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Structural and Functional Brain Alterations in Patients with Irritable Bowel Syndrome

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Biomedical Engineering

by

Jui-Yang Hong

2015

ABSTRACT OF THE DISSERTATION

Structural and Functional Brain Alterations in Patients with Irritable Bowel Syndrome

by

Jui-Yang Hong

Doctor of Philosophy in Biomedical Engineering

University of California, Los Angeles, 2015

Professor Danny Jiong Jiong Wang, Co-Chair

Professor Emeran A. Mayer, Co-Chair

Irritable Bowel Syndrome (IBS) is the most common chronic visceral pain syndrome characterized by chronic abdominal pain/discomfort associated with altered bowel habits. Cognitive, affective and psychosocial factors such as hypervigilance, hypersensitivity and selective attention of visceral pain are commonly seen in IBS patients. Many studies have demonstrated IBS-related biological alterations in the immune system, gut microbiota, gene polymorphisms and hypothalamic-pituitary-adrenal system. However, these findings are not able to explain the subjective pain experience of IBS patients. By applying different brain imaging technology (e.g. Magnetic Resonance Imaging [MRI]) in pain research, multiple brain regions involved in emotional arousal, reward, attentional and cognitive processes have been identified during the past decade. Based on the extensive epidemiological, psychophysiological and neurobiological information about pain mechanisms, there is a growing consensus that the central

nervous system plays an important role in the pathophysiology of IBS symptoms, in addition to multiple other peripheral factors.

This dissertation aims to identify IBS-related structural and functional brain signatures, and to integrate these signatures and clinical characteristics into a comprehensive disease model, which takes sex-related differences into account. The following analysis approaches were used to accomplish these aims: 1. Fractional amplitude of low frequency fluctuation was applied to measure the power spectrum intensity of spontaneous brain oscillations during task-free resting-state functional MRI. 2. Functional connectivity analysis was performed to examine how different parts of the brain work together and to identify dysfunction of the communication in the disease group. 3. Task-based functional MRI study approach was used to study how brain responses to certain stimuli differ between individuals with IBS and healthy controls. In addition, to determine the disease specificity of observed brain networks, brain structural changes were compared between IBS patients and patients with inflammatory bowel diseases. Altogether, with different aspects of research approaches to investigate IBS, progress has been made to better understand the pathophysiology of the disorder.

The dissertation of Jui-Yang Hong is approved.

Ichiro Nishimura

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2015

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SELECT PUBLICATIONS

1. Hong JY, Kilpatrick LA, Labus JS, Gupta A, Katibian D, Ashe-McNalley C, Stains J, Heendeniya N, Smith SR, Tillisch K, Naliboff B, Mayer EA (2014) Sex and disease-related alterations of anterior insula functional connectivity in chronic abdominal pain. J Neurosci 34:14252-14259.

2. Hong JY, Labus JS, Jiang Z, Ashe-Mcnalley C, Dinov I, Gupta A, Shi Y, Stains J, Heendeniya

N, Smith SR, Tillisch K, Mayer EA (2014) Regional neuroplastic brain changes in patients with chronic inflammatory and non-inflammatory visceral pain. PLoS One 9:e84564.

3. Hong JY, Kilpatrick LA, Labus J, Gupta A, Jiang Z, Ashe-McNalley C, Stains J, Heendeniya N, Ebrat B, Smith S, Tillisch K, Naliboff B, Mayer EA (2013) Patients with Chronic Visceral Pain Show Sex-Related Alterations in Intrinsic Oscillations of the Resting Brain. J Neurosci 33:11994-12002.

4. Mayer EA, Gupta A, Kilpatrick LA, **Hong JY** (2015) Imaging brain mechanisms in chronic visceral pain. Pain 156 Suppl 1:S50-S63.

5. Gupta A, Kilpatrick L, Labus J, Tillisch K, Braun A, **Hong JY**, Ashe-McNalley C, Naliboff B, Mayer EA (2014) Early adverse life events and resting state neural networks in patients with chronic abdominal pain: evidence for sex differences. Psychosom Med 76:404-412.

6. Labus JS, Dinov ID, Jiang Z, Ashe-McNalley C, Zamanyan A, Shi Y, **Hong JY**, Gupta A, Tillisch K, Ebrat B, Hobel S, Gutman BA, Joshi S, Thompson PM, Toga AW, Mayer EA (2014) Irritable bowel syndrome in female patients is associated with alterations in structural brain networks. Pain 155:137-149.

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Chapter 1

Introduction

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder in developed countries, with estimated prevalence rates ranging from 7% to 20% (Keszthelyi et al., 2012b; Canavan et al., 2014; Chey et al., 2015). IBS is characterized by recurrent lower abdominal discomfort or pain associated with altered bowel habits and stool forms. Although the etiology of IBS is incompletely understood, multiple factors have been suggested to attribute to the pathophysiology of IBS including enhanced inflammatory mediators, altered intestinal microbiota, dietary factors, gastrointestinal motility, abnormal enteroendocrine cells, gene expression and polymorphisms (El-Salhy et al., 2014; Lee and Park, 2014). The autonomic and central nervous systems are also shown to be involved in the development of IBS symptoms (Keszthelyi et al., 2012a). A theoretical model, 'brain-gut axis', is widely used to describe the pathogenesis of IBS (Ohman and Simren, 2007; Coss-Adame and Rao, 2014).

The brain-gut axis involves top-down modulation from the central nervous system and bottom-up signaling from the gut (Mayer, 2011; Mayer and Tillisch, 2011). The central nervous system down-regulates the viscera and gastrointestinal tract via autonomic nervous system, sympatho-adrenal axis and hypothalamic-pituitary-adrenal axis (Mayer, 2011). The information about the gut (e.g. mechanical and chemical stimuli, microbial signals, toxins, nutrients, and inflammatory factors) is directly or indirectly transferred to the spinal cord, brainstem, cortical and subcortical areas through the enteric nervous system, immune-related signaling, endocrine signaling and neural signaling pathways (Mayer, 2011; Al Omran and Aziz, 2014). The bi-directional communication can be influenced by biophysiological, psychological and environmental factors (Surdea-Blaga et al., 2012). The pathophysiology of IBS is currently viewed

as a dysregulation of the brain-gut axis which describes the abnormal bi-directional communication between the central nervous system and the gut (Ohman and Simren, 2007; Fichna and Storr, 2012; Kennedy et al., 2012).

Like many functional gastrointestinal disorders, IBS is reported more frequently in women (Chang et al., 2006; Lovell and Ford, 2012). Although this can be associated with factors such as access to health care, social pressure and daily life events, women with IBS usually have more severe symptoms and higher comorbidities (Mayer et al., 2004; Cain et al., 2009; Voss et al., 2012). Studies also show female IBS patients have higher prevalence of constipation-predominant subtype while male patients are more likely to have IBS with diarrhea (Lovell and Ford, 2012). Moreover, sex-related differences within IBS patients have been found in autonomic nervous system, gastrointestinal motility, drug treatment, gene expression, hypothalamic-pituitary-adrenal axis, brain structure and activation (Labus et al., 2008; Mulak and Tache, 2010; Voss et al., 2012; Coss-Adame and Rao, 2014; Yan et al., 2014).

In the United States, estimated total costs including clinical visits, medications, examinations, surgery, hospitalizations, and absences from work range between $2,353 and $15,284 annually per IBS patient (Nellesen et al., 2013). Although treatments such as change of diet, antidepressants, antibiotics, probiotics, complementary and alternative medicine have been shown to relieve some IBS symptoms (Ford and Talley, 2012; Soares, 2014), overall successful curative level is far from satisfactory. This can be the result of failure to establish a comprehensive disease model and failure to separate distinct subgroups of patients such as those based on sex. Therefore, the main focus of this dissertation aims to identify sex- and IBS-related brain alterations by using non-invasive neuroimaging approaches.

1.1. Task-Based Functional Magnetic Resonance Imaging (fMRI)

In cognitive neuroscience, the task-related fMRI is performed to understand the function of activated brain regions corresponding to external stimuli or designed tasks. Theoretically, the activation of particular brain regions requires more energy which induces increased blood flow transporting more oxygen and glucose to the needed regions. The fMRI measures the dynamics of blood oxygenation (i.e. oxygenated hemoglobin and deoxygenated hemoglobin) to indirectly reflect neuronal activities. Deoxygenated hemoglobin is paramagnetic and it has very strong magnetic susceptibility which strengthens the T2* shortening effect. During the task, because the blood flow brings oxygenated hemoglobin which reduces the T2* decay effect, the local MR signal increases. In sum, the task-based fMRI measures the hemodynamic changes in response to stimuli and generates the blood oxygenation level-dependent (BOLD) contrast images. The use of task-based fMRI has helped to elucidate the brain regions involved in somatosensory and visceral pain processing. In IBS research, visceral stimulation has been widely performed to study disease-related differences in brain activation. Several brain regions in the emotional-arousal network, the homeostatic afferent network and the salience network are more activated during rectal balloon inflation periods in IBS patients compared to healthy controls (Tillisch et al., 2011). Cognitive processing such as attention, anticipation, and learning has also been suggested to play an important role in IBS symptom generation (Labus et al., 2008; Lackner et al., 2008; Mayer et al., 2009a; Chapman and Martin, 2011). However, the cognitive mechanism in the pathophysiology of IBS is still unclear. In chapter 4, I discuss an abdominal pain expectation paradigm containing both cued threat and uncued threat. By using this experiment, I aim to identify disease- and sex-related differences in the emotional and cognitive processing of the two threats.

1.2. Resting-State fMRI

Resting-state fMRI has been increasingly used to investigate the intrinsic brain activity in clinical and basic neuroscience research. During this experimental paradigm, subjects are instructed to lie down in the scanner with their eyes either open or closed. Subjects do not receive any designated stimuli or perform tasks. The spontaneous fluctuations of BOLD signal are measured during this period. The resting-state BOLD signal have been suggested to be related to neural activity (Auer, 2008) despite the fact that the neurophysiological mechanisms are not completely understood.

Several brain networks have been identified with resting-state fMRI including the default mode network (Buckner et al., 2008), the salience network (Seeley et al., 2007), the executive control network (Spreng et al., 2013), and the sensorimotor network (Laird et al., 2011). Because of its simple experimental design, fewer limitations to patients, reliability and easy replication, resting-state fMRI have also been used to study brain development, neurodegenerative diseases, psychiatric diseases, as well as physiological and psychological disorders (Barkhof et al., 2014).

Pain perception is a complex process involving afferent pain pathways and the central nervous system. In some cases, chronic pain patients feel pain without any external stimuli or even during 'resting-state'. Therefore, resting-state fMRI has recently been used to investigate pain such as fibromyalgia, functional dyspepsia and chronic back pain (Baliki et al., 2006; Napadow et al., 2010; Liu et al., 2013). Malinen et al. (2010) observed aberrant brain activity in patients with chronic limb pain during resting state. In this work, I apply resting-state fMRI to investigate the neurophysiology of IBS. Two approaches are used to analyze spontaneous fluctuations of BOLD activity: frequency power analysis and seed-based connectivity analysis.

1.3. Fractional Amplitude of Low-Frequency Fluctuation

Low-frequency fluctuation of BOLD signals during resting-state fMRI (0.01-0.08 Hz or 0.01-0.1 Hz) has been widely used to study brain function and these spontaneous oscillations may contain useful neurophysiological information (Fox and Raichle, 2007; Zang et al., 2007; Shmuel and Leopold, 2008). For example, Biswal et al. (1995) observed that the synchronous low frequency fluctuation in the motor cortex was similar to the brain activation pattern during a finger tapping task. To detect the regional intensity of the spontaneous low-frequency oscillation, the amplitude of low-frequency fluctuation (ALFF) for each voxel is calculated by averaging the square root of the power spectrum across the low-frequency domain, and then standardized by dividing the global mean ALFF value (Zang et al., 2007). Fractional ALFF (fALFF) approach is introduced to reduce the confounding effects of physiological noise and to increase the sensitivity of detecting spontaneous brain activities (Zou et al., 2008). The fALFF is calculated as the ratio of the square root of the power spectrum across the low-frequency domain to the square root of the power across the entire frequency range (Zou et al., 2008). Studies have shown that some brain regions (e.g. insula and amygdala) tend to be involved in higher frequency fluctuations (Kopell et al., 2000; Salvador et al., 2008). Baliki et al. (2011) demonstrated that patients with chronic back pain had aberrant high-frequency BOLD oscillations correlated to spontaneous pain ratings. In chapter 2, I use fALFF and the alternative approach of assessing the relative power within multiple frequency bands (Malinen et al., 2010; Baria et al., 2011) to identify regional specific abnormalities in resting-state brain function of IBS patients, as well as to investigate possible sex-related differences.

1.4. Intrinsic Functional Connectivity

The intrinsic functional connectivity analysis calculates the correlation coefficients between resting-state BOLD signals over time in voxels or regions (Friston, 2011). Multiple large-scale distinct brain networks, sub-structural connectivity patterns (e.g. subregions of the insula), and region-to-region connections have been identified by using resting-state functional connectivity approach (Smith et al., 2009; Kim et al., 2010; Deen et al., 2011; Kim et al., 2011). Studies have suggested the resting-state functional connectivity is associated with brain anatomical connectivity (Skudlarski et al., 2008; Greicius et al., 2009). In addition, the strength of connectivity within and between each network or each region is thought to reflect task performances, behaviors or clinical symptoms (Lynall et al., 2010; Baldassarre et al., 2012; Cole et al., 2012). Therefore, this functional connectivity approach has been widely used to study neuropsychological diseases (Gu et al., 2010; Jones et al., 2010) and pain disorders (Cifre et al., 2012; Yu et al., 2014). In chapter 3, seed-driven resting-state functional connectivity approach (Whitfield-Gabrieli and Nieto-Castanon, 2012) is introduced to examine the functional connectivity of dorsal anterior insula which has been suggested to play a major role in pain matrix, salience processing and cognitive control (Deen et al., 2011; Legrain et al., 2011; Chang et al., 2013). Seed-based correlation coefficients are calculated between the averaged BOLD time series of all voxels within the seeds, and every voxel in the rest of the brain with the bivariate correlation linear measure (Whitfield-Gabrieli and Nieto-Castanon, 2012). Group and sex differences in the functional connectivity of dorsal anterior insula are tested on a whole-brain scale. In addition, I discuss how the altered functional connectivity patterns can be related to clinical characteristics, as a possible pathophysiological consideration.

1.5. Structural MRI

Although the cellular and molecular mechanisms underlying the brain structural changes are not completely understood, researchers have found close relationship between structural and functional brain MRI (Hagmann et al., 2010; Supekar et al., 2010; Uddin et al., 2011). For example, a systematic review suggested patients with fibromyalgia shared similar structural and functional brain alterations within the pain matrix (Cagnie et al., 2014). Recently, structural MRI and diffusion tensor imaging approaches have been applied to find possible biomarkers in patients with chronic pain such as rheumatoid arthritis, pelvic pain, inflammatory bowel disease (IBD) and IBS (Seminowicz et al., 2010; Wartolowska et al., 2012; Agostini et al., 2013; Piche et al., 2013; Bagarinao et al., 2014; Ellingson et al., 2013). However, most existing chronic pain studies investigated one single pain condition at a time and were conducted with different methodologies across those studies. Therefore, it is helpful to understand the pain mechanism by comparing different chronic pain conditions using the same study approach. Although IBD and IBS share similar gastrointestinal symptoms, IBD is characterized by inflammation and ulcerations. Differences in brain responses to visceral pain and in brain expression of neurotransmitter receptors have been found between patients with IBS and IBD (Mayer et al., 2005; Jarcho et al., 2013). In collaboration with the Laboratory on Neuroimaging, image processing pipelines and mathematical algorithms are employed to calculate brain cortical thickness. I aim to identify brain structural signatures in IBD and IBS patients as possible biomarkers and to understand the different mechanisms underlying chronic visceral pain.

Chapter 2

Patients with Chronic Visceral Pain Show Sex Related Alterations in Intrinsic Oscillations of the Resting Brain

This chapter is adapted from:

Hong JY, Kilpatrick LA, Labus J, Gupta A, Jiang Z, Ashe-McNalley C, Stains J, Heendeniya N, Ebrat B, Smith S, Tillisch K, Naliboff B, Mayer EA (2013) Patients with Chronic Visceral Pain Show Sex-Related Alterations in Intrinsic Oscillations of the Resting Brain. J Neurosci 33:11994-12002.

Abstract

Abnormal responses of the brain to delivered and expected aversive gut stimuli have been implicated in the pathophysiology of Irritable Bowel Syndrome (IBS), a visceral pain syndrome occurring more commonly in women. Task-free resting state fMRI can provide information about the dynamics of brain activity which may be involved in altered processing and/or modulation of visceral afferent signals. Fractional amplitude of low frequency fluctuation (fALFF) is a measure of the power spectrum intensity of spontaneous brain oscillations. This approach was used to identify differences in the resting state activity of the human brain in IBS subjects compared to healthy control subjects (HCs), and to identify the role of sex related differences. We found that both the female HCs and female IBS subjects had a frequency power distribution skewed towards high frequency (HF) to a greater extent in the amygdala and hippocampus compared to male subjects. In addition, female IBS subjects had a frequency power distribution skewed towards HF in the insula, as well as towards low frequency (LF) in the sensorimotor cortex to a greater extent than male IBS subjects. Correlations were observed between resting state blood oxygen level dependent (BOLD) signal dynamics and some clinical symptom measures (e.g. abdominal discomfort). These findings provide the first insight into sex-related differences in IBS subjects compared to HCs using resting-state fMRI.

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Introduction

Irritable Bowel Syndrome (IBS) is the most common chronic visceral pain disorder characterized by chronic abdominal pain, bloating, change in bowel habits and abdominal distension. It is estimated that 10-20% of the western population is affected and with a greater prevalence in women (Mykletun et al., 2010; Chang, 2011; Fukudo and Kanazawa, 2011; Kennedy et al., 2012). The sensation of abdominal pain is a complex, multidimensional mechanism that involves communication between the brain and gut. Thus, a theoretical model of top-down modulation and bottom-up signaling from visceral afferents has been widely accepted (Mayer, 2011; Mayer and Tillisch, 2011; Kennedy et al., 2012). Although the cause of IBS is still unknown, multiple affective factors such as anxiety, depression, early life stress and abuse have been suggested to be associated with IBS (Ringel et al., 2008; Kennedy et al., 2012). In addition, plausible central mechanisms involved in pain amplification include altered cognitive and emotional modulation have been reported (Melzig et al., 2008; Wiech and Tracey, 2009; Elsenbruch, 2011; Kennedy et al., 2012).

Functional brain imaging studies have been widely used to investigate regional brain activities while receiving visceral pain stimuli with healthy populations (Mayer et al., 2009b). The most consistently activated brain regions including anterior insula (INS), posterior INS, anterior cingulate cortex (ACC), primary sensory cortex (SI) and prefrontal cortex (PFC). Posterior INS is the primary interoceptive cortex, receiving visceral afferent information from thalamus. The information is relayed from the posterior INS to mid and anterior INS, where the interoceptive awareness and conscious experience of feelings are formed (Craig, 2009; Mayer et al., 2009a). In addition, the feelings and awareness can be mediated by cognitive, affective and motivational inputs from limbic system and higher level of frontal and cingulate cortex (Mayer et al., 2009a;

Wiech and Tracey, 2009). Anterior INS is interconnected with subcortical regions and ACC (Mayer et al., 2009b). ACC has been suggested to have multiple functions including regulation of negative emotion, conflict resolution, pain modulation, allocating attention and error processing (Wiech and Tracey, 2009). Different aspects of information from several brain regions integrate in the anterior INS comprising the consciously interoceptive feelings. Studying the brain regional function in the brain-gut network with healthy subjects is the first step to understand the functional gastrointestinal disorders including IBS.

Functional brain imaging studies have demonstrated abnormal blood oxygen level dependent (BOLD) responses in IBS subjects in regions of a homeostatic afferent, emotional arousal and cognitive modulatory networks compared to healthy populations (Wilder-Smith et al., 2004; Rapps et al., 2008; Mayer et al., 2009a; Tillisch et al., 2011). Although the activation comparison results between IBS and healthy subjects were slightly inconsistent which may be due to small sample size and different experimental designs, IBS subjects had greater activation in the thalamus, anterior midcingulate cortex (MCC), anterior and mid INS (Rapps et al., 2008; Tillisch et al., 2011).

