted to approximate daily mean chlorpromazine milligram equivalents (CPZ) dose using standard guidelines (Leucht et al., 2003). Symptom severity in people with schizophrenia was assessed using the Positive and Negative Syndrome Scale (PANSS) (Kay et al., 1987) by a psychologist or psychometrician trained in administration and scoring.

Additionally, twenty-three healthy adults were scanned by functional magnetic resonance imaging (fMRI). See Table 1b for demographics of the healthy adult group versus patients. Exclusion criteria for healthy controls included a personal history of or a first-degree relative with a DSM-IV Axis I diagnosis, history of uncontrolled diabetes or cardiovascular disease including hypertension, recent alcohol/substance abuse (within the past 5 years), head injury with loss of consciousness, epileptic seizures, structural brain abnormalities, developmental disorders, mental retardation and/or central nervous system infection.

All participants had normal vision or their vision was corrected to normal with MRI compatible lenses. All procedures were approved by the University of New South Wales and the South Eastern Sydney and Illawarra Area Health Service Human Research Ethic Committees. The procedure was explained and written informed consent was obtained from each participant before entry into the study.

2.3. Raloxifene treatment

During the first six-week phase of a thirteen week, randomized, placebo-controlled, double blind, cross- over trial schizophrenia patients received 120 mg encapsulated raloxifene (2 × 60 mg, oral administration) daily in the active condition or oral administration of two placebo (lactose) capsules daily in the placebo condition as an adjunctive treatment to their current antipsychotic medication. Following treatment in the first 6-week phase, patients entered a one-week wash-out, raloxifene half-life=27.7 h, (Lilly, 2007) followed by the second 6-week phase consisting of the alternate treatment (raloxifene or placebo). Several studies have shown beneficial effects of raloxifene treatment at a daily dosage of 120 mg orally to reduce symptom severity in postmenopausal females with schizophrenia (Kulkarni et al., 2010) or to improve or preserve cognition and maintain brain activity in healthy aging (Goekoop et al., 2006).

Patients were monitored on a weekly basis to assess potential adverse events and self-reported compliance (compliance was also determined by returned pill counts). Additional methods for monitoring treatment compliance were achieved through the collection of blood samples for circulating hormones and clotting factors affected by treatment at baseline, 6 and 13 week assessments. The trial was registered prior to initiation with the Australian and New Zealand Clinical Trials Registry (ANZCTR number: 12608000461392).

2.4. fMRI methods

2.4.1. Scanning procedure

A 3 T Phillips Achieva MRI scanner with an 8 channel birdcage head coil at Neuroscience Research Australia, Randwick, Australia was used to collect imaging data. In total, 162 whole-brain EPI images, TR/TE=3000/30; 45 interleaved slices, thickness=3 mm, gap=0.3 mm; voxel size 2.14 × 2.14 × 3 mm; flip angle=90°; FOV=240 mm were acquired. A T1-weighted high-resolution anatomical scan was

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obtained for each participant for registration purposes, TR/TE = 5.3/2.4; 180 slices, thickness = 1 mm, no gap; voxel size 1 × 1 × 1 mm; FOV = 256 mm.

2.4.2. Emotional Go/No-Go task

All participants received detailed verbal instructions before the fMRI scan and the experimenter verified that the participant had an adequate comprehension of the procedure. Details of the emotional go/no-go task have been reported elsewhere (Amin et al., 2006; Vercammen et al., 2012, 2013). The task included 4 conditions: 1) attend negative: responding to negative words while inhibiting responses to neutral words, 2) inhibit negative: responding to neutral words while inhibiting responses to negative words, 3) attend positive: responding to positive words while inhibiting responses to neutral words, and 4) inhibit positive: responding to neutral words while inhibiting responses to positive words. The words were selected from the "Affective Norms for English Words" stimulus set (Bradley and Lang, 1999).