Sex related differences in visceral perception, in the autonomic nervous system and in brain responses to visceral stimuli or their expectation have been demonstrated (Mayer et al., 2001; Chang and Heitkemper, 2002; Mayer et al., 2004; Mayer et al., 2005; Mayer et al., 2006; Lieberman et al., 2007; Wang et al., 2007; Labus et al., 2008; van Marle et al., 2009). A rectal distension study showed healthy women had greater total volume of activated voxels than healthy men. In addition, healthy females showed brain activation in the INS and ACC while healthy males showed localized clusters primarily in the sensorimotor cortex (Kern et al., 2001). Straube et al. studied the sex differences in brain activation during anticipated pain. 36 healthy participants

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(12 males) were given electrical stimuli to the finger. During an anticipation period after receiving the strongest pain stimulus, the activation of pregenual medial PFC (mPFC) was higher in women than men, while men had higher activation in the INS during moderately painful stimuli. The authors suggest that self-focused attention and anticipation about unpleasant stimuli may involve the pregenual medial PFC more in women than in men (Straube et al., 2009b). A positron emission tomography (PET) study in IBS populations showed males had greater activation of cortical region (dorsolateral PFC) while females had greater activation of emotional arousal brain regions (amygdala [AMYG] and ACC) during visceral stimuli (Naliboff et al., 2003). Similar to brain imaging studies in rodent (Wang et al., 2009), these studies have shown greater engagement of cortical regions in males (e.g. PFC) and greater engagement of emotional brain regions and circuits in females (e.g. AMYG and ACC) during rectal distension and expectation of abdominal pain.

Recently, resting-state fMRI have been used to investigate various physiological and psychological conditions such as chronic back pain, fibromyalgia, and social anxiety disorders. The BOLD signals during resting-state fMRI provide greater understanding of possible brain region abnormities and highlight anatomical and functional intrinsic connectivity associated with patients (Fox and Raichle, 2007; Baria et al., 2011). Investigating sex related differences associated with resting state have offered additional insights into the underlying pathophysiology associated with various diseases (Schoonheim et al., 2012). Independent component analysis (ICA) approach has been widely used to identify spontaneously synchronized BOLD activity across the brain (Beckmann et al., 2005; De Luca et al., 2006; Fox and Raichle, 2007). Many spatially coherent resting state maps have been investigated such as default mode network (Orliac et al., 2013), salient network (Otti et al., 2013) and sensorimotor network (Kalthoff et al., 2013).

Synchronized brain regional activities within the network tend to work functionally (Fox and Raichle, 2007).

Characterization of spontaneous intrinsic BOLD oscillations in the brain (resting-state fMRI) using frequency spectral analysis has also been used to identify functional BOLD oscillations and brain abnormalities in psychiatric, and in somatic pain conditions (Zang et al., 2007; Hoptman et al., 2010; Malinen et al., 2010; Zuo et al., 2010; Baria et al., 2011; Han et al., 2011; Davis and Moayedi, 2012; Farmer et al., 2012; Kwak et al., 2012; Wang et al., 2012). Multiple frequency ranges/bands have been suggested based on electroencephalographic (EEG) and physiologically neuronal activities in animal and human studies: slow-5 (0.01-0.027 Hz), slow-4 (0.027-0.073 Hz), slow-3 (0.073-0.198 Hz), slow-2 (0.198-0.25 Hz), low frequency (LF; 0.01-0.05 or 0.01-0.08 Hz), medium frequency 1 (MF1; 0.05-0.10 Hz), medium frequency 2 (MF2; 0.10-0.15 Hz), medium frequency (MF; 0.05-0.12 or 0.08-0.17 Hz), and high frequency (HF; 0.15-0.20 or 0.12-0.25 or 0.17-0.25 Hz) (Buzsaki and Draguhn, 2004; Fox and Raichle, 2007; Malinen et al., 2010; Raichle, 2010; Zuo et al., 2010; Baliki et al., 2011; Baria et al., 2011; Zhang et al., 2013a).

Abnormalities in amplitudes of low frequency fluctuations (ALFF) have been implicated in many neurobehavioral and psychiatric disorders (Hoptman et al., 2010; Huang et al., 2010; Kwak et al., 2012; Zang et al., 2007). For example, Parkinson's disease patients had greater ALFF at slow-5 frequency band in the caudate, hippocampus (HIPP) and temporal gyrus compared to HCs (Zhang et al., 2013b). Abnormal ALFF was also observed in patients with amnestic mild cognitive impairment (Han et al., 2011). ALFF analysis approach has also been implemented in several pain studies (Malinen et al., 2010; Baliki et al., 2011; Kim et al., 2013). Patients with chronic spinal and limb pain have been shown to exhibit greater HF (0.12-0.25 Hz) spectral power in the INS and ACC compared to HCs (Malinen et al., 2010). Regional abnormalities in spontaneous BOLD

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oscillations in the INS, mPFC and posterior cingulate cortex (PCC) have also been observed in chronic back pain patients (Baliki et al., 2011). These alterations in regional frequency spectral power have been suggested to reflect abnormal intrinsic neuronal activities, functional connectivity and spontaneous pain (Zou et al., 2008; Baliki et al., 2011).

Fractional amplitude of low frequency fluctuation (fALFF), another measure of the power spectrum intensity of spontaneous brain frequency oscillations, is a normalized ALFF (i.e. the ratio of specific range of LF power to the entire frequency spectrum power) (Zou et al., 2008). fALFF was suggested to minimize the physiological artifacts irrelevant to brain activity while ALFF is susceptible to the noises (Zou et al., 2008; Zuo et al., 2010).

In the current study, using fALFF, we aimed to identify disease related regional differences in intrinsic oscillatory dynamics of BOLD signal as previously reported for other persistent pain conditions, by comparing patients with IBS and HCs. In addition, we aimed to identify possible sex related differences, and to determine if the identified regions with altered oscillatory dynamics are associated with IBS symptoms and behavioral characteristics. Based on previous published studies in chronic pain patients (Malinen et al., 2010; Baliki et al., 2011; Farmer et al., 2012) we aimed to test the following hypotheses using a region of interest (ROI) approach: 1) Regarding disease related differences, there is a shift of regional frequency spectral power towards HF in the interoceptive related regions in the patient group. 2) Sex related differences in oscillatory dynamics exist in emotional, somatosensory and interoceptive regions. 3) Abnormal regional oscillatory dynamics are correlated with clinical symptoms.

Materials and Methods

Subjects.

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178 right-handed subjects were recruited through the UCLA Digestive Diseases Clinic and advertisements. The sample included 76 female HCs (mean age, 29.39; SD, 9.93 years), 42 male HCs (mean age, 35.95; SD, 12.97 years), 29 male IBS subjects (mean age, 37.28; SD, 10.75 years) and 31 female IBS subjects (mean age, 30.65; SD, 10.71 years). Diagnosis of IBS was made by a gastroenterologist or nurse practitioner with expertise in functional GI disorders based on the ROME II or ROME III symptom criteria during a clinical assessment (Drossman, 2000; Drossman, 2006). The diagnostic criteria include recurrent abdominal pain or discomfort associated with two or more of the following: 1) pain/discomfort is relieved/improved by defecation 2) the onset of pain/discomfort is related to a change in frequency of stool 3) the onset of pain/discomfort is related to a change in the form (appearance) of stool. All procedures were approved by the UCLA Medical Institutional Review Board, and all subjects provided informed consent. Exclusion criteria comprised pregnancy, substance abuse, abdominal surgery, tobacco dependence, and psychiatric illness. In addition, IBS subjects with current regular use of analgesic drugs (including narcotics, opioids and alpha2-delta ligands) were excluded. Use of medications such as antidepressants (low dose tricyclic antidepressants [TCAs], selective serotonin uptake inhibitors [SSRIs], NSRIs) was only allowed if patients had been on a stable dose for a minimum of 3 months. In our sample, only three IBS subjects were on low dose TCAs (less than 75mg per day) (one female) or SSRIs (one female and one male). Questionnaires were completed before scanning to determine IBS symptom type, severity, duration of symptoms, and abdominal sensation [UCLA Bowel Symptom Questionnaire, BSQ] (Chang et al., 2001), comorbid affective and mood disorders [Hospital Anxiety Depression Scale, HAD] (Mykletun et al., 2001), IBS-related fears and anxiety [Visceral Sensitivity Index, VSI] (Labus et al., 2004; Labus et al., 2007) and measurement of the big five personality traits [NEO Personality Inventory-Revised,

NEO PI-R] (Costa and McCrae, 1995).

Resting State MRI Data Acquisition.

All resting state scans were collected with subjects having their eyes closed while in the scanner. Noise cancelling headphones were used to help reduce the noise from the scanner. Images were acquired with echo planar sequence on Siemens 3 Tesla Trio and Allegra scanners: **(1)** Siemens 3 Tesla Trio using the following parameters: echo time (TE) = 28ms, repetition time (TR) = 2000ms, scan duration = 10m6s, flip angle = 77 degrees, FOV = 220, slice thickness = 4.0mm, 40 slices were obtained with whole-brain coverage. (27 female HCs, 28 male HCs, 19 male IBS subjects, 25 female IBS subjects); **(2)** Siemens 3 Tesla Trio using the following parameters: TE = 26ms, TR = 2500ms, scan duration = 5m8s, flip angle = 90 degrees, FOV = 200, slice thickness = 3.0mm, 38 slices were obtained with whole-brain coverage. (41 female HCs); **(3)** Siemens 3 Tesla Trio using the following parameters: TE = 28ms, TR = 2000ms, scan duration = 8m6s, flip angle = 77 degrees, FOV = 220, slice thickness = 4.0mm, 40 slices were obtained with whole-brain coverage. (8 female HCs, 6 female IBS subjects); **(4)** Siemens 3 Tesla Allegra using the following parameters: TE = 28ms, TR = 3000ms, scan duration = 6m6s, flip angle = 90 degrees, FOV = 200, slice thickness = 3.0mm, 38 slices were obtained with whole-brain coverage. (14 male HCs, 10 male IBS subjects)

Structural MRI.

A standard T1-weighted magnetization-prepared rapid acquisition gradient echo (MP-RAGE) scan was obtained before resting scan session using the following parameters: TR = 2200 ms, TE = 3.26 ms, slice thickness = 1 mm, 176 slices, 256 x 256 voxel matrices, and

1.0×1.0×1.0 mm voxel size.

Resting State MRI image preprocessing.

All image processing and data analysis were performed using Statistical Parametric Mapping

8 (SPM8) software (Wellcome Department of Cognitive Neurology, London, UK). Images were

first imported from DICOM into NIFTI-1 format followed by slice timing correction, spatial

realignment and motion correction. A native-to-MNI transformation matrix was obtained by

running the MP-RAGE scan through a segmentation procedure. This matrix was then used to bring

fMRI images, which were aligned to the MP-RAGE scan into MNI space. All scans were

resampled to a voxel size of 2x2x2mm.

Between group analyses of normalized fALFF.

We used fALFF, a power-density frequency spectrum approach applied to resting state time

course data, in order to identify region specific abnormalities in resting state brain function (Zou et

al., 2008). The examination of low frequency fluctuations of BOLD signal using this type of

approach has exhibited a close relationship to spontaneous neuronal activity, adequate gray

matter-white matter differentiation, and the improved fALFF algorithm specifically has been

shown to reduce the confounding effects of physiological noise in the data compared to earlier

frequency based methods (Goldman et al., 2002; Mantini et al., 2007; Zou et al., 2008; Biswal et

al., 2010). fALFF analysis was performed using in-house codes written in C++. Linear trends were

removed before the time course data from each voxel was subjected to Fast Fourier

Transformations into the frequency domain (Zou et al., 2008). Similar to many previous studies

(Wise et al., 2004; Duff et al., 2008; Zou et al., 2008; Baliki et al., 2011; Baria et al., 2011), we

subdivided the BOLD frequency into three different bands. We pooled resting state fMRI data with different TRs (2-3s) from multiple studies for the purposes of comparing patterns of BOLD oscillation in a large sample size. To make comparisons possible across multiple studies, we modified the fALFF by setting a cutoff frequency (0.16 Hz). The modified fALFF was calculated as the ratio of the square root of the oscillatory amplitude sum across the low frequency band (LF, 0.01-0.05Hz), middle frequency band (MF, 0.05-0.10 Hz) and high frequency band (HF, 0.10-0.16 Hz) to the square root of the power across the entire frequency band (0-0.16Hz). We normalized the fALFF for each voxel, transformed it to a Z score by subtracting the global mean fALFF value and then dividing by the standard deviation within a whole brain mask (provided in MRIcro, http://www.mccauslandcenter.sc.edu/mricro/mricron/). A 8mm isotropic Gaussian kernel was then used to smooth normalized fALFF maps (Zou et al., 2008).

Group differences in fALFF in the different frequency bands were tested using linear contrast analyses on estimates from a general linear model implemented in SPM8. Using the flexible factorial specification, normalized fALFF maps were entered as dependent variables and group (male HC, female HC, male IBS, and female IBS) and frequency band (LF, MF, and HF) were entered as factors. The main effect and interactions between group and frequency band were entered as effects in the model. Subject was also included as an effect in the model (Gläscher and Gitelman, 2008). Because chronic pain has been associated with shifts in BOLD frequency power distribution (Malinen et al., 2010; Farmer et al., 2012), we specified interaction contrasts (i.e., differences of differences) to localize regions that exhibited changes/shifts in frequency power distribution due to group, e.g. [female HCs (HF versus LF) – female IBS (HF versus LF)]. As a sensitivity analysis we entered subject age (in years), depression score and factors for scanning protocols as nuisance covariates, but this did not change the results, e.g. contrast maps for [female

IBS (HF versus LF) – male IBS (HF versus LF)] showed 95.99% overlap (see **Supplemental Figure 1**). As such, we do not report results for the model controlling for these potential nuisance covariates.

Based on the findings from previous studies (Labus et al., 2008; Malinen et al., 2010; Apkarian et al., 2011; Baliki et al., 2011; Tillisch et al., 2011; Van Oudenhove, 2011), we investigated the resting state signal in specific ROIs, including the sensorimotor cortex, INS, and affective regions (AMYG, and HIPP). ROIs were constructed in WFU PickAtlas tool using the aal human brain atlas with a two-dimensional dilation factor of one (Maldjian et al., 2003; Maldjian et al., 2004). ROI analyses was applied by threshholding whole-brain fALFF activation maps at an uncorrected significance threshold of $p<0.001$ and using small volume correction procedure in SPM8 and considering results as significant at cluster threshold of $p<0.05$ corrected for family wise error correction (FWE). To better quantify the differences between groups, we extracted normalized fALFF values (Z scores) averaged over the significant voxels within each ROI by MarsBaR (http://marsbar.sourceforge.net) and plotted them using Statistical Package for the Social Sciences (SPSS) software (version 19).

Independent sample T-tests were conducted to examine bowel symptom severity (BSQ) between male and female IBS subjects. Analysis of variance (ANOVA) was performed to examine differences in non-BSQ clinical and behavioural characteristics including visceral sensitivity (VSI), and anxiety and depression (HAD), and personality traits (NEO). Significant group effects were further examined using linear contrasts using false-discovery rate (FDR) for the four comparisons at 5% (Benjamini and Hochberg, 2000; Benjamini et al., 2006). Associations between clinical characteristics (BSQ, symptom duration, VSI and HAD) and frequency oscillation shifts were conducted in SPSS by correlating clinical scores for each of the variables of

interest with the frequency shifts between averaged Z score of different fALFF maps for each

voxel within the significant ROIs and correcting for multiple comparisons by FDR.

Results

Clinical Characteristics

Subjects' clinical data are summarized in **Table 1**. Female IBS subjects tended to have greater

IBS-related symptom scores compared to male IBS subjects (p >0.05). Although within normal

clinical ranges, IBS subjects had higher anxiety and depression symptom scores on the HAD than

HCs, with male IBS having significantly higher scores than male HCs for anxiety (adjusted p (q)

=.006), and female IBS having significantly higher anxiety (adjusted p (q) =.006) and depression

(adjusted p (q) =.002) scores compared to female HCs. Additionally, male and female IBS subjects

had significantly higher VSI scores than male and female HCs, respectively (males: adjusted p (q)

<.001; females: adjusted p (q) <.001).

IBS related differences in frequency power distribution

Male HCs showed greater HF versus MF and LF power distribution in the left anterior INS,

bilateral mid INS, and left posterior INS compared to male IBS subjects (**Table 2 and Figure 1**).

These results indicate that male HCs had a frequency power distribution skewed towards HF to a

greater extent than male IBS subjects in the left anterior INS, bilateral mid INS, and left posterior

INS (**Figure 5a**).

Female IBS showed greater HF and MF versus LF power distribution in the left AMYG, right

HIPP and anterior INS compared to female HCs (**Table 3 and Figure 2a**). ROI analysis also

revealed that female IBS subjects had greater frequency distribution of LF versus MF and HF

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power in the sensorimotor regions (precentral, postcentral, and paracentral cortex and supplementary motor area) compared to female HC subjects (**Table 3 and Figure 2b**). The results indicate that female IBS had a frequency power distribution skewed towards HF in the left AMYG, right HIPP and anterior INS and towards LF in the sensorimotor regions to a greater extent compared to female HCs (**Figure 5b**).

There were no significant differences between IBS subjects and HC subjects when males and females were combined in each group.

Sex related differences in frequency power distribution

Healthy controls. Female subjects had larger HF and MF versus LF power distribution in AMYG and HIPP, compared to males (**Table 4 and Figure 3a**). Male subjects showed greater LF versus HF power distribution in sensorimotor cortical regions (bilateral precentral, and right postcentral cortex) compared to female subjects (**Table 4 and Figure 3b**). These results indicate that male HCs had a frequency power distribution skewed towards LF in the sensorimotor cortex to a greater extent than female HCs, while female HCs had a distribution skewed towards HF in affective regions to a greater extent than in male HCs (**Figure 5c**).

IBS group. Female compared to male IBS subjects had greater HF versus LF power distribution in all INS subregions (anterior INS, mid INS, posterior INS) and several affective regions (AMYG and HIPP) (**Table 5 and Figure 4a**). In addition, female subjects showed significantly greater LF versus HF power distribution in the left precentral, postcentral cortex and supplementary motor area (**Table 5 and Figure 4b**). These results indicate that female IBS subjects had a frequency power distribution skewed towards LF in sensorimotor regions, as well as towards HF in the interoceptive and emotional arousal regions to a greater extent than in male IBS

subjects (**Figure 5d**).

Correlations of BOLD signal dynamics with clinical and behavioral characteristics

Correlation analyses were performed separately in male and female subjects between clinical symptom scores and the frequency power distributions from regions displaying sex specific IBS-related alterations. In female IBS subjects, IBS symptom related discomfort level during the past week was positively correlated with the degree to which the frequency power distribution was skewed toward HF (HF versus LF) in the left anterior INS (r=.506, p=.003, q=.04, FDR corrected) (**Figure 6**). In male IBS subjects, IBS symptom related discomfort level was associated with the degree of frequency power distribution (HF versus LF) in the left HIPP. However, this correlation did not survive FDR correction (r=.52, p=.008, q=.086). No statistically significant correlations between regional frequency power abnormalities in a priori brain regions (e.g. posterior INS in males, or anterior INS in females) and any other clinical variables (including IBS symptom duration, neuroticisms, HAD anxiety or symptom related anxiety) were identified.

Discussion

Spontaneous BOLD signal has great clinical application values and has been widely used to investigate neurological, psychiatric diseases and chronic pain (Fox and Raichle, 2007). In the current study, we examined group differences between patients with persistent abdominal pain (IBS) and HC subjects in the distribution of intrinsic BOLD oscillations across three frequency bands using a ROI approach, with a special emphasis on detecting sex related differences in these patterns. In particular, we studied the frequency spectrum power shift/change between the three frequency bands in the AMYG, HIPP, subregions of INS, and sensorimotor areas resulting in the

following main findings: 1) Significant disease related differences were observed when female

IBS subjects were compared to female HCs, with patients showing a greater skew in the frequency

power distribution towards HF in AMYG and anterior INS, and a greater skew towards LF in the

sensorimotor regions. 2) Significant sex related differences were observed both within the HC and

the IBS groups: Female HC subjects (compared to males) showed a greater skew in the frequency

power distribution towards HF in AMYG and HIPP. Within the IBS group, female IBS subjects

(compared to males) showed similar patterns as the observed sex differences within the HCs in

regions of an emotional arousal circuit, in addition to a greater skew toward HF in all INS

subregions. 3) Altered regional frequency power distribution was correlated with subjective

abdominal discomfort ratings in female patients, but not symptom duration or anxiety ratings. To

our knowledge, this is the first report on sex and disease related differences in spontaneous brain

oscillations in a large sample of male and female subjects with and without IBS.

The finding that some regions were dominated by LF oscillations while others were skewed

towards HF in particular groups is consistent with previous reports in resting state studies and

other chronic pain conditions (Salvador et al., 2008; Malinen et al., 2010; Baliki et al., 2011). In

addition, our results indicate that sex and disease can contribute to the degree to which frequency

power distribution is skewed. Baria et al. found distinct BOLD oscillation power spatially

distributed across different brain regions. The results showed LF band with highest magnitude

power was mostly localized to the prefrontal, parietal and occipital cortex and LF band with lowest

magnitude power was mainly localized to the temporal and subcortical areas. Reversely, HF band

with highest magnitude power was distributed in the cingulate and subcortical areas while HF

band with lowest magnitude power in parietal and occipital cortex. They reported that unimodal

brain regions receiving and sending information limited to cortical regions (such as the

sensorimotor cortex) tended to be dominated by LF oscillations, while more complex multimodal regions having more complicated multifaceted connection (such as INS and limbic cortices) displayed a shift of power to HF bands (Mesulam, 1998; Baria et al., 2011). Similar findings were also revealed by Salvador et al. (2008). They examined resting state connectivity among 45 brain regions by calculating mutual information in three different time series frequency domains. In the HF band (0.17-0.25 Hz), the INS and AMYG had greatest coherence values while in the LF band (0.002-0.08 Hz), dorsolateral superior frontal gyrus had highest coherence value (Salvador et al., 2008). Different frequency spectrum power varies across the brain functionally and anatomically which can also reflect local neuronal activities (Mukamel et al., 2005). Usually the regions work functionally together have synchronized frequency spectrum oscillations locally or distally (Buzsaki and Draguhn, 2004). In the followings, we discuss about different frequency oscillation power distributions underlying disease and sex differences as well as in terms of the functional roles and abnormalities in our selected regions.

Sex-specific alterations in oscillatory dynamics between IBS compared to HCs

In contrast to previous reports from other chronic pain conditions, no disease related differences in regional oscillation frequencies were identified when male and female subjects were combined, despite the significantly larger sample size compared to previous reports. Even though these earlier studies in patients with different persistent pain conditions have provided evidence for abnormal resting state oscillatory dynamics (Malinen et al., 2010; Baliki et al., 2011), the majority of studies were limited by small sample size, sometimes heterogeneous patient populations and by the combination of male and female subjects.

When female and male subjects were compared separately, robust differences between IBS

subjects and HCs were observed. Female IBS showed oscillatory alterations in affect related brain regions (anterior INS, AMYG) and sensorimotor regions compared to female HCs. In contrast, male IBS subjects showed alterations in viscerosensory regions (mainly in mid INS and posterior INS) compared to male HCs. Similar to previous observations in the temporomandibular disorder, chronic back pain and fibromyalgia patients (Malinen et al., 2010; Napadow et al., 2010; Baliki et al., 2011; Ichesco et al., 2011), we observed disease related alterations in oscillatory dynamics in the INS, even though the affected INS subregions differed between males and females. INS cortex is believed to involve in converging interoceptive information, monitoring self-recognition and body state, processing emotional feelings and modulating attention (Paulus and Stein, 2006; Lewis et al., 2008b; Craig, 2009). Interoceptive sensation especially for pain which is the most common characteristic in IBS has been widely studied. Consistent findings from different pain stimuli have suggested that INS is the hub of gathering multimodal information and generating the perception of pain feeling (Peyron et al., 2000; Apkarian et al., 2005; Salvador et al., 2008; Cauda et al., 2011; Tillisch et al., 2011). While posterior INS and mid INS receive primary interoceptive and somatosensory inputs, the anterior INS functions is considered a multimodal association cortex, receiving interoceptive and affective inputs (Craig, 2009). One may speculate that an upregulation of emotional arousal/salience circuits involving the anterior INS and AMYG contributes more to symptoms in female patients, while an increased engagement of networks related to sensorimotor integration and interoceptive processing may play a greater role in male patients.

Sex related differences in HCs

The current study found significant sex related differences associated with spontaneous BOLD fluctuation patterns in the HC group. Female subjects showed different oscillation

dynamics compared to males in affect related regions and in sensorimotor regions. The pattern of the observed sex related difference in intrinsic BOLD oscillations are similar to those reported from studies on brain responses to emotional (Kilpatrick et al., 2006; Dickie and Armony, 2008; Stevens and Hamann, 2012) and pain stimuli (Derbyshire et al., 2002; Moulton et al., 2006) in healthy subjects. For example, in response to negative emotional stimuli, female subjects had greater activation in HIPP and AMYG compared to male subjects (Stevens and Hamann, 2012). In response to noxious heat, male HCs had greater activations in somato- and viscerosensory regions, as well as prefrontal and parietal regions compared to female HCs (Derbyshire et al., 2002; Moulton et al., 2006). Sex differences in AMYG have also been reported in fMRI studies using emotional stimuli and resting state functional connectivity (Kilpatrick et al., 2006; Dickie and Armony, 2008). Kilpatrick et al. (2006) showed that healthy females had stronger functional connectivity between left AMYG and subgenual PFC. The similarity between sex differences in resting brain measures and task-based measures is in line with previous studies demonstrating a relationship between rest and task-evoked responses (Mennes et al., 2011), even though the precise relationship between task-based BOLD signals and resting state frequency fluctuations remains to be determined (Mennes et al., 2011). In summary, our findings suggest a fundamental difference in the central processing of affective and nociceptive stimuli in the healthy male and female brain, which can be detected in the absence of any stimulus.

Sex related differences in IBS subjects

Similar to the healthy group, female IBS subjects (compared to males) had greater oscillatory dynamics in affect related regions, AMYG and HIPP, as well as in all INS subregions which were not observed in HCs. The current findings suggest some similarities with previously published sex

related differences observed in evoked brain responses in IBS subjects (Berman et al., 2000; Naliboff et al., 2003; Labus et al., 2008). Berman et al. (2000) performed the first PET study of sex-related brain responses to rectal distension. The study involved 30 IBS patients (13 females) who received visceral stimuli. The overall regional cerebral blood flow (rCBF) activity was stronger in male IBS and the activation of the bilateral INS was only observed in male IBS (Berman et al., 2000; Mayer et al., 2004). Naliboff et al. (2003) measured rCBF in 42 IBS patients (23 female) during rectal stimuli of moderate intensity and during expectation by using H215O-PET. Both male and female patients showed activation in dorsal ACC and anterior INS, areas involved in the homeostatic brain matrix, and in the PFC, brainstem regions in response to the visceral stimuli. However, female patients showed greater activation in AMYG, ventromedial PFC (BA11), subgenual ACC (BA25) and dorsal ACC while male IBS showed greater activation in dorsolateral PFC (BA9/46), midposterior INS and dorsal pons/periaqueductal gray (Naliboff et al., 2003). Labus et al. (2008) suggested that during expectation of rectal stimuli (EXP condition), male patients showed normal feedback inhibition resulting in weaker connectivity between the AMYG and pons/locus coeruleus complex (LCC) and subgenual ACC (BA25). In contrast, IBS women showed stronger connectivity between the pons/LCC and the medial orbital frontal cortex and between AMYG and subgenual ACC without feedback inhibition (Labus et al., 2008).

Spontaneous BOLD fluctuation patterns have been widely studied, and suggested to be able to reflect the task-relevant brain activity and functional structure of brain (De Luca et al., 2005; Vincent et al., 2006; Fox and Raichle, 2007; Baria et al., 2011; Tagliazucchi et al., 2011). Abnormal power spectral density shifts from LF towards HF especially at 0.16 Hz in the INS had previously been reported in patients with chronic musculoskeletal pain (Malinen et al., 2010). Our study also showed IBS subjects had similar aberrant frequency power distributions in all the INS

subregions, with female IBS subjects showing greater fluctuation shifts from LF to HF. Even though the reason behind the greater frequency dynamics in female IBS subjects remains unclear, one can speculate that increased activity in affective regions (e.g. AMYG and HIPP) contributed to this sex related difference in frequency power distributions of the anterior aspects of the INS.

Correlation of frequency dynamics with clinical/behavioral parameters

In female patients, a significantly positive correlation between aberrant frequency power distribution in the left anterior INS and the subjective report of abdominal discomfort was observed, consistent with previous reports on correlations of the anterior INS with subjective ratings of visceral stimuli (Dunckley et al., 2005; Lowen et al., 2013). In view of the close bidirectional interactions of anterior INS with the AMYG, it is conceivable that tonically increased input to the anterior INS from emotional arousal circuits plays a role both in the generation of subjective symptoms, as well as in the observed abnormalities in intrinsic oscillations. The lack of a significant correlation between symptom duration and altered oscillatory dynamics makes it less likely that the observed findings are a consequence of longstanding alteration in gut function and visceral afferent signaling, and one could speculate that it represents a primary, possibly genetically/epigenetically determined abnormality of central sensory processing. Alternatively, the lack of significant correlations with clinical symptoms, including duration, anxiety or neuroticisms may be related to sample size, or to the fact that such complex clinical variables are more correlated with alterations in brain networks, rather activity in individual regions.

Possible pathophysiological implications

The findings of this study are consistent with a dysregulation of at least two resting state

networks involving affective and sensory brain regions. Evidence has been provided for the existence of two distinct and anti-correlated intrinsic resting state networks associated with different INS subregions that function as hubs: One network centered in the ventral aspect of the anterior INS with connections to the ACC, AMYG and temporal lobe regions, which is primarily related to attentional and affective regions, and which may play a role in salience detection and other emotional aspects. The other network links the mid INS and posterior INS to sensorimotor, premotor, MCC and pACC indicating a role in sensorimotor integration (Cauda et al., 2011). The fact that previous work has shown good correspondence of networks derived from resting-state studies and from task-relevant brain activation studies (De Luca et al., 2005; Vincent et al., 2006; Fox and Raichle, 2007; Smith et al., 2009; Baria et al., 2011), is consistent with our observation that disease and sex- related findings in the current resting state study are similar to previously published activation studies in IBS.

Conclusions

By using a fALFF approach to examine disease and sex differences between IBS subjects and HCs in the interoceptive and limbic brain regions, we found several patterns of altered frequency distribution between different bands. To our knowledge, this is the first large scale resting-state fMRI analysis performed in IBS subjects, identifying disease related oscillation frequencies which are sex specific. The findings are consistent with sex specific dysregulations in INS centric networks engaged in emotional arousal/salience detection and in sensorimotor processing (Cauda et al., 2011; Cauda et al., 2012), confirming previous findings obtained in studies using evoked brain responses to nociceptive stimuli and their expectation. The lack of identified correlations between these frequency abnormalities and symptom duration, together with the observation that

several of the observed sex related differences in IBS were also observed in the HCs, suggests that

some of these alterations may be primary and not a consequence of longstanding symptoms.

Table 1. Clinical and behavioral characteristics

	HC males			IBS males			HC females			IBS females			F	Sig.
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD		
Neuroticism[1]	37	47.03	10.40	29	52.72	14.78	72	43.60	10.14	31	49.06	9.52	5.259	.002
Anxiety[2]	42	3.26	2.60	29	5.69	4.45	76	3.04	2.436	31	5.03	3.67	7.123	.000
Depression[2]	42	1.64	1.56	29	2.79	3.40	76	1.09	1.73	31	2.42	2.50	5.475	.001
Overall Symptoms[3]	-	-	-	24	9.71	4.24	-	-	-	31	9.97	4.38	0.058	.826
Abd Pain[4]	-	-	-	25	8.92	4.70	-	-	-	31	9.23	4.90	0.410	.814
Abd Discomfort[5]	-	-	-	25	8.80	5.66	-	-	-	31	11.58	5.15	0.668	.060
Duration[6]	-	-	-	24	12.83	9.77	-	-	-	30	11.30	8.75	0.039	.546
Symptom related worries[7]	41	3.41	5.85	29	32.14	17.39	72	3.29	5.77	31	34.94	14.83	107.76	.000

F= main effect of group from ANOVA and t-tests for four and two group comparisons, respectively; Statistically significant p<0.05

1 NEO Personality Inventory (Costa and McCrae, 1995);

2 HAD: Hospital Anxiety and Depression (Mykletun et al., 2001);

BSQ: Bowel Symptom Questionnaire (Chang et al., 2001);

3 BSQ Overall Symptoms in the Past week (0-20)

4 BSQ Abdominal Pain in the Past week (0-20)

5 BSQ Discomfort in the Past week (0-20)

6 BSQ Duration in years, derived from onset of symptom

7 VSI: Visceral Sensitivity Index (Labus et al., 2004; Labus et al., 2007).

Table 2. Regions showing altered frequency power distribution in male IBS patients compared to male HCs

ROI		Cluster size (Voxels)	p value	T	Z score	x	y	z
Male HCs versus male IBS (Amplitude of HF versus MF)								
aINS	L	23	0.024	3.48	3.45	-34	14	-14
mINS	L	18	0.022	3.55	3.52	-40	-4	0
pINS	L	92	0.008	4.02	3.97	-40	-8	0
mINS	R	26	0.017	3.9	3.85	40	-8	0
Male HCs versus male IBS (Amplitude of HF versus LF)								
pINS	L	22	0.032	4.02	3.97	-38	-10	6

ROI analyses was applied by threshholding whole-brain fALFF activation maps at an uncorrected significance threshold of p<0.001 and using small volume correction procedure in SPM8 and considering results as significant at cluster threshold of p<0.05 corrected for voxel wise error rate. MNI coordinates (x,y,z) for peak voxel showing significance.

Table 3. Regions showing altered frequency power distribution in female IBS patients compared to female HCs

ROI		Cluster size (Voxels)	p value	T	Z score	x	y	z
Female IBS versus female HCs (Amplitude of MF versus LF)								
aINS	L	51	0.013	4.64	4.57	-28	20	-18
aINS	R	33	0019	4.1	4.05	28	22	-16
HIPP	R	23	0.011	3. 69	3.65	34	-24	-14
Female IBS versus female HCs (Amplitude of HF versus LF)								
aINS	L	6	0.042	3.4	3.37	-28	20	-18
AMYG	L	6	0.016	3.27	3.24	-26	-4	-24
Female IBS versus female HCs (Amplitude of LF versus MF)								
paCC	L	28	0.041	3.63	3.60	-6	-40	62
paCC	R	18	0.041	3.58	3.30	10	-42	68
Female IBS versus female HCs (Amplitude of LF versus HF)								
paCC	L	113	0.008	4.14	4.09	-16	-14	78
paCC	R	81	0.011	4.26	4.20	10	-44	70
poCC	L	166	0.010	4.15	4.10	-34	-30	70
poCC	R	197	0.007	4.26	4.20	10	-44	70
preCC	L	444	0.000	4.18	4.12	-18	-14	78
SMA	L	35	0.049	3.95	3.91	-14	-12	76

ROI analyses was applied by threshholding whole-brain fALFF activation maps at an uncorrected significance threshold of p<0.001 and using small volume correction procedure in SPM8 and considering results as significant at cluster threshold of p<0.05 corrected for voxel wise error rate. MNI coordinates (x,y,z) for peak voxel showing significance. paCC: Paracentral Cortex

Table 4. Brain regions showing sex differences in frequency power distribution in HCs

Female HCs versus male HCs (Amplitude of MF versus LF)

ROI		Cluster size (Voxels)	p value	T	Z score	x	y	z
AMYG	L	34	0.007	4.23	4.17	-22	-10	-12
HIPP	L	28	0.01	4.50	4.44	-26	-18	-16
AMYG	R	50	0.005	4.91	4.83	24	-10	-12
HIPP	R	52	0.006	5.3	5.19	28	-16	-14

Female HCs versus male HCs (Amplitude of HF versus LF)

ROI		Cluster size (Voxels)	p value	T	Z score	x	y	z
AMYG	L	13	0.012	3.55	3.51	-20	-10	-12
HIPP	L	15	0.015	3.78	3.74	-26	-18	-16
AMYG	R	9	0.002	3.85	3.80	26	-10	-12
HIPP	R	67	0.004	4.64	4.57	28	-16	-20

Male HCs versus female HCs (Amplitude of LF versus HF)

ROI		Cluster size (Voxels)	p value	T	Z score	x	y	z
poCC	R	149	0.013	4.42	4.35	66	-20	40
preCC	L	109	0.021	4.39	4.33	-48	4	54
preCC	R	131	0.015	5.39	5.28	50	14	38

ROI analyses was applied by threshholding whole-brain fALFF activation maps at an uncorrected significance threshold of $p<0.001$ and using small volume correction procedure in SPM8 and considering results as significant at cluster threshold of $p<0.05$ corrected for voxel wise error rate. MNI coordinates (x,y,z) for peak voxel showing significance. poCC: Postcentral cortex; preCC: Precentral cortex; SMA: Supplementary motor area.