"Negative target/neutral distracter" and "neutral target/negative distracter" conditions were identical with respect to stimuli; and likewise for the "positive target/neutral distracter" and "neutral target/positive distracter" conditions. At the start of each condition, the words "neutral," "positive" or "negative" appeared in capital letters to indicate to which valence the participant was to attend and respond to. For the fMRI scan, participants were instructed to respond as quickly as possible to stimuli of the specified valence (targets), but to ignore stimuli of other valences (distracters). Each trial consisted of a fixation cross for 300 ms, a word stimulus presented in the centre of the screen for 300 ms and a 900 ms response interval. The 4 conditions were presented in pseudo-randomized order in a block design. Each block consisted of 10 stimulus presentations and each condition was presented 4 times for a total of 160 stimulus presentations. There were 30 seconds of fixation to a crosshair at the initiation and conclusion of the task. Stimuli were presented on an inverted computer screen that participants viewed in a mirror mounted in the head coil and responses were recorded using a fibre optic response pad (Lumina Systems) that recorded accuracy (percent correct) and reaction time (ms) data.

2.4.3. fMRI processing

All processing and analyses were performed with SPM8 (Wellcome Trust Centre for Neuroimaging) and MATLAB. We focused on words with negative valence, as negative emotion has been shown to have stronger interference effects on cognitive processing in schizophrenia, with robust activation differences between healthy controls and patients in the PFC (Vercammen et al., 2012) and sex steroid hormones have been shown to modulate PFC activity during inhibition of negative words (Vercammen et al., 2013).

All data sets were screened for excessive motion (>3 mm in x, y or z directions or $>3^{\circ}$ rotation) and magnetic resonance imaging artifacts. Movement parameters were included as regressors in the first-level model. Three dummy scans were obtained before each fMRI data acquisition to allow for the equilibration of the MRI signal. Functional images were realigned to the first image in the time series and co-registered to the anatomical image. All images were normalized to the Montreal Neurological Institute (MNI) anatomical template using a nonlinear 12-parameter affine transformation. Images were smoothed with a 10-mm full width half maximum Gaussian kernel.

2.5. Genotyping and fMRI analyses based on ESR1 genotype

Genotyping was performed using Applied Biosystems (Mulgrave, VIC, Australia) TaqMan SNP assays designed for use with an ABI Prism 7900HT Fast Real Time quantitative PCR system for the ESR-1 SNPs rs2234693 (T/C) and rs9340799 (A/G). All SNPs were in Hardy-Weinberg equilibrium. The genotype distribution in patients (n=21) for rs9340799 was AA n=9, AG n=9, GG n=3, and for rs2234693: CC n=6, TC n=11, TT n=4. Thus, for rs9440799, homozygous G and heterozygous AG were combined to form a G carrier group, and for rs2234693, homozygous T and heterozygous TC were combined to form a T carrier group.

DNA was isolated from 8 ml samples of whole blood collected in EDTA tubes using a PUREGENE DNA purification kit (QIAGEN, Chadstone Centre, VIC, Australia) following the manufacturer's protocols. Genomic DNA from each individual was prepared at a dilution of 10 ng/μl. A PCR solution consisting of 2.5 μl of 2 × Universal mastermix with ROX, 0.125 μl genotyping probe and 0.375 μl double-distilled H2O was prepared, added into a 384-well plate containing 1 μl of genomic DNA from each sample and pipetted up and down to ensure the genomic DNA and PCR solution were sufficiently mixed. After PCR amplification, all SNP genotyping results were analysed with Sequence Detection Software version 2.3 (ABI, Life Technologies, Mulgrave, VIC, Australia).

2.6. Statistics

2.6.1. Demographic, symptom and behavioral analyses

Fisher's exact tests were calculated for nominal data, paired t-tests for continuous and normally distributed data comparing raloxifene and placebo, and independent two sample t-tests for continuous and normally distributed data comparing healthy controls separately to raloxifene or placebo conditions. Paired t-tests were performed on the mean percentage correct, mean percentage of false alarms and omissions, and on mean reaction times (RTs) for 'GO' trials between placebo and raloxifene conditions. Independent two sample t-tests were performed on the mean percentage correct, mean percentage of false alarms and omissions, and on mean RTs during 'GO' trials comparing healthy controls separately to patients receiving placebo or raloxifene. Symptom severity as measured by PANSS total, positive and negative symptom severity scores during raloxifene and placebo conditions were compared with paired t-tests. Results were false discovery rate (FDR) corrected for multiple testing. Significance level was set at $p<0.05$, two-tailed. Statistics were performed in SPSS 20.0.