Table 5. Brain regions showing sex differences in frequency power distribution in IBS

Female IBS versus male IBS (Amplitude of HF versus LF)

ROI		Cluster size (Voxels)	p value	T	Z score	x	y	z
aINS	L	52	0.013	3.89	3.84	-32	20	-16
pINS	L	53	0.016	3.47	3.44	-36	-6	-12
aINS	R	44	0.015	4.54	4.48	30	22	-16
mINS	R	82	0.005	4.00	3.95	36	6	-12
pINS	R	115	0.005	4.36	4.30	38	-4	-14
AMYG	L	27	0.008	3.44	3.41	-30	-2	-18
HIPP	L	46	0.007	4.02	3.98	-26	-26	-10
Female IBS versus male IBS (Amplitude of LF versus HF)								
poCC	L	287	0.002	4.56	4.50	-34	-16	40
preCC	L	544	0.000	4.93	4.84	-18	-20	68
SMA	L	67	0.026	4.51	4.44	-14	-14	72

ROI analyses was applied by threshholding whole-brain fALFF activation maps at an uncorrected significance threshold of p<0.001 and using small volume correction procedure in SPM8 and considering results as significant at cluster threshold of p<0.05 corrected for voxel wise error rate. MNI coordinates (x,y,z) for peak voxel showing significance.
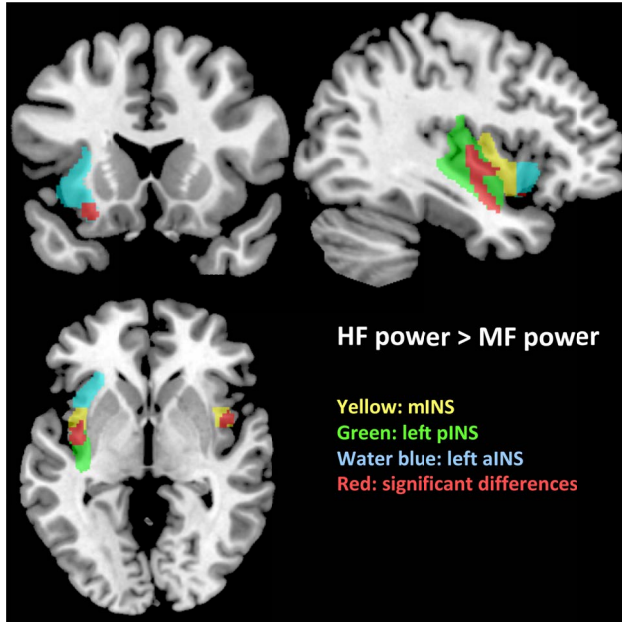
Figure 1. Altered BOLD frequency power distribution of INS subregions in male subjects.
Significant differences of HF versus MF power distribution in male HCs compared to male IBS
were shown in red ($p<0.05$, FWE corrected). For display purposes only, all statistical results,
$p<0.001$ uncorrected, were overlapped on a MRIcron ch2better template.
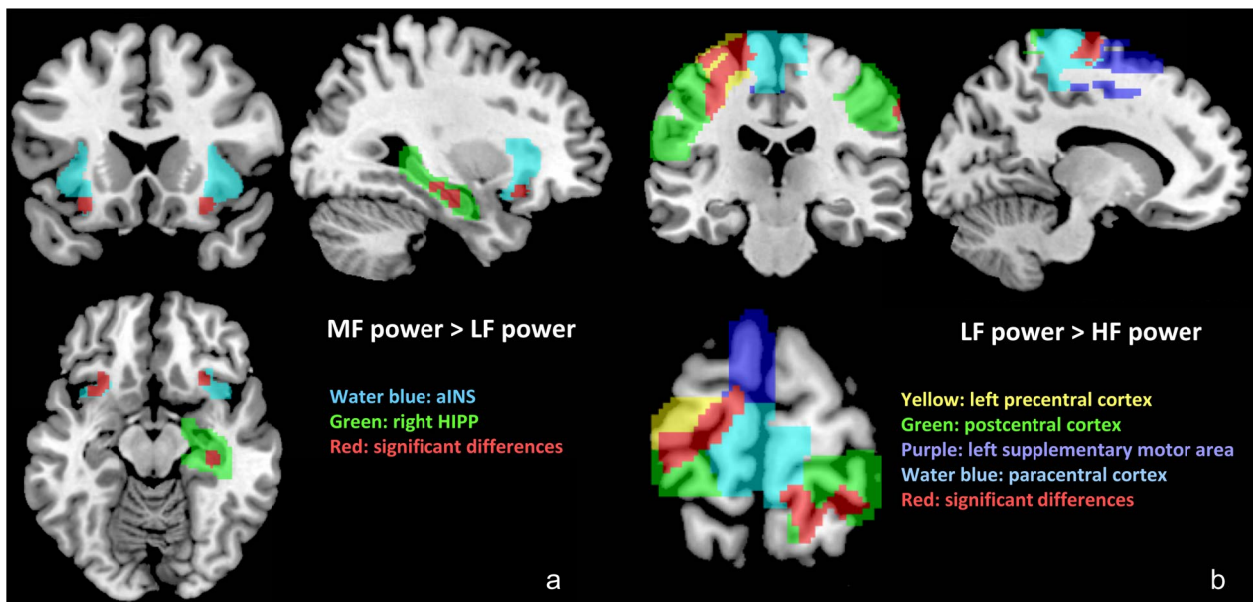
**Figure 2. Altered BOLD frequency power distribution in female IBS compared to female
HCs.** a) Significant differences of MF versus LF power distribution in female IBS compared to
female HCs were shown in red ($p<0.05$, FWE corrected). b) ROIs with significant differences
between LF versus HF power distribution in female IBS compared to female HCs were shown in
red ($p<0.05$, FWE corrected).
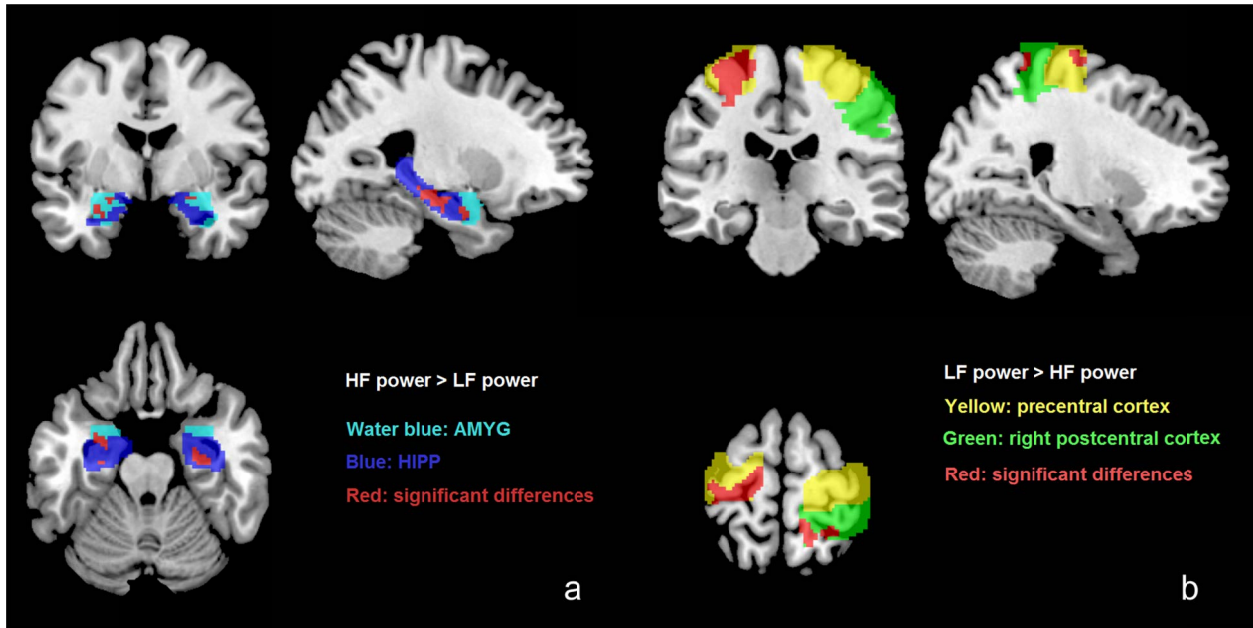
Figure 3. Altered regional BOLD oscillations distribution between female HCs and male HCs. a) Significant differences of HF versus LF power distribution in female HCs compared to male HCs are shown in red (p<0.05, FWE corrected). b) ROIs with significant differences between LF versus HF power distribution in male HCs compared to female HCs are shown in red (p<0.05, FWE corrected).
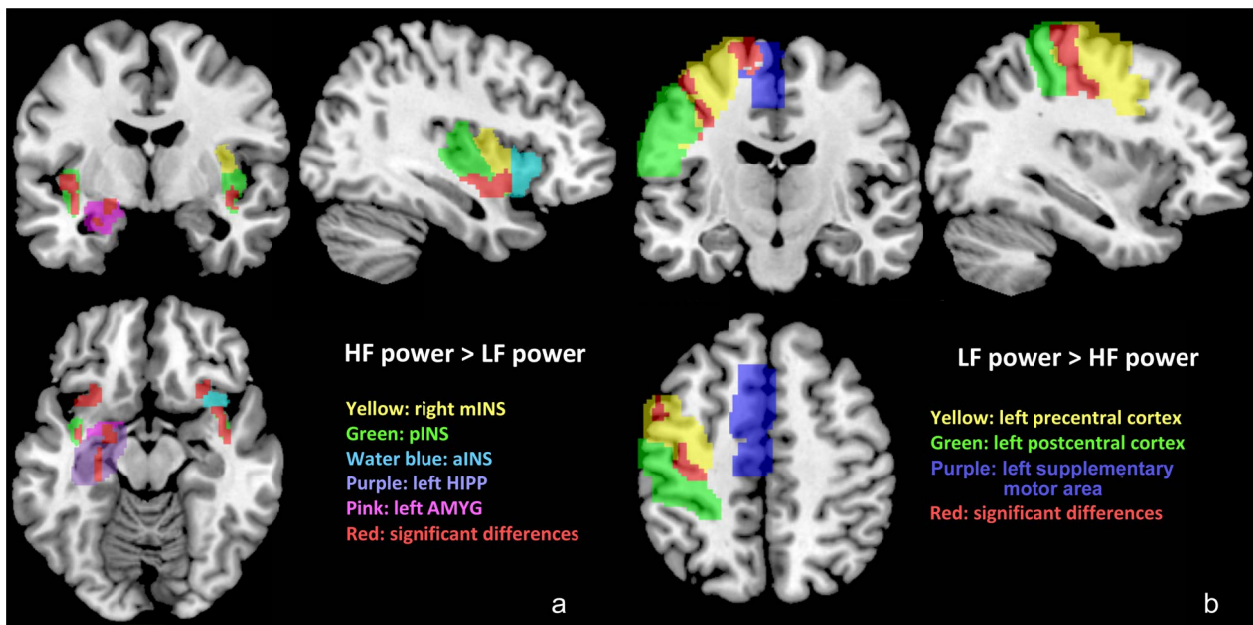
Figure 4. Altered regional BOLD oscillations distribution in female IBS compared to male IBS. a) Significant differences of HF versus LF power distribution in female IBS compared to male IBS subjects are shown in red (p<0.05, FWE corrected). b) ROIs with significant differences between LF versus HF power distribution in female IBS compared to male IBS are shown in red (p<0.05, FWE corrected).
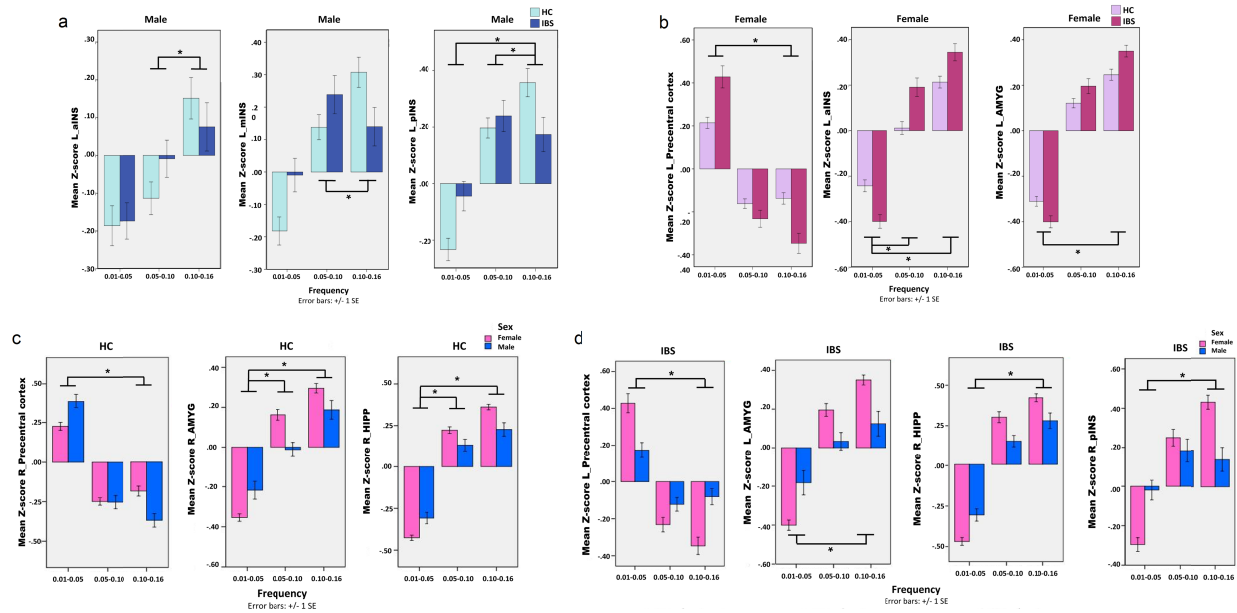
Figure 5. Comparison of frequency power in represented ROIs across groups. Graphs show normalized mean z-score of frequency power distribution for selected ROIs. a) Male HCs showed greater frequency power distributions skewed from MF toward HF in left INS subregions. b) Female IBS showed greater frequency power distributions skewed toward HF in left AMYG and anterior INS, and toward LF in left precentral cortex compared to female HCs. c) Female HCs had greater frequency power shifts from LF toward higher frequencies in right AMYG and right HIPP compared to Male HCs. Male HCs had a greater skew from HF toward LF in right precentral cortex compared to female HCs. d) Female IBS showed greater frequency power oscillation skewed toward HF in right posterior INS, right HIPP and left AMYG, and toward LF power in left precentral cortex compared to male IBS. *: Significant difference, p<0.05, FWE corrected.
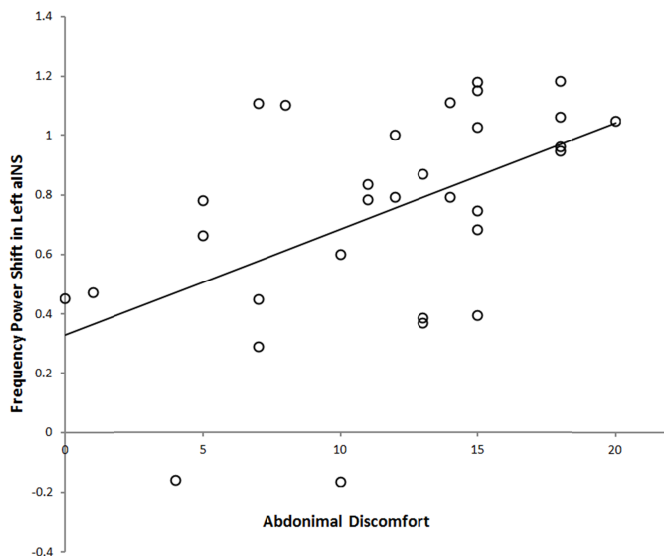
Figure 6. Correlations of BOLD signal dynamics with symptom. Graphs show significantly positive correlation (p<0.05, FWE corrected) between frequency power distribution skewed from LF toward HF in left anterior INS with abdominal discomfort in IBS females.

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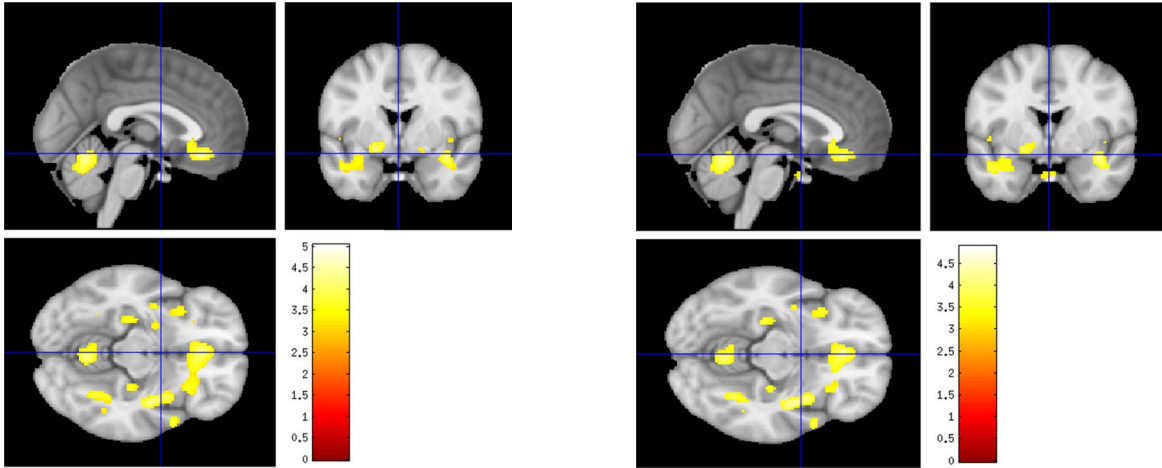
Supplemental Figure 1. Comparison between two contrast maps with and without controlling protocol. We reanalyzed our data controlling for different protocols as a nuisance covariate by adding it as a factor and specifying it as a main effect in our model. Based on the sensitivity analysis, the results with and without protocol as a nuisance variable showed no significant differences, as can be seen from this represented contrast. (Left: without protocol as a covariate, Right: with protocol as a covariate). Both maps are shown with adjusted threshold at p=0.001.

Chapter 3

Sex and Disease-Related Alterations of Anterior Insula Functional Connectivity in Chronic Abdominal Pain

This chapter is adapted from:

Hong JY, Kilpatrick LA, Labus JS, Gupta A, Katibian D, Ashe-McNalley C, Stains J, Heendeniya N, Smith SR, Tillisch K, Naliboff B, Mayer EA (2014b) Sex and disease-related alterations of anterior insula functional connectivity in chronic abdominal pain. J Neurosci 34:14252-14259.

Abstract

Resting-state functional magnetic resonance imaging has been used to investigate intrinsic brain connectivity in healthy subjects and patients with chronic pain. Sex-related differences in the frequency power distribution within the human insula (INS), a brain region involved in the integration of interoceptive, affective and cognitive influences, have been reported. Here we aimed to test sex and disease-related alterations in the intrinsic functional connectivity of dorsal anterior INS. The anterior INS is engaged during goal-directed tasks, and modulates the default mode and executive control networks. By comparing functional connectivity of dorsal anterior INS in age-matched female and male healthy subjects and patients with irritable bowel syndrome, a common chronic abdominal pain condition, we show evidence for sex and disease-related alterations in the functional connectivity of this region: 1) Male patients compared to female patients had increased positive connectivity of dorsal anterior INS bilaterally with medial prefrontal cortex (PFC) and dorsal posterior INS; 2) Female patients compared to male patients, had greater negative connectivity of left dorsal anterior INS with left precuneus; 3) Disease-related differences in the connectivity between bilateral dorsal anterior INS and dorsal medial PFC were observed in female subjects; 4) Clinical characteristics were significantly correlated to the insular connectivity with dorsal medial PFC in male IBS subjects and with precuneus in female IBS subjects. These findings are consistent with the INS playing an important role in modulating the intrinsic functional connectivity of major networks in the resting brain, and show that this role is influenced by sex and diagnosis.

Introduction

Resting-state functional magnetic resonance imaging (fMRI) has been increasingly used to examine intrinsic brain connectivity in both healthy subjects (Spreng et al., 2013; Wang et al., 2014) and various disease populations (Mainero et al., 2011; Woodward et al., 2012). Alterations in the connectivity of regions comprising major intrinsic brain networks at rest have been reported in patients with chronic pain disorders including fibromyalgia (Napadow et al., 2010), chronic back pain (Kong et al., 2013), and headache (Xue et al., 2012; Qiu et al., 2013). Irritable bowel syndrome (IBS), the most common visceral pain disorder, is characterized by chronically recurrent abdominal pain associated with changes in bowel habits (Drossman, 2006). Like many other chronic pain syndromes, IBS is more prevalent in women (Adeyemo and Chang, 2008; Chang, 2011). Disease-related differences in the activity and responses of several brain regions including insula (INS), anterior cingulate cortex, and prefrontal cortex (PFC) have consistently been observed in IBS subjects, using resting-state and task-based fMRI (Rapps et al., 2008; Mayer et al., 2009b; Tillisch et al., 2011; Hong et al., 2013). Sex-related differences in both healthy controls (HCs) and IBS subjects have been found in numerous studies, including task-based (Labus et al., 2008; Labus et al., 2013b), morphometric (Luders and Toga, 2010; Jiang et al., 2013; Ingalhalikar et al., 2014), and resting-state imaging studies (Allen et al., 2011; Filippi et al., 2013; Hong et al., 2013). We recently reported sex and disease-related differences in spontaneous blood-oxygen-level dependent signal oscillations in IBS, with female patients showing greater high frequency power in the INS compared to female HCs and male IBS subjects (Hong et al., 2013).

Cognitive factors, including selective attention and pain prediction, play an important role in the subjective experience of pain in both HCs and chronic pain patients (Dunckley et al., 2007;

Wiech and Tracey, 2013), and sex-related differences in these cognitive factors have been reported (Straube et al., 2009b; Popescu et al., 2010). Engagement of dorsal anterior INS during pain perception (Lutz et al., 2013), executive control and attention processing (Dosenbach et al., 2007; Chang et al., 2013) and prediction (Preuschoff et al., 2008) have consistently been observed. In the current study, we aimed to characterize sex and disease-related differences in resting-state functional connectivity of dorsal anterior INS in age-matched HCs and IBS patients. Specifically, we wanted to test the following hypotheses: 1) Disease-related differences exist in the way that the dorsal anterior INS is functionally connected to other brain regions (e.g. PFC). 2) Sex-related differences exist in the functional connectivity of the dorsal anterior INS in both HCs and IBS subjects. 3) The functional connectivity differences are correlated with clinical characteristics.

Materials and Methods

Subjects.

96 age-matched and right-handed subjects were recruited through the UCLA Digestive Diseases Clinic and advertisements. The sample included 24 male HCs (mean age=34.33 years, standard deviation=11.19), 24 female HCs (mean age=30.67 years, standard deviation=9.9), 24 male IBS subjects (mean age=34.71 years, standard deviation=10.38) and 24 female IBS subjects (mean age=33.58 years, standard deviation=9.74). 52 subjects have been used in previous study from our group (Hong et al., 2013). All procedures were approved by the UCLA Medical Institutional Review Board, and all subjects provided informed consent. Exclusion criteria included substance abuse, pregnancy, tobacco dependence, abdominal surgery, and psychiatric illness as determined by the Mini International Neuropsychiatric Interview (Sheehan et al., 1998). Diagnosis of IBS was made by a gastroenterologist or nurse practitioner with expertise in

functional gastrointestinal (GI) disorders based on the ROME III symptom criteria during a clinical assessment (Drossman, 2006). The diagnostic criteria included recurrent abdominal pain or discomfort associated with two or more of the following: 1) pain/discomfort is relieved/improved by defecation 2) the onset of pain/discomfort is related to a change in frequency of stool 3) the onset of pain/discomfort is related to a change in the form (appearance) of stool.

Materials.

Questionnaires were completed before scanning to determine GI symptom type, duration of symptoms, severity, and abdominal sensation during the past week [UCLA Bowel Symptom Questionnaire, BSQ] (Chang et al., 2001), levels of anxiety and depression [Hospital Anxiety Depression Scale, HAD] (Mykletun et al., 2001), GI symptom-specific perception associated with prediction, fear and worry [Visceral Sensitivity Index, VSI] (Labus et al., 2004; Labus et al., 2007) and intensity of gastrointestinal symptoms over the past 24 hours with using the Gracely Pain Scale (Heft et al., 1980).

Image Data Acquisition.

All resting state images were acquired with subjects resting with eyes closed during a 10 minute scan in a Siemens 3 Tesla Trio scanner with echo planar sequence, repetition time (TR): 2000ms, echo time (TE): 28ms, flip angle: 77 degrees, slice thickness: 4mm, 40 slices were obtained with whole-brain coverage. High resolution structural images were collected with standard T1-weighted magnetization-prepared rapid acquisition gradient echo (MP-RAGE), TR: 2200ms, TE: 3.26ms, slice thickness: 1 mm, 176 slices, 256 x 256 voxel matrices, and 1.0×1.0×1.0 mm voxel size.

Resting State fMRI Image Preprocessing.

Resting state image preprocessing was performed using Statistical Parametric Mapping 8 (SPM8) software (Wellcome Department of Cognitive Neurology, London, UK). The first three volumes were removed to allow for scanner stabilization. Images were transformed from DICOM into NIFTI format, slice-time corrected, spatially normalized to the MNI template with the MP-RAGE scan and resampled to a voxel size of 2x2x2mm.

Resting State fMRI Seed-to-Voxel Analysis.

Preprocessed and normalized functional images were imported into the CONN-fMRI functional connectivity toolbox v13 (Whitfield-Gabrieli and Nieto-Castanon, 2012) (http://www.nitrc.org/projects/conn) for further preprocessing and for seed-to-voxel connectivity analysis. A component-based noise correction method, CompCor, was used to remove non-neural noises without regressing out the global signal in order to increase sensitivity and specificity of connectivity analysis (Behzadi et al., 2007; Whitfield-Gabrieli and Nieto-Castanon, 2012). Three dimensional confounds for both white matter and cerebrospinal fluid, as well as six realignment parameters and first-order temporal derivatives of motion were removed using regression. Resting state images then were bandpass filtered between 0.008 and 0.08 Hz in the CONN toolbox. Bilateral dorsal anterior INS were selected as the regions of interests (ROIs) based on previous research (Deen et al., 2011; Chang et al., 2013). The first author of this study delineated the left and right dorsal anterior INS on the Destrieux Atlas template (Fischl et al., 2004) by following the boundaries of anterior limiting sulcus, short insular sulcus and by using the coordinate z = -1 in MNI space (Naidich et al., 2004; Craig, 2009; Kurth et al., 2010). Connectivity correlation

coefficients representing the association between average BOLD time series across all voxels within the left and right dorsal anterior INS and every voxel in the brain were calculated using a general linear model. Each ROI Fisher's r to z transformed bivariate correlation maps were smoothed with a 4mm isotropic Gaussian kernel and submitted into group level analyses implemented in SPM8.

Conjunction analysis.

For right and left dorsal anterior INS, a second-level random effects full factorial model was specified with group (male IBS, female IBS, male HCs and female HCs) as a factor and age, anxiety and depression scores as covariates. A cerebral cortex explicit mask was applied. To examine the functional connectivity patterns (positive and negative) of the dorsal anterior INS among all four groups, conjunction null tests were performed in SPM8 (Friston et al., 2005). After applying voxel-wise threshold p-value of 0.005, significance was considered at a cluster threshold of $p<0.05$ corrected for multiple comparisons using family wise error (FWE).

ANOVA.

For second level group comparison, a voxel-wise one-way ANOVA test was performed to determine differences among the four groups (male IBS, female IBS, male HCs and female HCs) in left and right dorsal anterior INS correlation maps. To account for multiple comparisons, results were corrected by performing Monte Carlo simulation implemented in AlphaSim (http://afni.nimh.nih.gov/afni/doc/manual/AlphaSim) (Song et al., 2011) at 10,000 iterations, cluster connection radius=3.46mm and FWHM=4mm. The simulation demonstrated that clusters with a voxel threshold of $p<0.005$ with a contiguous cluster sized greater than 29 voxels within a

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brain mask had a corrected p value <0.05. To further describe the significant group differences detected in SPM8, we extracted the connectivity correlation coefficient (z value) averaged over the voxels within each significant cluster and performed post-hoc tests in SPSS version 19 software with four different contrasts (male HCs versus female HCs, male HCs versus male IBS, female HCs versus female IBS, and male IBS versus female IBS). A sex and disease interaction analysis was also performed. False-discovery rate (FDR) correction at p<0.05 (Benjamini and Hochberg, 2000; Benjamini et al., 2006) was applied to correct for multiple comparisons. We also plotted the mean of the z values for each group.

Clinical Characteristics Analysis.

ANOVA with post-hoc comparisons were employed to test for differences in age, VSI and HAD anxiety and depression. All p values were corrected for multiple comparisons using FDR correction at 5% (Benjamini and Hochberg, 2000; Benjamini et al., 2006). Independent sample t tests were performed to examine BSQ measures between male and female IBS subjects.

Correlation Analysis.

To examine the possible associations between clinical characteristics and altered functional connectivity, we performed correlation analyses in SPSS software. Averaged z values within the significant clusters were correlated with VSI, BSQ and past 24 hours symptom intensity for the IBS groups separately. Significant correlations were defined at p value <0.05.

Results

Clinical characteristics

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The clinical data are summarized in **Table 1**. Anxiety and depression symptom scores were greater in male IBS patients compared to male HCs (anxiety: p=.033; depression: p=.021), and in female IBS patients compared to female HCs (anxiety: p<.001; depression: p<.001). Higher anxiety and depression symptom scores were observed in female patients compared to male patients, even though the differences did not reach statistical significance. Male and female patients had significantly higher VSI scores than male and female HCs, respectively (p<.001 and p<.001). In addition, female IBS subjects showed significantly higher VSI scores than male IBS subjects (p=.003). There was no significant difference in IBS-related symptom scores (i.e. BSQ) between male and female IBS subjects. Intensity of gastrointestinal symptoms over the past 24 hours was higher in female IBS subjects compared to male IBS subjects, but it did not reach significance (p=.18). In the study, 87.5% women were in premenopausal stage (22 female HCs and 20 female IBS subjects who were well balanced in terms of luteal and follicular phases during scanning).

Connectivity of dorsal anterior INS among all groups

We first investigated the functional connectivity patterns of dorsal anterior INS shared by all subjects (IBS+HCs). Significant results from the whole-brain connectivity analyses with dorsal anterior INS seeds are shown in **Figure 1**. Bilateral dorsal anterior INS showed positive functional connectivity with widespread brain regions including cingulate subregions (mid and anterior cingulate cortex), INS, supramarginal gyrus, somatosensory (thalamus, putamen), motor (supplementary motor area, precentral gyrus), frontotemporal (inferior frontal gyrus, rolandic operculum, middle frontal gyrus, superior temporal gyrus), and affective (amygdala) regions (**Figure 1a and 1b**). We also observed bilateral dorsal anterior INS to be negatively connected to

bilateral precuneus and angular gyrus (**Figure 1c and 1d**).

Group differences in dorsal anterior INS connectivity

As shown in **Table 2**, significant group differences were identified for several regions that were functionally connected to dorsal anterior INS, including bilateral dorsal medial PFC [Brodmann area 9, BA 9], medial PFC (BA 10), left dorsal posterior INS and left precuneus. Post hoc tests were performed to examine between group functional connectivity differences. Male HCs and male IBS subjects showed significantly greater positive connectivity between bilateral dorsal anterior INS and left dorsal posterior INS compared to female HCs and female IBS subjects, respectively (**Table 2 and Figure 2 and 3**). Similarly, male HCs and male IBS subjects showed greater right dorsal anterior INS connectivity to right medial PFC than female HCs and IBS subjects, respectively. This was due to the right dorsal anterior INS being positively connected to right medial PFC in male subjects while negatively connected in female subjects (**Table 2 and Figure 3**). Disease-related differences in the connectivity of left dorsal anterior INS with left medial PFC (BA 10) and left dorsal medial PFC (BA 8/9) were observed within the female subjects (**Table 2 and Figure 2**). While negative connectivity between dorsal anterior INS and these PFC regions were observed in female HCs and female IBS subjects, female IBS subjects showed greater negative connectivity than female HCs as well as male IBS subjects (**Table 2 and Figure 2 and 3**). Similarly, left dorsal anterior INS was negatively connected to left precuneus in all subjects, but female IBS subjects showed significantly greater negative connectivity compared to female HCs and male IBS subjects (**Table 2 and Figure 2**). Sex and disease interactions were observed in the connectivity of left dorsal anterior INS with left medial PFC (p=.032) and precuneus (p=.015).

Correlations of dorsal anterior INS functional connectivity with clinical characteristics.

We correlated functional connectivity measures with symptom scores for male and female IBS subjects separately. In male IBS subjects, VSI significantly correlated with the connectivity between bilateral dorsal anterior INS and dorsal medial PFC (left: r=.442, p=.031; right: r=.405, p=.05); and between left dorsal anterior INS and left medial PFC (r=.41, p=.047). In female IBS subjects, ratings of past 24 hours symptom intensity significantly correlated with the connectivity between left dorsal anterior INS and precuneus (r=.597, p=.007); and between bilateral dorsal anterior INS and dorsal medial PFC (left: r=.474, p=.04; right: r=.466, p=.044).

Discussion

Using resting state functional connectivity analysis, we investigated the disease and sex-related differences in the connectivity of the dorsal anterior INS in 96 male and female HCs and IBS subjects. The main findings of this study were: 1) Among all subjects, dorsal anterior INS showed positively functional connectivity with the entire INS, as well as with frontal, sensorimotor and affective regions, and with cingulate subregions. 2) Disease-related differences in the connectivity were seen in female subjects, and included greater negative dorsal anterior INS connectivity with left precuneus and frontal regions. 3) Sex-related differences in the dorsal anterior INS connectivity to medial PFC and dorsal medial PFC were observed in both HCs and IBS subjects, and these sex-related differences were magnified in IBS subjects. 4) Clinical measures were correlated with the connectivity between dorsal anterior INS and dorsal medial PFC in male IBS patients as well as between dorsal anterior INS and precuneus in female IBS subjects. These findings in a chronic visceral pain population for the first time demonstrate

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differential intrinsic brain connectivity of dorsal anterior INS depending on sex and diagnosis.

Functional connectivity of large-scale networks among all groups

Studies have used resting-state functional connectivity networks to characterize interactions of brain regions associated with specific functions. Several robust resting state networks have been revealed including the default mode network (DMN) (Buckner et al., 2008), the salience network (Seeley et al., 2007), and the executive control network (ECN) (Dosenbach et al., 2007; Laird et al., 2011; Touroutoglou et al., 2012; Spreng et al., 2013). However, discrepancies in the inclusion of certain brain regions in a particular network still exist between different studies depending on analysis strategies, network definitions and study samples. Converging evidence from functional imaging studies suggests that dorsal anterior INS is involved in attention, cognitive control, prediction and pain perception (Touroutoglou et al., 2012; Chang et al., 2013). In accordance with earlier studies (Deen et al., 2011; Moran et al., 2013), we observed in the combined data set (male + female; IBS + HCs) that the dorsal anterior INS was positively connected to several task-positive resting state networks (Di and Biswal, 2013) including the ECN and the salience network. We also observed negative connectivity of dorsal anterior INS with precuneus and angular gyrus, both of which are part of the DMN. Our findings of positive and negative functional connectivity of the dorsal anterior INS with regions of different brain networks are similar with previous reports on anti-correlations between task-negative and task-positive networks (Fox et al., 2005). Recently, a triple network model has been proposed in which the anterior INS plays a critical role in switching between the ECN and DMN (Sridharan et al., 2008; Menon and Uddin, 2010). In line with these reports, our findings are consistent with a role for dorsal anterior INS functioning as a major hub mediating the interactions between task-positive and task-negative networks.

Disease-related differences in the functional connectivity

Altered intrinsic functional connectivity in resting state networks have been reported in several chronic pain conditions (Baliki et al., 2008; Napadow et al., 2010; Qiu et al., 2013). Given that IBS-related differences in functional brain activity involving the anterior INS have also been reported (Elsenbruch et al., 2010; Hong et al., 2013), we aimed to test the hypothesis that the dorsal anterior INS shows disease-related alterations in functional connectivity. Female IBS patients compared to female HCs displayed greater negative functional connectivity of dorsal anterior INS with medial PFC and precuneus which are key components of the DMN. Napadow et al. (2010) also found greater functional connectivity in female fibromyalgia patients (compared to HCs) between the DMN and regions involved in pain perception, including dorsal anterior INS. The reason for the difference in the direction of the functional connectivity between the two studies is unclear, but may be due to differences in study techniques. For example, we used dorsal anterior INS as a seed and correlated to all voxels within the brain mask, while Napadow et al. performed independent component analysis and compared the selected DMN maps. Although DMN is usually anti-correlated with task-positive network, heterogeneity of sub DMN components may exist and be activated or deactivated depending on the task (Leech et al., 2011; Anticevic et al., 2012). Similar to our findings, alterations in the connectivity of the anterior INS have also been reported in subjects with migraine (Xue et al., 2012) and schizophrenia (Manoliu et al., 2013a; Moran et al., 2013).

Sex-related differences in the functional connectivity

A comprehensive resting-state imaging study has suggested sex-related differences exist in

functional connectivity within both the task-positive network and DMN (Biswal et al., 2010). Similarly, in the current study, several sex-related differences in functional connectivity of the dorsal anterior INS with other brain regions were observed:

Intrainsular connectivity. Male subjects showed greater functional connectivity between dorsal anterior INS and dorsal posterior INS compared to females. As the posterior INS functions as primary interoceptive representation area receiving inputs from somatosensory and viscerosensory regions (Craig, 2009; Farb et al., 2012), one may speculate that male subjects exert greater cognitive modulation of primary sensory information than females.

Connectivity with precuneus. Compared to male HCs, female HCs showed greater negative connectivity between dorsal anterior INS and precuneus and this sex-related difference was magnified in IBS subjects. Failures of down-regulation within precuneus and shifting away from DMN in response to pain stimuli have been observed in female IBS subjects (Hall et al., 2010). One may speculate that the negative connectivity of precuneus with dorsal anterior INS is related to modulation of DMN by the INS in female subjects, and is enhanced in female IBS patients.

Connectivity between dorsal anterior INS and medial PFC. Sex-related differences for functional connectivity between dorsal anterior INS and medial PFC were observed, with males showing positive, and females showing negative connectivity. The medial PFC plays roles in social cognition, self-relevance and emotion regulation (Etkin et al., 2011; Menon, 2011), and can engage either in DMN or ECN depending on the condition-oriented function (Daniels et al., 2010). A recent study proposed that anterior INS may play a role in this condition-dependent switching and modulation of function (Menon and Uddin, 2010). Our results suggest that this modulation mechanism can be influenced by sex even during the resting state: medial PFC appears to be more engaged in cognitive function for males given the positive connectivity with dorsal anterior INS,

while medial PFC may be more involved in self-referential mental activity for females, especially female patients, given the negative connectivity with dorsal anterior INS. Similarly, female subjects with temporomandibular disorder showed strong correlations between medial PFC to precuneus functional connectivity and pain rumination (Kucyi et al., 2014). Even though sex hormone levels were not determined in the current study, 87.5% of female subjects were in the premenopausal state, and female groups were well balanced in terms of menstrual cycle phase they were studied in. Thus we feel that a confounding effect of sex hormone levels is unlikely.

Symptoms correlation with dorsal anterior insular connectivity

In male IBS patients, higher scores of symptom-specific worries and prediction of negative outcomes was associated with increased functional connectivity between dorsal anterior INS and dorsal medial PFC which are suggested to be involved in cognitive functions such as anticipation, error prediction, probability assessment and expectation of uncertainty (Lovero et al., 2009; Holtz et al., 2012; Grupe and Nitschke, 2013). For female IBS patients, the functional connectivity between dorsal anterior INS and precuneus was positively correlated with the reported intensity of recent gastrointestinal symptoms. This is consistent with findings of previous studies (Napadow et al., 2010; Loggia et al., 2013) in which chronic pain patients (female-predominant) with greater DMN-INS anti-correlation demonstrated less clinical pain and patients with more positive DMN-INS connectivity demonstrated greater clinical pain. When viewed together, the altered DMN-INS connectivity may play a role in the subjective symptom generation in female chronic pain patients, regardless of specific diagnosis. However, the positive association between DMN-INS connectivity and pain symptoms needs additional study. Collectively, our results suggest that male IBS subjects recruited dorsal medial PFC during negatively biased predictions

about expected GI symptoms, whereas female IBS subjects engaged regions in the DMN to generate self awareness of symptoms. In order to more closely link pain state to the intrinsic brain activity during scanning, symptom measurements such as spontaneous pain scores and cognitive-specific task performances should be included in future studies.

Conclusions

The results of this study are consistent with previous reports in healthy subjects and in other disease populations, suggesting that the dorsal anterior INS plays an important role in modulating the intrinsic functional connectivity of major networks in the resting brain, and that this role is influenced by sex and diagnosis. Sex-related differences in the connectivity patterns of the dorsal anterior INS suggest that the female brain shows greater resource allocation to interoceptive awareness, whereas the male brain relies more on cognitive function. As these sex-related differences are enhanced in IBS patients, one may speculate that these mechanisms play a role in the pathophysiology of IBS.

Table 1. Clinical characteristics

	HCM			HCF			IBSM			IBSF			
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	F/t	Sig.
Age	24	34.33	11.19	24	30.67	9.9	24	34.71	10.38	24	33.58	9.74	.76	.52
Anxiety[1]	24	3	2.7	24	1.91	1.77	24	5.83	4.88	24	7.96	5.55	11.13	<.001
Depression[1]	24	1.38	1.53	24	.46	.78	24	3.17	2.88	24	3.79	3.37	10.15	<.001
VSI[2]	23	3.39	4.46	22	2.14	4.64	24	32.08	15.72	24	43.04	16.67	68	<.001
Overall bowel symptoms[3]	-	-	-	-	-	-	24	9.96	4.23	24	8.83	4.8	.97	.39
Abdominal pain[4]	-	-	-	-	-	-	24	9.17	4.48	24	8.75	4.81	.41	.76
Abdominal discomfort[5]	-	-	-	-	-	-	24	9.79	5.18	24	11.21	5.89	.89	.38
Duration of symptoms[6]	-	-	-	-	-	-	24	13	9.31	22	13.14	9.86	.05	.96
Symptom intensity[7]	-	-	-	-	-	-	15	5.67	4.91	19	7.95	4.7	1.38	.18

F= main effect of group from ANOVA and t-tests for four and two group comparisons, respectively.