2.6.2. fMRI analyses

Emotional Go/No-Go task, scanning procedure and fMRI preprocessing were identical for healthy controls and patients with schizophrenia. In the patients, scans during active and placebo conditions were obtained at the conclusion of weeks 6 and 13. We excluded one patient because of incidental findings of abnormalities on structural MRI, three patients because of excessive movement, and five patients due to insufficient behavioral data, yielding 21 patients in the within-subjects design analysis. To avoid circularity between our main analysis (raloxifene treatment in schizophrenia) and our functional ROI analysis, the functional ROIs were derived from an independent sample of twenty-three healthy controls using a one-sample t-test of activated regions during the inhibition of negative versus neutral words in healthy adults to design the bilateral PFC mask region of interest (ROI) consisting of BA10 and BA9, see Figure 2 and Vercammen et al. (2012). At the first level of analysis, the contrast of interest was defined as condition 2 (inhibit responses to negative words) minus condition 1 (inhibit responses to neutral words) to assess the magnitude of the difference in blood oxygenation level-dependent (BOLD) signal for inhibiting responses to negative words. Significance level was set at $p<0.05$, FDR corrected.

At the second level analysis, a paired t test was performed comparing the raloxifene condition to the placebo condition with performance as a covariate. Significant differences were threshold free cluster enhancement (TFCE) family wise error (FWE) and small volume corrected ($p<0.05$) with a mask for the region of interest

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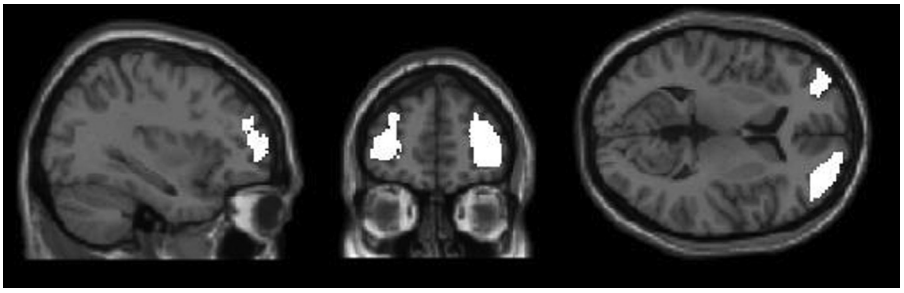

Figure 2 PFC mask BA 9 and 10 based on regions activated during inhibition of negative versus neutral words in healthy adults. DLPFC mask in white. The mask was designed from the one sample *t*-test of healthy participants, FDR corrected, $p<0.05$, confined to prefrontal cortex (Vercammen et al., 2012).

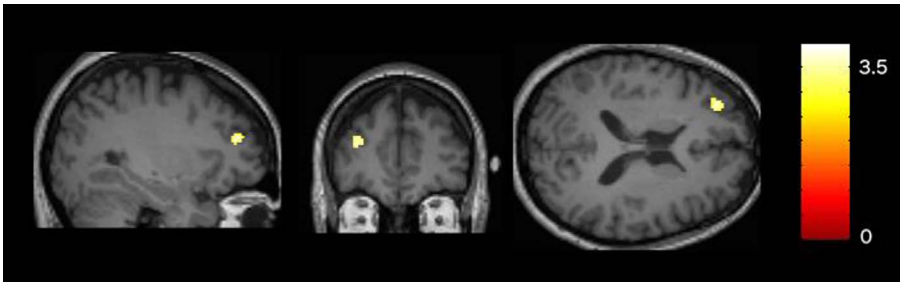

Figure 3 Effects of raloxifene relative to placebo on BOLD activity during inhibition of negative words in people with schizophrenia. Raloxifene > placebo, $p<0.005$, cluster size > 50 (whole brain, uncorrected, for visualization). x/y/z= −30/44/20, $T=4.17$, $Z=3.47$, BA10, 59 voxels, left dorsolateral prefrontal cortex. Yellow areas show significantly increased BOLD signal in the raloxifene treatment group as compared to placebo.