1. HAD: Hospital Anxiety and Depression (Mykletun et al., 2001);
2. VSI: Visceral Sensitivity Index (Labus et al., 2004; Labus et al., 2007);
BSQ: Bowel Symptom Questionnaire (Chang et al., 2001)
3. BSQ overall symptoms in the past week (0-20)
4. BSQ abdominal pain in the past week (0-20)
5. BSQ discomfort in the past week (0-20)
6. BSQ duration in years, derived from onset of symptom
7. Intensity of gastrointestinal symptoms over the past 24 hours (0-20)
Statistically significant p<0.05; SD: standard deviation; M: male; F: female

Table 2. Significant differences of the functional connectivity with dorsal anterior INS among male and female HCs and IBS subjects

ANOVA							Post hoc (Pcorr)
	Peak F-value	Z	Voxel	x	y	z	
L_daINS with							
L_mPFC (BA10)	11.31	4.58	108	-8	52	10	IBSM>IBSF (<.001); HCF>IBSF (.018)
L_dpINS	8.08	3.77	61	-40	-10	8	IBSM>IBSF (<.001); HCM>HCF (.03)
L_dmPFC (BA8/9)	7.7	3.66	52	-16	34	54	IBSM>IBSF(<.001);HCM>HCF(.008); HCF>IBSF (.025)
L_precuneus	6.82	3.39	32	-4	-44	40	IBSM>IBSF (<.001); HCF>IBSF (.006)
	7.42	3.58	30	-14	-54	26	
R_daINS with							
R_mPFC (BA 10)	9.25	4.09	78	4	54	12	IBSM>IBSF (<.001); HCM>HCF (.007)
L_dpINS	9.15	4.06	60	-46	-12	12	IBSM>IBSF (<.001); HCM>HCF (<.001)
R_dmPFC (BA 9)	7.42	3.58	54	20	48	40	IBSM>IBSF (.006); HCF>IBSF (.006)

MNI coordinates (x,y,z) for peak voxel; M: male; F: female; L: left; R: right; daINS: dorsal anterior insula; dpINS: dorsal posterior insula; dmPFC: dorsal medial prefrontal cortex; mPFC: medial prefrontal cortex; Pcorr: statistical significance at p<0.05, corrected
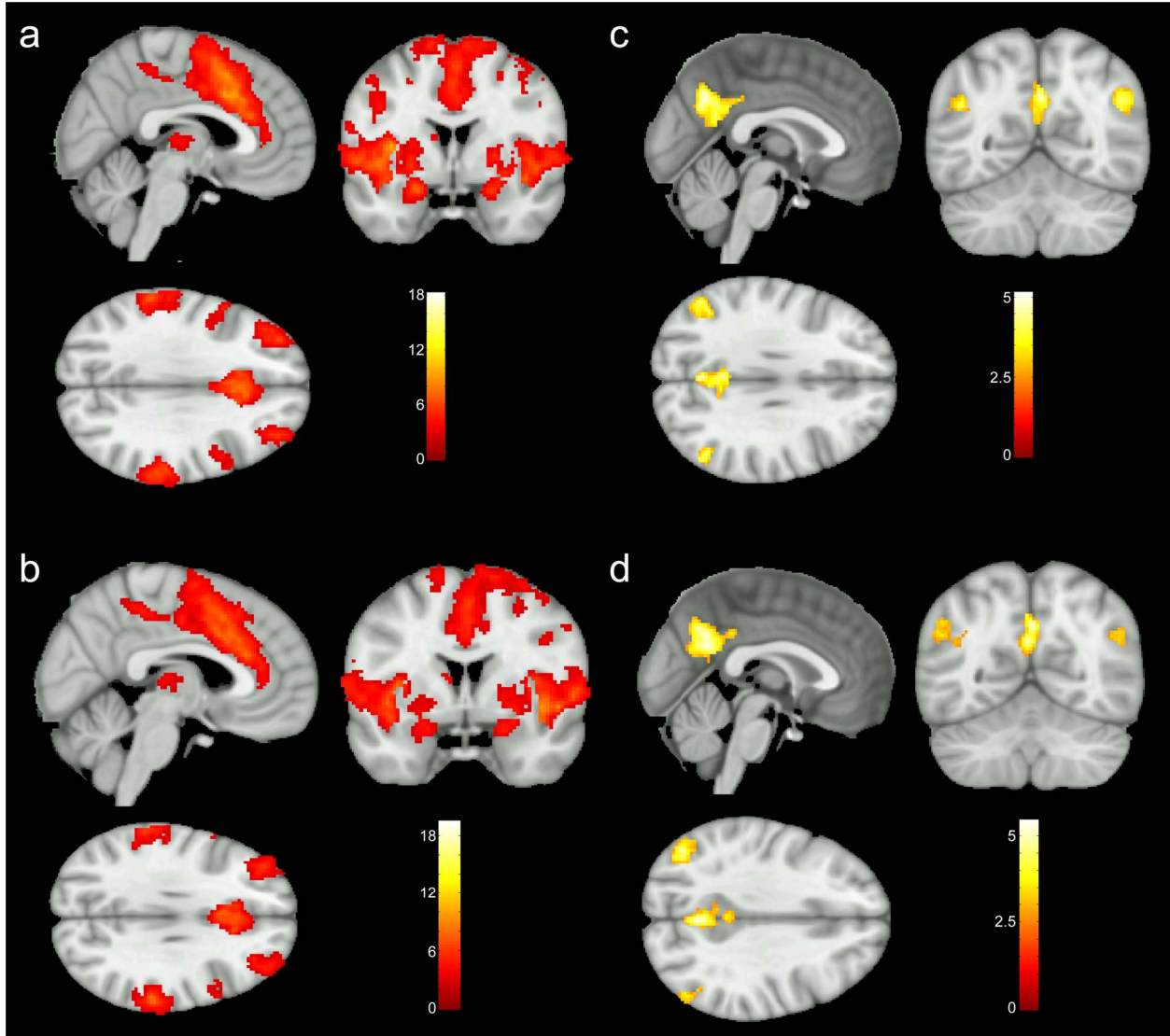
Figure 1. Significant clusters connected to the dorsal anterior INS among all subjects.
Regions showed positive functional connectivity to a) left dorsal anterior INS and b) right dorsal anterior INS. Regions were negatively connected to c) left dorsal anterior INS and d) right dorsal anterior INS. Images were thresholded at a voxel-wise p-value of 0.005 and cluster-wise corrected for multiple comparisons using FWE correction at a p-value<0.05.
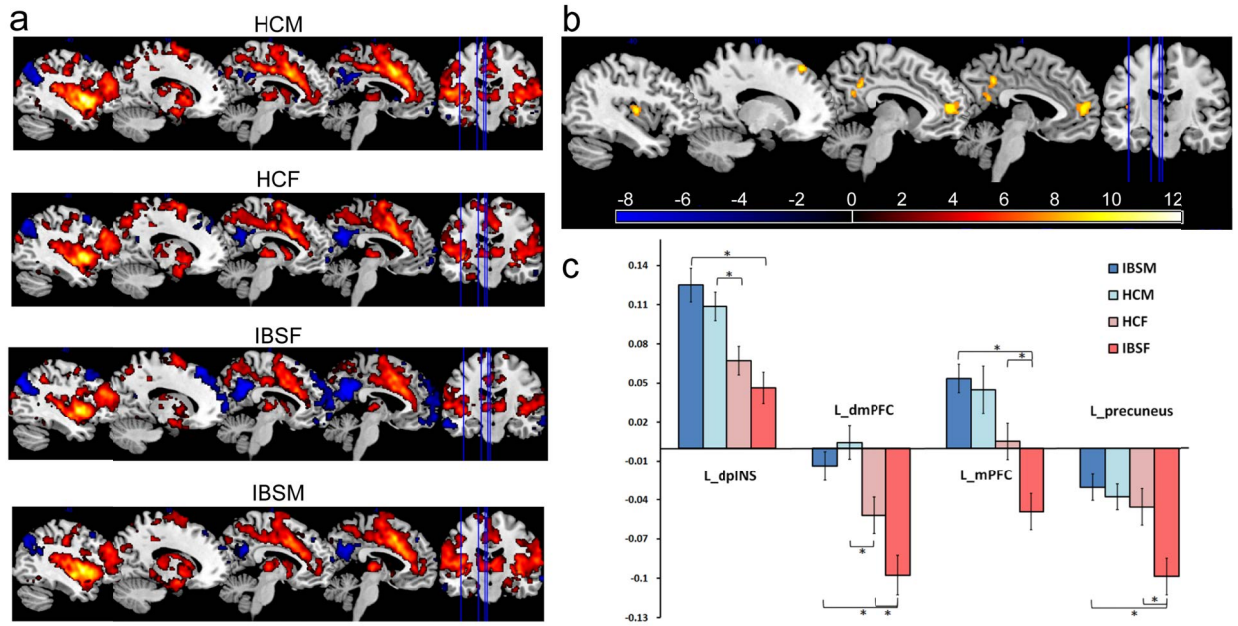
Figure 2. Group differences in functional connectivity of left dorsal anterior INS. a) Single group connectivity maps of left daINS; For display purposes only, all statistical results, thresholding at p<0.005 uncorrected and cluster>29 voxels, were overlapped on a MRIcron ch2better template; Red: positive connectivity; Blue: negative connectivity. b) Regions showed significant functional connectivity differences between groups (p<0.05, corrected). c) Graph showed mean Z scores of significant regions connected with left dorsal anterior INS for each group. *: Significant difference after controlling for age, anxiety and depression, p<0.05, corrected; Error bars reflect standard error; L: left; dpINS: dorsal posterior insula; dmPFC: dorsal medial prefrontal cortex; mPFC: medial prefrontal cortex
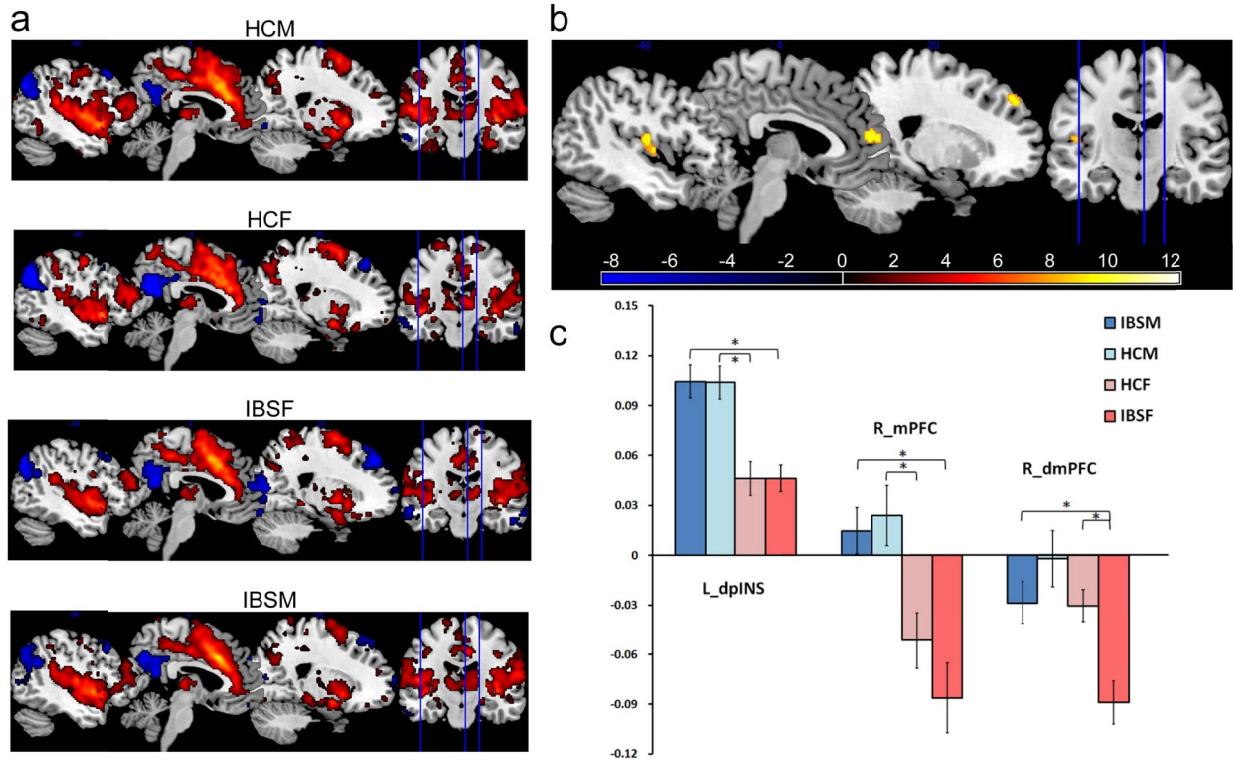
Figure 3. Group differences in functional connectivity of right dorsal anterior INS. a) Single group positive and negative connectivity maps of right dorsal anterior INS (all statistical results were overlapped on a MRIcron ch2better template). b) Regions showed significant functional connectivity differences between groups (p<0.05, corrected). c) Graph showed average Z scores of significant regions connected with right dorsal anterior INS for each group. *: Significant difference after controlling for age, anxiety and depression, p<0.05, corrected for multiple comparison; R: right; dpINS: dorsal posterior insula; dmPFC: dorsal medial prefrontal cortex; mPFC: medial prefrontal cortex

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Chapter 4

Altered Brain Activity in Patients with Chronic Abdominal Pain during Cued and Uncued Pain Expectation

This chapter is adapted from:

Abstract

A majority of patients with irritable bowel syndrome (IBS) show increased behavioral and brain responses to expected and delivered aversive visceral stimuli during controlled rectal balloon distension, and during palpation of the sigmoid colon. We aimed to determine if altered brain responses to cued and uncued pain expectation are also seen in the context of a noxious somatic pain stimulus applied to the same dermatome as the sigmoid colon. A task-dependent functional magnetic resonance imaging technique was used to investigate the brain activity of 37 healthy controls (18 females) and 37 IBS patients (21 females) during: 1) a cued expectation of an electric shock to the left lower abdomen versus a cued safe condition; and 2) an uncued cross-hair condition in which the threat is primarily based on context versus a cued safe condition. Regions within the salience, attention, default mode, and emotional arousal networks were activated by the cued abdominal threat and the cross-hair condition versus the cued safe condition. During the uncued cross-hair condition versus the cued safe condition, IBS patients showed greater brain activations in affective (amygdala, anterior insula), sensory (thalamus), and attentional (middle frontal gyrus, including dorsolateral prefrontal cortex) regions, and in the precuneus than healthy subjects. These disease-related differences were primarily seen in female subjects. The observed greater engagement of cognitive and emotional brain networks in IBS patients during contextual threat may reflect the propensity of IBS patients to overestimate the likelihood and severity of future abdominal pain.

Introduction

The brain computes expected future outcomes in an uncertain world and selects optional coping strategies (Grupe and Nitschke, 2013) based on complex interactions between brain networks concerned with salience evaluation, attention and recall of past memories (Mineka and Zinbarg, 2006). Hypervigilance and anticipatory anxiety are often associated with prediction of future pain and can increase the subsequent subjective experience of a delivered pain stimulus (Porro et al., 2002; Keltner et al., 2006), presumably by the engagement of endogenous pain facilitation systems (Porro et al., 2002; Paulus and Stein, 2006; Wiech et al., 2008; Straube et al., 2009a; Grupe and Nitschke, 2013). Predictions about future pain can be manipulated using designs that vary the predictability of the aversive stimulus. Prior studies have used cued designs in which a specific cue gives the subject information about the probability of an aversive stimulus (Ploghaus et al., 1999; Berman et al., 2006; Schunck et al., 2008; Straube et al., 2009a; Atlas et al., 2010; Kano et al., 2013; Seifert et al., 2013). It is hypothesized that illness specific anxiety may be most strongly related to uncued or contextual threat, in which there is no explicit threat cue or known threat interval, but instead the experimental environment itself is associated from prior experience with aversive stimulation (Grillon et al., 2004; Grillon et al., 2006; Grillon, 2008; Sarinopoulos et al., 2010; Schmitz et al., 2011). Cued expectation of painful stimuli is associated with activation in the regions of salience (insula [INS], anterior cingulate cortex [ACC]), sensorimotor (pre and post central gyrus) and attentional control (parietal and frontal cortex) networks (Yaguez et al., 2005; Carlsson et al., 2006; Larsson et al., 2012; Seidel et al., 2014). The INS, amygdala, hippocampus and ACC also play important roles in responding to an uncertain aversive stimulus and contextual threat (Carlsson et al., 2006; Alvarez et al., 2008; Simmons et al., 2008; Sarinopoulos et al., 2010). These cued, uncued or contextual paradigms have been used to characterize brain abnormalities in

patients with anxiety disorders and post traumatic stress disorder (Grillon et al., 2008; Grupe and Nitschke, 2013; Simmons et al., 2013).

Irritable bowel syndrome (IBS), a common gastrointestinal pain disorder, is characterized by chronically recurrent abdominal pain and discomfort associated with altered bowel habits. The majority of patients exhibit increased trait anxiety (Longstreth et al., 2006) and gastrointestinal symptom-related worries, a measure of hypervigilance to gastrointestinal symptoms and gastrointestinal-related contexts (Labus et al., 2007). Many patients also show increased perceptual hypersensitivity to experimental visceral stimuli (Elsenbruch, 2011; Kanazawa et al., 2011; Keszthelyi et al., 2012a) as well as altered responses in salience and pain processing regions both during cued expectation (Berman et al., 2008; Labus et al., 2008; Larsson et al., 2012) and delivery of rectal pain stimuli, most consistently in INS, ACC and thalamus (Sheehan et al., 2011; Tillisch et al., 2011). Greater engagement of an emotional arousal network including anterior INS, amygdala and ACC has been suggested as a key neurobiological mechanism underlying hypervigilance and hypersensitivity in IBS patients (Keszthelyi et al., 2012a).

Sex-related differences of brain activation have also been observed in response to experienced visceral pain and somatosensory stimuli (Kern et al., 2001; Berman et al., 2006; Henderson et al., 2008; Benson et al., 2014) and during cued expectation of unpleasant stimuli (Straube et al., 2009b; Benson et al., 2012; Kano et al., 2013). During cued expectation, healthy women relative to men showed greater brain activation of medial prefrontal cortex (Straube et al., 2009b), precuneus, supplementary motor area (Kano et al., 2013), dorsolateral prefrontal cortex (Benson et al., 2012), and decreased activation of the INS (Berman et al., 2006).

In the current study, by using an electrical stimulation delivered to the abdomen, we aimed to test three main hypotheses regarding brain responses to pain expectation: 1) Healthy controls

(HCs) and IBS subjects will show altered brain responses to both cued and uncued threat of somatic pain versus a cued safe condition, similar to the responses previously reported for experienced experimental visceral pain. 2) Disease differences in brain responses between IBS patients and HCs will be more pronounced with the increased uncertainty of a contextual threat. 3) Sex-related differences will be seen in these altered brain responses.

Materials and Methods

Subjects.

Seventy-four right-handed subjects were recruited through UCLA Digestive Disease Clinic and community advertisements. 37 subjects were diagnosed with IBS including 16 male IBS patients and 21 female IBS patients. IBS subtypes for the IBS males included 5 constipation predominant, 8 diarrhea predominant, 2 unspecified and 1 mixed. For females, subtypes included 8 constipation predominant, 3 diarrhea predominant, 2 unspecified and 8 mixed. 37 healthy subjects were also recruited including 18 females. Exclusion criteria in all subjects comprised pregnancy, substance abuse, abdominal surgery, tobacco dependence, medications that affect the central nervous system (e.g. narcotics and opioids), oral contraceptives or psychiatric illness as determined by the Mini International Neuropsychiatric Interview (Sheehan et al., 1998). Diagnosis of IBS was made by a gastroenterologist or nurse practitioner with expertise in functional gastrointestinal disorders based on the ROME III symptom criteria during a clinical examination (Drossman, 2006). The diagnostic criteria include recurrent abdominal pain or discomfort in the past 3 months associated with two or more of the following: 1) pain/discomfort is relieved/improved by defecation 2) the onset of pain/discomfort is related to a change in frequency of stool 3) the onset of pain/discomfort is related to a change in the form (appearance) of stool. All

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procedures were approved by the UCLA Medical Institutional Review Board, and all subjects provided informed consent.

Questionnaires.