(PFC). To evaluate sex differences on fMRI BOLD signal, a sex-by-drug treatment interaction was assessed in SPM, using a flexible factorial design. Sex differences with respect to the response to raloxifene (raloxifene - placebo treatment) were also evaluated for behavioral measures (mean percent correct and mean reaction time).

2.6.3. fMRI, genotype and raloxifene treatment analyses

Genotype effects were modeled and tested within SPM performing a two-sample *t*-test (raloxifene-placebo difference). Allelic combinations were grouped in order to increase the sample size of the minor allele frequency groups for comparison, rs9340799: AA (n=9) versus G carriers (n=12), rs2234693: CC (n=6) versus T carriers (n=15). The results of an independent whole-brain analysis, representing the largest sample of healthy adults (N=43) performing the emotional go/no-go task in fMRI to date, contrasting the conditions "inhibit negative-attend neutral" (Vercammen et al., 2014) showed increased activation in healthy controls during the inhibition of responses to negative words relative to neutral words in a predominantly prefrontal network of brain regions with a peak at x/y/z = 28/52/10 (right middle frontal gyrus, BA 10). Thus, a small volume correction of our genotype results was performed at x/y/z = 28/52/10 with a sphere of 10 mm. Additionally, effects of rs2234693 and rs9340799 genotypes on behavioural measures (mean percent correct and mean reaction time) were also evaluated (raloxifene - placebo treatment, difference score).

2.6.4. fMRI healthy control versus schizophrenia raloxifene and placebo condition analyses

Given the increased activity in the PFC with raloxifene treatment (see Results), BOLD signal beta weights were also extracted from healthy participants (n=23) for separate comparisons of healthy controls with patients receiving raloxifene and the same patients during placebo treatment. Paired t-tests of raloxifene versus placebo and independent two sample t-tests of healthy controls versus patients receiving raloxifene and healthy controls versus patients receiving placebo were performed. All results were FDR corrected for multiple testing.

3. Results

3.1. Demographics

For demographics and symptom severity of the patients in the raloxifene treatment trial see Table 1a. All patients were chronically ill and displayed mild to moderate symptom severity based on their PANSS scores. Demographic comparison of the patients with schizophrenia and healthy adults are provided in Table 1b.

3.2. Effects of raloxifene

3.2.1. Raloxifene increases PFC BOLD activity during emotional inhibition

Relative to placebo, raloxifene treatment was associated with increased fMRI BOLD activity in the left PFC (BA10, x/y/z= −30/44/20, T=4.17, Z=3.47, df=20, TFCE FWE small volume corrected, $p<0.05$, see Figure 3) during inhibition of responses to negative words in people with schizophrenia. Within the region of interest, patients receiving placebo did not display significantly greater BOLD activity than the same patients receiving raloxifene. No significant differences were detected between male and female patients for raloxifene versus placebo conditions in relation

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Table 2 Group means and standard deviations for behavioural performance parameters on the emotional Go/No-Go task in patients with schizophrenia during raloxifene and placebo treatment.

	Placebo treatment Inhibit negative-respond neutral	Raloxifene treatment Inhibit negative-respond neutral	*t*-value	*p*-value
RT	1075 (280)	1098 (310)	−0.70	0.49
Accuracy	73 (9)	67 (10)	1.80	0.34
False alarms	0.7 (0.7)	1.0 (0.9)	−1.44	0.34
Omissions	2.0 (0.9)	2.2 (1.0)	−0.77	0.49

Reaction time (RT), mean (sd) in milliseconds; Accuracy: mean (sd) in percent correct; False alarm errors: mean number (sd); Omissions: mean number (sd); df=20.

Table 3 Group means and standard deviations for behavioral performance parameters on the emotional Go/No-Go task at week 6 versus week 13 regardless of treatment condition, testing for order effects.

	Time 1 Inhibit negative-respond neutral	Time 2 Inhibit negative-respond neutral	t-value	p-value
RT	1087 (303)	1086 (288)	0.02	0.98
Accuracy	66 (7)	74 (11)	−3.3	0.006
False alarm errors	0.8 (0.9)	0.9 (0.8)	−0.42	0.91
Omissions	2.6 (0.9)	1.7 (0.7)	5.3	0.004

Notes: Reaction time (RT), mean (sd) in milliseconds; Accuracy: mean (sd) in percent correct; False alarm errors: mean number (sd); Omissions: mean number (sd) paired *t*-test, df=20.

to BOLD signal in the ROI (no significant voxels at $p<0.001$ in F or t contrasts within the ROI).

3.2.2. Symptom severity and behavior analyses

Similar to our finding in our larger sample, raloxifene treatment had no significant effects on PANSS total, PANSS negative or PANSS positive symptom severity scores in this subset of participants (Table 1a). In relation to the emotional go/no-go task, raloxifene treatment had no significant effect on reaction time, no significant effect on accuracy, omissions or false alarms during inhibition of negative words (see Table 2 for means and standard deviations in relation to performance). A separate analysis of behavior performance at week 6 versus week 13 regardless of treatment condition (raloxifene or placebo) was performed to test for order effects (see Table 3). A significant improvement between week 6 and week 13, regardless of treatment condition, was obtained in relation to performance (see Table 3). Patients receiving raloxifene and patients receiving placebo were also compared to healthy controls in relation to percent correct, false alarms, omissions, and RT during "Go trials." There were significant differences between healthy controls and patients receiving raloxifene and between healthy controls and patients receiving placebo in relation to the performance variables (see Table 4). No significant differences were found between male and female participants in raloxifene versus placebo conditions in relation to performance accuracy (raloxifene - placebo difference score, males versus females, t=0.313, df=19, $p=0.76$) or reaction time (raloxifene-placebo difference score, males versus females, t=1.18, df=19, p=0.25).

3.2.3. ESR-1 genotype predicts increased PFC BOLD activity to raloxifene treatment

In the raloxifene relative to placebo comparison, patients with schizophrenia who had ESR-1 genotype rs9340799 A/A (n=9) showed significantly increased fMRI BOLD activity in bilateral PFC (middle frontal gyrus, BA 10) relative to G allele carriers (n=12) during inhibition of responses to negative words (see Figure 4). The signal in the right middle frontal gyrus (x/y/z = 32/58/14) remained significant after small volume and FWE correction ($p < 0.041$). Concurrent with the increased PFC activity in response to raloxifene, we also showed that those patients who were ESR-1 genotype rs9340799 A/A homozygotes showed significantly greater accuracy relative to G-carriers ($z=-2.6$, $p=0.04$). There was no significant genotype difference in relation to reaction time ($z=0.07$, $p=0.97$).

In contrast, patients with schizophrenia who varied on ESR-1 genotype rs2234693 did not show a significant difference in raloxifene related BOLD response, reaction time ($z=1.4$, $p=0.32$), or accuracy ($z=0.01$, $p=0.97$).

3.3. Comparison of healthy controls and patients during raloxifene and placebo treatment

To determine the extent to which the raloxifene-related BOLD activity approached normal levels, fMRI BOLD signal beta estimates in the left PFC (BA10, x/y/z=−30/44/20), were compared separately in healthy controls and patients with schizophrenia during raloxifene and placebo treatments (see Figure 5). When comparing healthy controls to patients receiving raloxifene, no significant differences were found ($t=0.36$, df=42, $p=0.72$); however, significantly less fMRI BOLD activity (deactivation) was found in these same patients receiving placebo relative to raloxifene treatment ($t=3.82$, df=20, $p=0.003$) and in the same patients receiving placebo compared to healthy controls ($t=3.14$, df=42, $p=0.005$).