Questionnaires were completed before scanning to determine IBS symptom classification, severity, duration of symptoms, and abdominal sensation during the past week [UCLA Bowel Symptom Questionnaire, BSQ] (Chang et al., 2001), levels of anxiety and depression [Hospital Anxiety Depression Scale, HAD] (Mykletun et al., 2001), as well as 21 point verbal descriptor anchored numerical rating scales for intensity and unpleasantness of pain associated with the abdominal stimulus (Heft et al., 1980).

Pain threshold assessment procedure.

Individual pain threshold was assessed before the imaging data acquisition. Abdominal electrical stimulation was performed using two electrode stimulation pads placed on the left side of subjects' lower abdomen in the region overlaying the sigmoid colon. Transcutaneous electrical stimulation to the abdomen was delivered with a Digitimer constant-current stimulator (model DS7A; Digitimer). Each stimulus consisted of a pulse train lasting 750ms with 2ms pulse width and frequency of 37 Hz. Shock level for the abdominal stimulation was individually set based on an ascending method of limits work-up procedure. The work-up began with a mild current intensity of 0.5 mA and increased 0.5 mA steps until subjects described the stimulation as 'aversive but tolerable'. After a brief rest period, a test stimulus at the aversive but tolerable threshold was given to subjects. Immediately after the stimulation, subjects were asked to rate the maximum intensity and unpleasantness of the pain they just felt using the Gracely Pain Scales (Heft et al., 1980).

Expectation of abdominal pain paradigm.

Before the anticipation of abdominal pain experimental paradigm began, subjects were told that they would see three types of visual images. One image consisted of an animation that included a blue circle with a colored bar moving to the right of the blue circle. The moving bar incrementally filled with gradient colors of light purple to blue, indicating how much time was left in this condition (**Figure 1**). During this cued safe condition, the subjects were told that they would definitely not receive an electrical stimulation. A second cued period was signaled by an animation that included a red circle and a colored bar moving to the right of the red circle. The moving bar was elongating while adding gradient colors, from yellow to red, indicating how much time was left in this condition (**Figure 1**). During this cued threat condition, the subjects were informed that they may receive an electrical stimulation at any time, the magnitude of which would match the level that they previously described as aversive but tolerable during the pain thresholding procedure. The third image was a stationary cross-hair shown in the middle of the screen with a white background. During this condition, the subjects were asked to focus on the cross-hair. Although subjects were told nothing would happen during this period, two electrodes were still attached on the subjects' lower abdomen and there was no ongoing explicit safety cue. In addition, there was not a moving bar to indicate how much time was left in this condition. We hypothesize that these cross-hair periods represent a contextual threat due to the prior history of shock and the continued presence of the abdominal electrodes, coupled with an absence of explicit ongoing cues for either safety or imminent shock or period duration. These periods will therefore be referred to as uncued or contextual threat periods below. The anticipation of abdominal pain procedure included two separate scanning runs. Each run contained seven cued threat and six cued safe conditions, and each of these conditions lasted 29 seconds. A 10-second cross-hair condition was in between each cued condition. At the beginning and end of each run, a 29 second cross-hair

period was also presented. Although subjects were told they would receive an electrical stimulation at any time during the cued threat condition, in fact, the one-second shock was only delivered once in each run. For run 1, the shock was delivered at the twelfth second of the forth cued threat condition; for run 2, the shock was administrated at the fifth second of the second cued threat condition. The presentations of conditions and timings were generated by E-Prime v2 (Psychology Software Tools, Inc, USA) and were shown in **Figure 1**.

Functional magnetic resonance imaging (fMRI) data acquisition.

Brain activity during the task was measured using a Siemens 3 Tesla Trio scanner with echo planar sequence, repetition time: 2000ms, echo time: 28ms, flip angle: 77 degrees, slice thickness: 4mm, and 40 slices obtained with whole-brain coverage. For registration purposes, high resolution structural images were collected with standard T1-weighted magnetization-prepared rapid acquisition gradient echo, repetition time: 2200ms, echo time: 3.26ms, flip angle: 9 degrees, slice thickness: 1 mm, 176 slices, 256 x 256 voxel matrices, and $1.0 \times 1.0 \times 1.0$ mm voxel size.

fMRI preprocessing.

Prior to statistical analysis, all imaging data were preprocessed using Statistical Parametric Mapping 8 (SPM8; Wellcome Trust Centre for the Study of Cognitive Neurology, London, UK). Images were first converted from DICOM into NIFTI format followed by slice timing correction to adjust for differences in slice acquisition times. Realignment was performed to estimate the 6 variable parameters of rigid body transformation and control for superfluous motion. High resolution T1-weighted magnetization-prepared rapid acquisition gradient echo images were used to align with functional images and then normalized into standard Montreal Neurological Institute brain space (2mm isotropic). All images were smoothed with 8mm full width of the kernel at half its maximum height.

Statistical analysis.

The experiment was analyzed as a block design. The general linear model was applied in SPM8 to determine brain activity during the three conditions (cued threat, cued safe and cross-hair). At the subject-level, regressors for the three conditions were convolved with a canonical hemodynamic response function. In avoidance of contamination from the brain responses of the shocks, the two cued threat periods with shocks were excluded from our analysis. Motion realignment parameters were also included as covariates. Individual brain responses for the cued threat condition and the cross-hair condition were determined by contrasting the estimated parameters with the cued safe condition (i.e. cued threat vs. cued safe and cross-hair vs. cued safe, respectively). The first level contrast maps (one map was cued threat vs. cued safe and the other map was cross-hair vs. cued safe) were used as the dependent variable in second level whole brain group analyses. Using the full factorial model option in SPM8, we specified group (male HCs, female HCs, male IBS and female IBS) as a factor and age, anxiety and depression scores as covariates. The cerebellum was excluded using an explicit mask. To examine the overall effect of the experimental conditions across groups, global conjunction analysis was performed for the contrasts of cued threat vs. cued safe and cross-hair vs. cued safe. To examine group differences, linear contrasts were specified to test main effect of disease (IBS vs. HCs). Specifically, linear contrasts were specified to examine whether the disease effect differed by sex (female IBS vs. female HCs and male IBS vs. male HCs) as well as whether there are sex differences in the brain activity within IBS patients (male IBS vs. female IBS). Whole brain statistical parametric maps were first thresholded at voxelwise p=0.005 uncorrected. To account for multiple comparisons and avoid Type I error, Monte Carlo simulation implemented in AlphaSim program (http://afni.nimh.nih.gov) was performed at 10,000 iterations. Statistical

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significance (p<0.05, corrected) was achieved with a minimum cluster size of 120 contiguous voxels.

Clinical characteristics analysis.

Specifying group as a four level factor (female HCs, male HCs, female IBS, male IBS), a one way ANOVA with post-hoc comparisons were performed to test for group differences in age, anxiety and depression, and abdominal pain ratings using SPSS version 19 software. Post hoc comparisons included female HCs vs. male HCs, female HCs vs. female IBS, male HCs vs. male IBS, and female IBS vs. male IBS. All p values were corrected for multiple comparisons using FDR correction at 5% (Benjamini and Hochberg, 2000; Benjamini et al., 2006). Independent sample t tests were performed to examine BSQ measures between male IBS and female IBS patients.

Results

Clinical characteristics

Detailed clinical and psychological characteristics of the four groups are summarized in **Table 1**. No significant differences between the four groups were observed in terms of age, abdominal pain threshold, pre- and post abdominal pain intensity and unpleasantness. However, significant group differences were observed for anxiety and depression symptom scores (F=5.96, p=.001 and F=5.5, p=.002, respectively). Post-hoc analysis revealed that IBS patients had significantly greater anxiety and depression scores than HCs (t=3.94, q<.001 and t=3.58, q<.001, respectively, corrected). In addition, amongst women, female IBS patients showed significantly greater anxiety and depression scores than female HCs (t=3.87, q<.001 and t=3.66, q<.001, respectively, corrected). However, no significant IBS-related difference was observed in

anxiety and depression scores in males (q=.215, t=1.65 for anxiety and q=.214, t=1.26 for depression, corrected). There were no significant differences in anxiety, depression and bowel symptom scores between male and female IBS subjects.

Brain responses associated with cued pain expectation and contextual threat

For the contrast of cued threat versus cued safe, the conjunction analysis showed significant brain responses in subregions of the frontal cortex including bilateral dorsal medial frontal cortex (BA8/32) and middle frontal gyrus (MFG) (BA46/45/10), inferior parietal lobe, ACC, mid cingulate cortex, posterior cingulate cortex (PCC), anterior INS, supplementary motor area, thalamus, basal ganglia, amygdala, supramarginal gyrus, occipital cortex as well as right precentral gyrus and precuneus (**Figure 2a and Supplemental Table 1**). The same brain regions were activated in response to the cross-hair condition compared to cued safe condition, with additional activation of postcentral gyrus (**Figure 2b and Supplemental Table 2**) supporting the hypothesis of this contrast representing an uncued contextual threat.

Disease-related differences in brain responses associated with contextual threat and cued pain expectation

During the cross-hair condition contrasted to the cued safe condition, IBS subjects as a group (compared to HCs) showed significantly greater brain responses in the right amygdala, ventral anterior INS, the right MFG (BA46 extending ventrally to BA10), left thalamus (including anterior, ventral anterior, ventral lateral, dorsomedial and medial nuclei), PCC and precuneus as well as right inferior occipital cortex, regardless of sex, (**Table 2 and Figure 3**). In the contrast of cued threat versus cued safe, IBS subjects had greater activation in the left inferior occipital cortex

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compared to HCs (**Table 2**). There was no significantly greater response in HCs compared to IBS subjects in the two contrasts.

Sex-related differences in brain responses associated with contextual threat and cued pain expectation

Female IBS patients showed greater brain signals in right amygdala, ventral anterior INS, PCC and precuneus, compared to female HCs in response to the uncued contextual threat (**Table 3 and Figure 4a**). In the contrast of cued threat versus cued safe, male IBS patients showed significantly greater brain responses in right supramarginal gyrus compared to female IBS patients (**Table 3 and Figure 4b**). In addition, female IBS patients had greater activation in the left angular gyrus relative to female HCs (**Table 3**).

Discussion

The current study examined brain responses to cued and uncued abdominal threat in HCs and IBS patients, and characterized disease and sex-related differences in these responses. As hypothesized, brain regions associated with salience, attention, cognitive evaluation and sensory processing were activated during the cued and uncued threat conditions compared to the cued safe condition. However, disease-related differences in the brain responses were mainly seen in the contrast of cross-hair versus cued safe, where IBS subjects showed significantly greater activations in affective (amygdala, ventral anterior INS), sensory (thalamus), attentional (MFG) and self-referential (precuneus) regions. In addition, these differences in brain responses to uncued contextual threat were primarily observed in females.

Brain responses associated with cued pain expectation

The conjunction analysis findings during cued expectation of an aversive stimulus to the left lower abdomen versus the cued safe condition were consistent with previous reports from studies using an analogous paradigm of pain expectation (Yaguez et al., 2005; Kano et al., 2013; Seifert et al., 2013; Loggia et al., 2014). Confirming previously reported similarities of brain activation patterns during both delivered and expected aversive stimuli (Koyama et al., 2005; Seifert et al., 2013), most of the regions activated during cued abdominal pain expectation in the current study have been found to be activated during experimental visceral pain paradigms (Tillisch et al., 2011). The reasons underlying these similarities are not known. However, as stimulus ratings differ significantly between the actual and the expected pain stimuli (Straube et al., 2009b), one may speculate that the similar activation of INS and ACC seen under both conditions is more reflective of the engagement of the salience network, rather than to activation of a "pain matrix" related to processing of aversive sensory information.

Brain responses associated with contextual threat

When the potential for an aversive stimulus application becomes ambiguous, and the stimulus is not paired with a specific cue, the brain tries to make predictions about the stimulus based on the context in which a previous stimulus has been experienced (Bouton, 1994; Whalen, 1998; Grillon, 2008). Such an ambiguous environmental state can be conceptualized as an uncertain threat about the pending event (Lake and Labar, 2011; Bach and Dolan, 2012). In the current study, we hypothesize that the period with a cross-hair created an ambiguous threat, as it occurred in the same environmental context where the subject had previously experienced the abdominal shock, including continued presence of the electrodes attached to the abdomen. Studies have suggested

that ambiguity regarding type or occurrence of the stimulus can lead to heightened behavioral and brain responses especially in patients with anxiety (Grillon et al., 2008; Lake and Labar, 2011; Shankman et al., 2014), and these enhanced responses are likely related to the brain's effort to predict the stimulus. Fixation on a cross-hair during fMRI scanning has been suggested to create an uncertain environment, leading to a greater emotional brain response relative to neutral stimuli (Heinz et al., 2007). When faced with such an ambiguous situation, the brain will respond with risk assessment, enhanced attention and prediction of likely outcomes (Ploghaus et al., 2003; Grupe and Nitschke, 2013). Brain regions implicated in this threat assessment include affective regions (INS, amygdala, striatum), attentional regions (ACC, frontal and parietal cortex) and the thalamus (Alvarez et al., 2008; Bach and Dolan, 2012; Grupe and Nitschke, 2013).

Disease-related differences in brain responses

Although similar brain regions were activated during the cued and uncued pain expectation, disease-related group differences in degree of activation were mainly observed during the cross-hair condition versus the cued safe condition. There are several possible reasons to explain these findings. In the current study, the intensity of the abdominal shock stimuli was adjusted to the individual pain threshold, which did not show significant differences between IBS patients and HCs. It is therefore possible that during the cued pain expectation, the brain of both IBS subjects and HCs compute a similar salience regarding the somatic pain experience, resulting in similar expectancy and perception of threat. Even though the stimulus was given to the patients over the lower abdomen, a region to which the majority of IBS patients have pain referral, the type of pain stimulus may carry a similar salience for both groups. Similar observations with electric stimulation of the gut have previously been reported (Azpiroz, 2002). During the cross-hair

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periods without strong cues, the IBS patients show a significant difference in response in affective and attentional brain regions likely due to greater affect in the presence of ambiguity and lack of predictive cues (Carlsson et al., 2006). This interpretation is consistent with findings in patients with anxiety disorder who showed significantly greater startle responses and activation of the amygdala and anterior INS during unpredictable threat condition, but not under a predictable threat condition (Grillon et al., 2008; Grupe and Nitschke, 2013; Simmons et al., 2013; Gorka et al., 2014). The amygdala and ventral anterior INS are key regions in interoception, emotion, pain and salience processing (Seeley et al., 2007; Bushnell et al., 2013) and the anterior INS has been shown to be involved in outcome prediction (Preuschoff et al., 2008). Consistent with such a role in the prediction of future events, both brain regions were also found to be activated during expectation of unpredictable negative events (Herry et al., 2007; Sarinopoulos et al., 2010; Shankman et al., 2014). In summary, considerable evidence supports the concept that responses of amygdala and anterior INS in IBS patients during unpredictable threat are a reflection of altered salience and emotional arousal network engagement in IBS patients (Borsook et al., 2013; Grupe and Nitschke, 2013).

In addition to INS and amygdala, we observed greater activation of the PCC and precuneus in patients compared to HCs during uncued contextual threat. Some studies showed that during pain expectation, precuneus and PCC were deactivated in HCs (Ter Minassian et al., 2013) and exhibited decreased activation associated with expected pain intensity (Koyama et al., 2005). People with social anxiety had increased precuneus activity during reward anticipation, suggesting that these subjects were unable to direct away from self-attention and this lead to an increased self-focused hypervigilance (Maresh et al., 2014). When viewed together, these reports are in line with the current findings that IBS patients showed activation of PCC and precuneus while HCs

showed deactivation of these regions in response to contextual threat. The salience network, especially the anterior INS has been suggested to play a role in switching between the default mode network and task-related networks (Menon and Uddin, 2010; Bonnelle et al., 2012). Along with these findings, we speculate that IBS patients have altered salience network function, affecting the regulation of PCC and precuneus activity (Hong et al., 2014a) during contextual threat.

IBS patients also showed heightened MFG activation during contextual threat. MFG is involved in decision making (Fleck et al., 2006) and self-generated attention processes (Burgess et al., 2007). Activation of the MFG has been linked to attentional processes related to pain(Dunckley et al., 2005; Dunckley et al., 2007) and to unpredictable pain expectation (Carlsson et al., 2006). Healthy subjects showed robust activation in MFG during expectation of heat pain (Koyama et al., 2005), electric shocks to the wrist (Drabant et al., 2011), and laser pain on the right forearm (Watson et al., 2009), and these responses were positively correlated with magnitude of anticipated pain intensity (Lobanov et al., 2014). When viewed together we speculate that the observed greater activation of the MFG in IBS patients during contextual threat reflects greater allocation of attentional resources to the task, and that this abnormality may be related to the observed alterations in salience network engagement (Simmons et al., 2011; Wiech and Tracey, 2013).

Greater activity in several thalamic subregions, including the medial nucleus, was observed in IBS patients. Activation of the medial nucleus of thalamus has previously been reported in HCs in response to pain anticipation which was associated with emotional arousal and vigilance (Porro et al., 2003). A meta-analysis suggested IBS patients had heightened medial thalamic responses to supraliminal rectal distension (Tillisch et al., 2011), and a recent study in IBS patients showed

activation of the thalamus during both rectal distension and expectation of such stimuli (Larsson et al., 2012). Although the role(s) of the thalamus in IBS is still not clear, recent evidence has identified microstructural alterations in the thalamus with the ACC and INS, and these alterations were associated with symptom severity (Ellingson et al., 2013).

Sex-related differences in brain responses

In response to contextual threat, female IBS patients showed greater amygdala, anterior INS, PCC and precuneus activity compared to female HCs, while no such differences were observed between male IBS patients and male HCs. These sex-specific disease differences have also been demonstrated in our two previous resting-state fMRI studies in which female IBS subjects showed altered dynamics of the amygdala (Hong et al., 2013) and altered functional connectivity between anterior INS and precuneus (Hong et al., 2014a) relative to female HCs, while these differences were not seen in males. Interestingly, female patients also had higher anxiety and depression symptom scores compared to female HCs, and these differences were not significant in males. Although we have controlled for symptoms of anxiety and depression in our analysis, it is possible that female IBS patients had other affective characteristics (e.g. pain catastrophizing) contributing to the differences in brain responses.

Conclusions

The findings of this study emphasize an IBS-related abnormality in brain responses in the context of ambiguous expectation of an abdominal somatic pain stimulus. The findings suggest a central role of stimulus appraisal and salience detection in IBS related brain and presumably symptom responses. While both groups had brain responses to the cued pain expectation which presumably carries similar salience for both groups, the patients showed relatively larger

responses in brain regions involved in salience detection, emotional arousal and self consciousness during contextual threat. We speculate that these brain findings are related to hypervigilance and heightened reactivity particularly to uncertainty of threat previously reported in IBS (Mayer et al., 2009a; Kilpatrick et al., 2010b; Wilder-Smith, 2011; Tkalcic et al., 2014) and anxiety disorders (Paulus and Stein, 2006; Grillon et al., 2008; Grupe and Nitschke, 2013). Future studies that directly manipulate contextual cues and predictability will be important to follow up on these findings and compare these results across other chronic pain conditions.