4. Discussion

The results of this study demonstrate that estrogen receptor modulation with raloxifene increases PFC activity in schizophrenia during an emotional response inhibition task. Importantly, both performance accuracy and the fMRI BOLD

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Table 4 Group means and standard deviations for behavioral performance parameters of healthy controls and schizophrenia patients receiving raloxifene or placebo on the emotional Go/No-Go task.

	Healthy Controls	Schizophrenia placebo	t-value	*p*-value	Schizophrenia raloxifene	t-value	p-value
RT	942(163)	1075(280)	1.9	0.058	1098 (310)	2.1	0.055
Accuracy	82(11)	73(9)	−3.2	0.006	67 (10)	−4.7	0.002
False alarms	3.7(3.0)	0.7(0.7)	−4.5	0.001	1.0 (0.9)	−3.9	0.002
Omissions	3.4(2.9)	2.0(0.9)	−2.1	0.044	2.2 (1.0)	−1.8	0.086

Notes: Reaction time (RT), mean (sd) in milliseconds; Accuracy: mean (sd) in %; False alarm errors: mean number (sd); Omissions: mean number (sd), 2 sample *t*-test, df= 42.

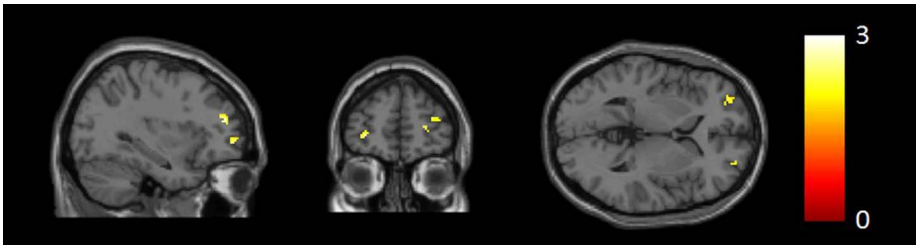

Figure 4 Raloxifene effects (raloxifene-placebo difference) on the basis of ESR-1 rs9340799 genotypes, 2 sample t-test, AA (n=9) versus G carriers (n=12). Yellow areas show significantly higher BOLD signal in AA homozygotes relative to G carriers (BA 9 & 10, bilaterally, $p < 0.005$, uncorrected).

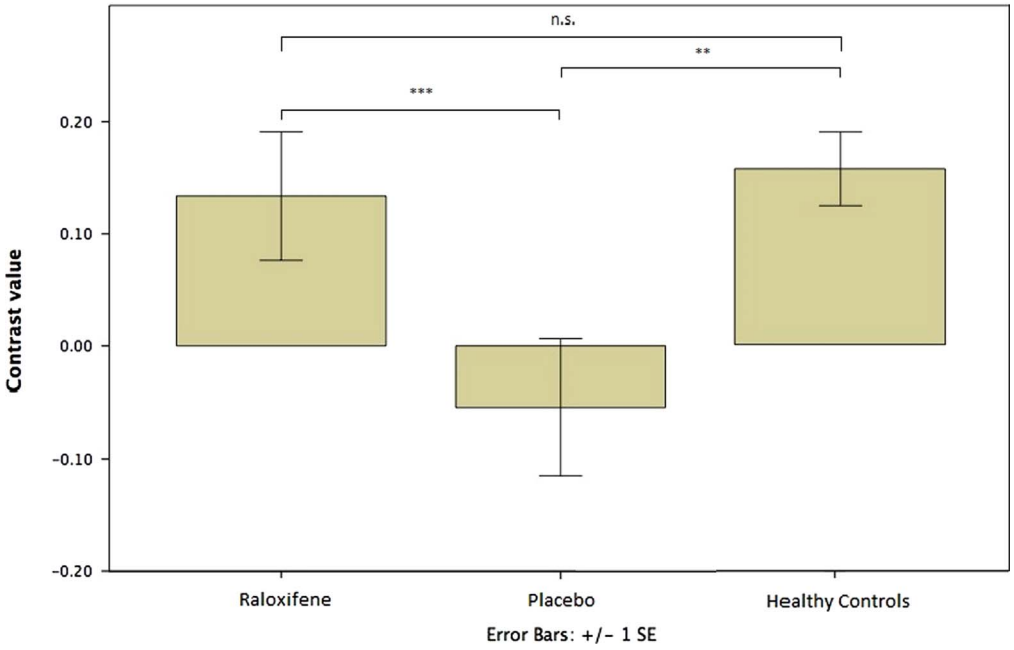

Figure 5 fMRI BOLD signal beta estimates (mean+/-sd) in dorsolateral prefrontal cortex, BA10, x/y/z= −30/44/20, showing raloxifene, n=21 (beta values 0.134±0.262), placebo, n=21 (beta values −0.054±0.278) and healthy controls, n=23 (beta values 0.157±0.158) comparisons. During raloxifene treatment patients activated the PFC (not significantly different than healthy controls) whereas during placebo treatment patients showed PFC deactivation. **p=0.005, ***p=0.003.

signal increased during inhibition of response to negative words while receiving raloxifene for the ESR-1 rs9340799 A/A genotype, demonstrating a pharmacogenetic interaction. However, we did not find a behavioral difference in response accuracy to negative words in the overall sample of people with schizophrenia during raloxifene administration, suggesting that assessing neuronal responses at the level of cortical physiology may either be a more sensitive indicator of treatment response (Bookheimer et al., 2000) or the physiological effect may be large enough to detect prior to any noticeable change in behavior and without taking genotype effects into account.

We have previously demonstrated that patients with schizophrenia show significantly attenuated BOLD response in the PFC

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during inhibition of negative words compared with healthy controls (Vercammen et al., 2012). The increase of BOLD signal with raloxifene treatment in the present study suggests restoration of PFC neuronal activity. The comparative analysis between patients and healthy controls performed here, demonstrates decreased BOLD signal in the PFC in patients receiving placebo relative to healthy controls, but an increase of PFC BOLD activity during raloxifene treatment relative to placebo, which we interpret here to be potentially restorative.

This supports our previous work showing that raloxifene administration increases activity in brain regions relevant to specific cognitive domains in schizophrenia, such as the inferior frontal gyrus during emotional face recognition (Ji et al., 2016). During implicit probabilistic association learning, raloxifene also increases activity in brain regions (e.g., hippocampus) of people with schizophrenia that are not typically elicited in healthy participants. However, people with schizophrenia who learn the associations without raloxifene treatment show increased activity in medial temporal lobe structures (Weickert et al., 2009) and this region overlaps with the region of activity during probabilistic association learning with raloxifene administration in schizophrenia (Kindler et al., 2015). Taken together, our results suggest that while widespread areas of the telencephalon can become more activated with raloxifene treatment, not all brain areas appear equally responsive to raloxifene in people with schizophrenia.

Our larger raloxifene trial demonstrated beneficial effects of raloxifene on attention in schizophrenia (Weickert et al., 2015). Both, attention and response inhibition are dependent on healthy PFC function (Asplund et al., 2010; Vercammen et al., 2012). As our trial used a within-subjects design, the brain response to raloxifene cannot be explained by differences in age, sex, baseline cognitive performance, or chlorpromazine equivalent dose, which did not differ significantly between treatment arms. There is a very limited number of reports on treatments that improve PFC activity/function in schizophrenia, such as transcranial direct current stimulation (Vercammen et al., 2011) or adjunctive medication (Minzenberg et al., 2015), whereas antipsychotics appear to have negative effects on PFC (Keedy et al., 2015).

Our results provide the first support for the use of ESR1 genotype to predict neural response to raloxifene. We find increased PFC activity with raloxifene treatment in people with schizophrenia who carry A/A (non-risk) genotype at rs9340799. This suggests that the rs9340799 AA genotype group may be more amenable to treatment via the ESR1 signaling pathway. The majority of case-control studies indicated an increased risk of cognitive impairment for both the G and C alleles of rs9340799 and rs2234693 respectively (Sundermann et al., 2010). At a molecular level, reductions in ESR-α mRNA in the frontal cortex of patients with schizophrenia have been reported in rs2234693 C carriers (Perlman et al., 2005; Weickert et al., 2008); however, both SNPs appear to control ESR-α transcription in vitro (Maruyama et al., 2000) and our results suggest that rs9340799 may have more penetrative effects in a raloxifene treatment context in humans. While these preliminary genotype based treatment response results may help to formulate beneficial treatment predictions to raloxifene based on ESR1 genotypes, further work confirming the utility of this particular functional ESR1 SNP in larger clinical trials and encompassing more diverse treatment outcomes would be needed to begin to tailor treatments to those likely to derive the most beneficial response based on this potential biomarker.

In addition to the limited sample size for a genetic analysis of this first pharmacoimagingenomics report of an ESR1 genetic impact on the brain response to raloxfiene treatment, there are some additional limitations to the present study. One concern with using a cross-over clinical trial design pertains to potential carryover effects. Although raloxifene has a half-life of only 27 hours, it may have long-term effects such as changing gene expression, thereby promoting neuronal protection, synaptic growth and a reduction of neuroinflammation (Wang et al., 2004). A learning effect or carryover effect (Yaffe et al., 2005) might explain the behavioral improvement that is sustained in the total group between week 6 and week 13, regardless of treatment condition. Future studies may want to use a parallel group design to assess potential long lasting effects of raloxifene. However, the groups were balanced for order of administration (i.e., active or placebo first) and any potential carryover effects would have reduced the effect of interest, i.e., comparing raloxifene to placebo. Thus, it seems unlikely that a putative carryover effect of raloxifene on BOLD response would be responsible for our findings of significantly increased PFC activity with raloxifene treatment. We did not detect differences in performance between raloxifene and placebo treatment in the overall sample. However, importantly, the effects of estrogen therapy may be more manifest at the physiological level as is supported by the concordance between BOLD activity and performance in the subgroup of patients with a greater response to raloxifene (ESR1 rs9340799 AA genotype). Pharmacoimaging directly assesses neurophysiological changes and may have a greater sensitivity to detect effects of raloxifene on brain activity, relative to behavioral tests alone. Previous studies have shown that BOLD activity can be more sensitive to neural change than behavioral measures alone and can predict cognitive decline (Bookheimer et al., 2000).

Overall, the results of the present study demonstrate that six weeks of the SERM raloxifene treatment increases PFC activity during emotional response inhibition in people with schizophrenia. The study provides direct evidence for the impact of the SERM raloxifene on brain activity in regions known to be important in the pathophysiology of cognitive deficits in schizophrenia. The strength of the response was modulated by specific genotypes as patients homozygous for the A allele of the ESR-1 rs9340799 genotype showed increased PFC activity with raloxifene compared to G carriers. Together, these results suggest the potential for a personalized pharmacotherapy aimed at using estrogen receptor targeting compounds to restore brain activity and cognition in schizophrenia.

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Contributors

Jochen Kindler performed the analyses and wrote the first draft of the manuscript. Cynthia Shannon Weickert designed the study (in part), and revised and edited the manuscript, Peter R. Schofield revised and edited the manuscript. Rhoshel Lenroot revised and edited the manuscript. Thomas Weickert designed the study (in part), wrote the protocol, and revised and edited the manuscript. All authors contributed to and approved the final manuscript.

Conflict of interest

Cynthia Shannon Weickert has consulted for pharma company Lundbeck and has received financial support for projects other than the project described in this report from pharma company Astellas. Peter R. Schofield reports receipt of speaker fees from Gartner Group. Thomas W Weickert's partner (Cynthia Shannon Weickert) has consulted for pharma company Lundbeck and has received financial support for projects other than the project described in this report from pharma company Astellas. All other authors declare they have no conflict of interest.

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