Table 1. Clinical characteristics

	HCM			HCF			IBSM			IBSF			
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	F/t	Sig.
Age	19	36.37	12.12	18	30.11	10.99	16	33.69	11.71	21	31	9.39	1.24	.302
Anxiety[1]	19	3.63	2.14	18	2.11	1.64	16	5.75	5.12	21	6.9	5.02	5.96	.001
Depression[1]	19	1.74	1.45	18	.67	1.14	16	2.56	2.25	21	3.14	2.67	5.5	.002
Overall bowel symptoms[2]	-	-	-	-	-	-	15	10.6	3.52	21	8.1	4.84	1.71	.097
Abdominal pain[3]	-	-	-	-	-	-	16	10.13	3.9	21	7.86	4.21	1.68	.103
Abdominal discomfort[4]	-	-	-	-	-	-	16	9.25	5.71	21	10.86	5.98	.83	.414
Duration of symptoms[5]	-	-	-	-	-	-	12	13.58	9.03	19	11.26	8.96	.7	.49
Threshold[6]	19	3.37	1.74	18	3.11	1.45	16	3.91	2.37	21	2.79	1.63	1.22	.309
Pre intensity[7]	19	12.16	4.03	18	12.33	2.93	16	12.31	2.6	21	12.33	3.44	.01	.998
Pre unpleasant[7]	19	9.16	3.79	18	9.33	2.2	16	9.81	2.14	21	10.43	2.64	.82	.485
Post intensity[8]	19	11.16	3.89	18	11.78	3.49	16	12.31	2.75	21	11.29	4.33	.35	.79
Post unpleasant[8]	19	9.05	3.54	18	9.72	2.87	16	10.13	2.5	21	10.29	3.26	.6	.614

F/t: F values and t values from ANOVA and t-tests for four and two group comparisons, respectively. 1: Hospital Anxiety and Depression; 2: BSQ overall symptoms in the past week (0-20; 0: no symptoms; 20: the most intense symptoms imaginable); 3: BSQ abdominal pain in the past week (0-20; 0: no pain; 20: the most intense pain imaginable); 4: BSQ discomfort in the past week (0-20; 0: no sensation; 20: the most intense sensation imaginable); 5: BSQ duration in years, derived from onset of symptom; 6: Averaged abdominal stimulation threshold (mA) which was described as aversive but tolerable; 7: Pre abdominal threat of pain intensity and unpleasantness (for intensity of pain: range 0-20, with 0 representing no sensation and 18 signifying extremely intense; for unpleasantness of pain: range 0-20, with 0 representing neutral unpleasantness and 17 signifying very intolerable unpleasantness); 8: Post abdominal threat of pain intensity and unpleasantness (0-20); Statistically significant p<0.05; SD: standard deviation

Table 2. Disease-related differences in brain responses

Contrast	H	Region	Voxels	Z	x	y	z
Cross-hair vs. cued safe							
IBS>HC		PCC/precuneus	500	3.35	4	-42	34
	R	lingual/Inf occipital	319	3.44	24	-84	-2
	R	amygdala/vaINS	235	3.57	30	2	-22
	R	MFG (BA46/10)	206	3.4	30	48	16
	L	thalamus	178	3.1	-18	-22	14
cued threat vs. cued safe							
IBS>HC	L	Inf occipital	294	3.37	-28	-94	-6

MNI coordinates (x,y,z) for peak voxel; Z: Z-values of peak voxels within the significant clusters (voxel threshold p<.005; cluster-corrected p<.05); H: hemisphere; L: left; R: right; vaINS: ventral anterior insula; PCC: posterior cingulate cortex; Lingual: lingual gyrus; Inf occipital: inferior occipital cortex; IBS: patients with irritable bowel syndrome; HC: healthy controls

Table 3. Sex-related differences in brain responses

Contrast	H	Region	Voxels	Z	x	y	z
Cross-hair vs. cued safe							
IBSF>HCF		PCC/precuneus	278	3.69	2	-40	32
	R	amygdala/vaINS	246	4.08	32	14	-12
cued threat vs. cued safe							
IBSM>IBSF	R	supramarginal gyrus	155	3.83	62	-34	40
IBSF>HCF	L	angular gyrus	147	3.45	-46	-60	36

MNI coordinates (x,y,z) for peak voxel; Z: Z-values of peak voxels within the significant clusters (voxel threshold $p<.005$; cluster-corrected $p<.05$); H: hemisphere; L: left; R: right; PCC: posterior cingulate cortex; vaINS: ventral anterior insula; HCF: healthy females; HCM: healthy males; IBSM: male IBS patients; IBSF: female IBS patients
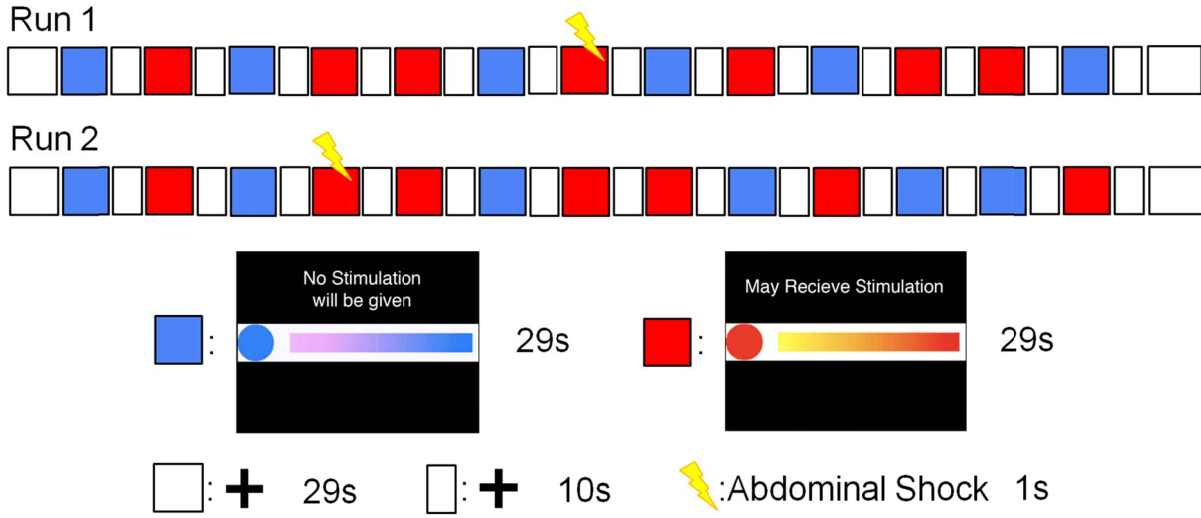
Figure 1. The experimental paradigm of anticipation to abdominal shock. There were two scanning runs, each run contained six cued safe conditions (lasted 29 second), seven cued threat conditions (lasted 29 seconds) and a shock within a threat condition. At the start and end of each run as well as between each 29-second condition, a crosshair with white background was presented for 29 seconds and 10 seconds, respectively. The blue block represents a cued safe condition with a description, a blue circle and a moving colored blue bar indicating how much time is left. The red block represents a cued threat condition with a description, a red circle and a moving colored red bar also indicating how much time is left in this trial. The white block represents a cross-hair condition.

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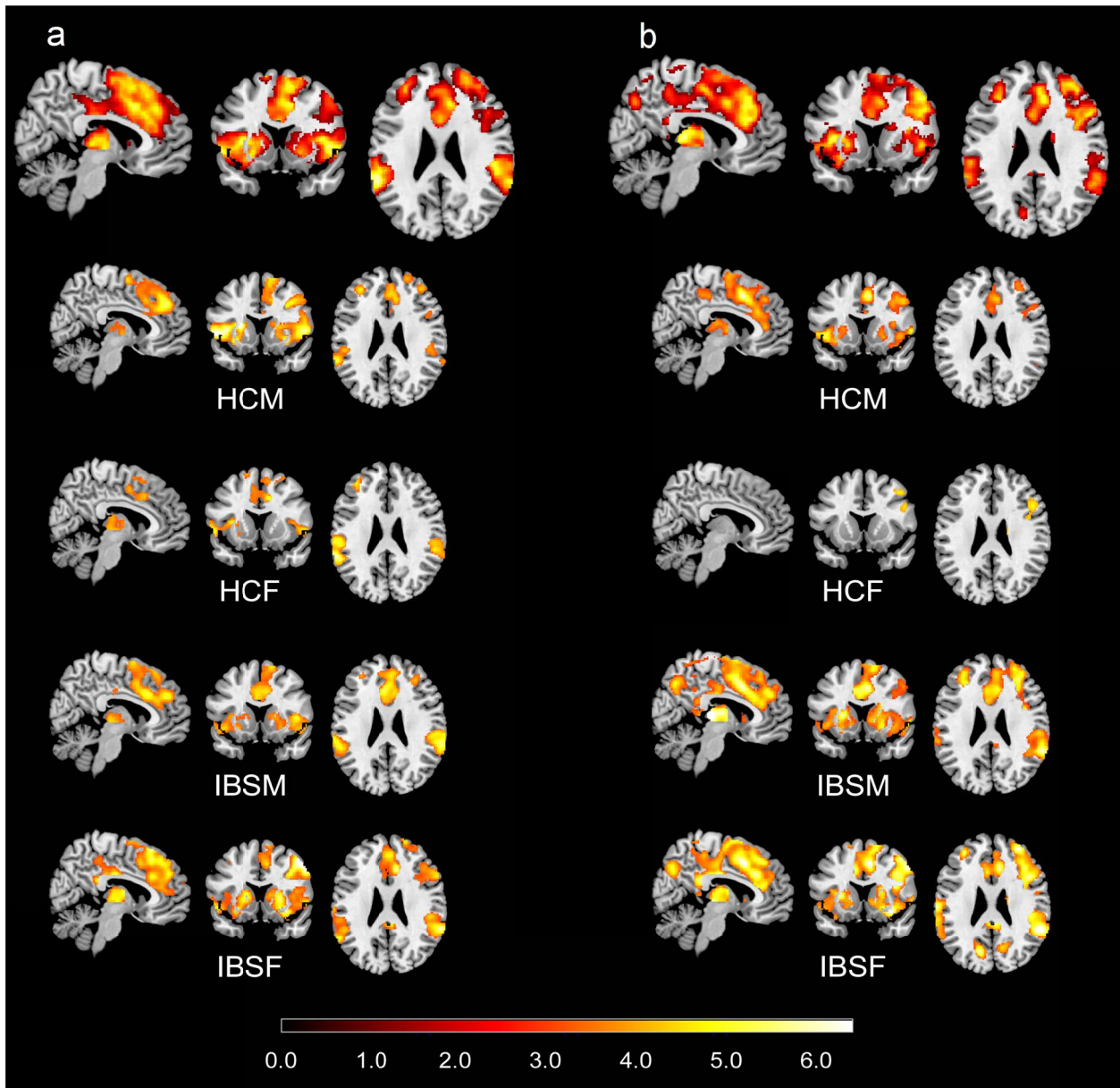
Figure 2. Brain activation maps of conjunction analysis. a) Brain responses associated with the contrast of cued threat versus cued safe. b) Brain responses associated with contextual threat. All significantly statistical results (p<.05, corrected) were overlapped on a MRIcron ch2better template. The color bar indicates Z-scores. HCM: healthy male subjects; HCF: healthy female subjects; IBSM: male IBS subjects; IBSF: female IBS subjects.
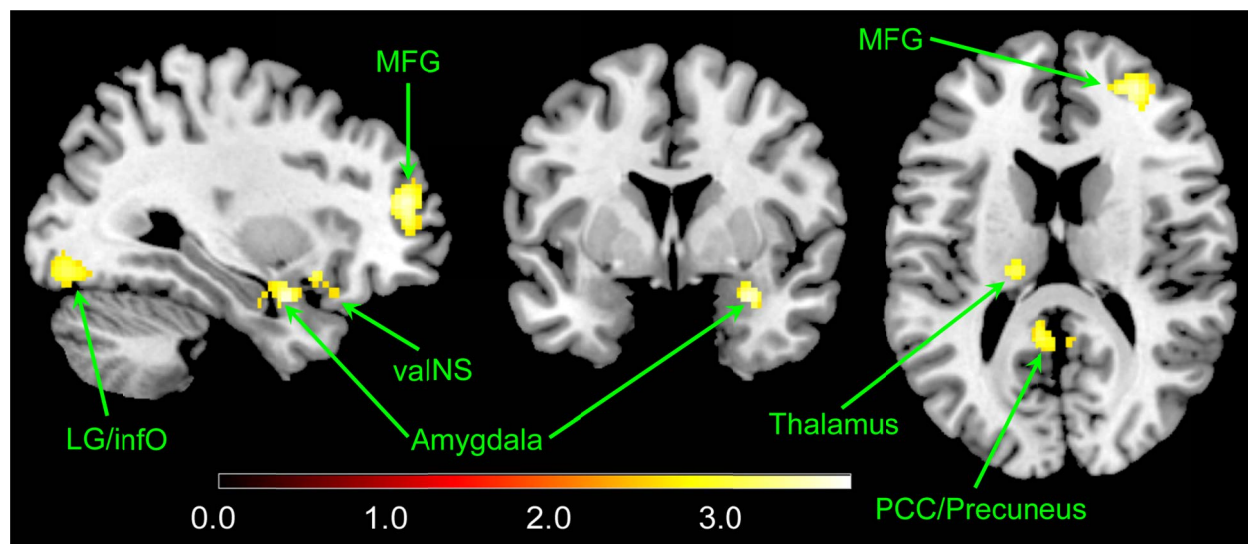
Figure 3. Disease-related differences in brain responses during contextual threat.
Significantly greater responses were observed in patients with IBS compared to HCs (p<.05, corrected). All significantly statistical results (p<.05, corrected) were overlapped on a MRIcron ch2better template. The color bar indicates Z-scores. MFG: middle frontal gyrus; vaINS: ventral anterior insula; PCC: posterior cingulate cortex; LG: lingual gyrus; infO: inferior occipital cortex
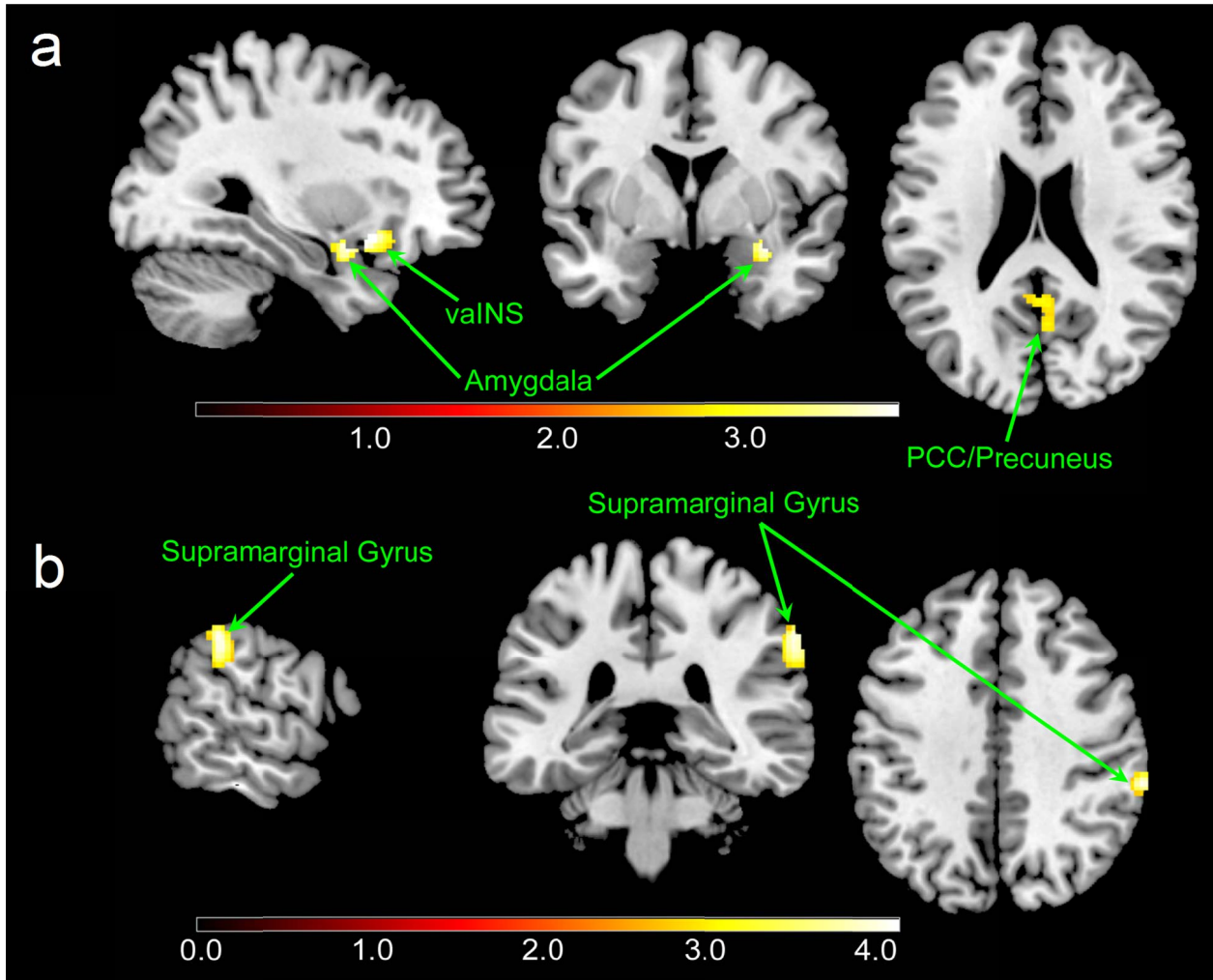
Figure 4. Different brain responses associated to sex. a) Significantly greater activations were shown in female IBS patients compared to female HCs in response to the cross-hair versus the cued safe. b) During cued abdominal pain expectation, male IBS patients showed significantly greater supramarginal gyrus activation compared to female IBS patients. All significantly statistical results (p<.05, corrected) were overlapped on a MRIcron ch2better template. The color bars indicate Z-scores. vaINS: ventral anterior insula; PCC: posterior cingulate cortex

Supplemental Table 1. Brain responses associated with cued threat

Region	Voxels	Z	x	y	z
bilateral ACC/MCC/PCC/SMA/dMFC/thalamus/ amygdala/aINS/ basal ganglia/ right precentral gyrus/MFG	21661	>8	-32	24	4
		7.77	46	18	0
		7.74	22	48	18
right supramarginal gyrus/IFL	3586	>8	58	-38	38
		>8	56	-46	36
		>8	64	-42	32
left supramarginal gyrus/ IFL	2058	>8	-56	-44	30
		7.61	-60	-38	26
		6.53	-58	-28	22
left MFG	973	6.69	-32	44	22
		4.46	-36	32	32
		3.42	-36	58	-4
right superior and middle occipital cortex	927	6.86	20	-98	6
		6.02	28	-96	2
		5.62	18	-92	-2
right precuneus	227	5.16	16	-64	42
left middle occipital cortex/lingual gyrus	452	4.58	-22	-100	4
		3.76	-32	-96	6
		3.7	-20	-94	-14

MNI coordinates (x,y,z) for peak voxel; Z: Z-values of peak voxels within the significant clusters (voxel threshold p<.005; cluster-corrected p<.05); ACC: anterior cingulate cortex; MCC: mid cingulate cortex; PCC: posterior cingulate cortex; SMA: supplementary motor area; dMFC: dorsal medial frontal cortex; MFG: middle frontal gyrus; aINS: anterior insula; IFL: inferior parietal lobe

Supplemental Table 2. Brain responses associated with contextual threat

Region	Voxels	Z	x	y	z
bilateral ACC/MCC/PCC/SMA/dMFC/	19002	7.44	42	12	42
right precentral gyrus/MFG/amygdala/aINS/		7	42	4	52
basal ganglia		6.84	-4	0	48
bilateral thalamus/	4691	7.34	-32	20	6
left amygdala/aINS/basal ganglia		7.2	14	-4	18
		7.04	6	-22	8
bilateral supramarginal gyrus/IFL/	4146	>8	58	-38	34
postcentral gyrus		7.15	54	-48	34
		7.11	42	-54	44
right precuneus	619	7.42	10	-68	42
		3.4	16	-48	40
left precuneus	607	4.9	-12	-66	34
left MFG	552	6.13	-32	38	22
		3.38	-32	52	14
left inferior and middle occipital cortex	303	7.05	-26	-98	-6
right inferior and middle occipital cortex	218	6.05	26	-98	-4
		4.56	38	-90	-6

MNI coordinates (x,y,z) for peak voxel; Z: Z-values of peak voxels within the significant clusters (voxel threshold p<.005; cluster-corrected p<.05); ACC: anterior cingulate cortex; MCC: mid cingulate cortex; PCC: posterior cingulate cortex; SMA: supplementary motor area; dMFC: dorsal medial frontal cortex; MFG: middle frontal gyrus; aINS: anterior insula; IFL: inferior parietal lobe

Chapter 5

Regional Neuroplastic Brain Changes in Patients with Chronic Inflammatory and non-Inflammatory Visceral Pain

This chapter is adapted from:

Hong JY, Labus JS, Jiang Z, Ashe-Mcnalley C, Dinov I, Gupta A, Shi Y, Stains J, Heendeniya N, Smith SR, Tillisch K, Mayer EA (2014a) Regional neuroplastic brain changes in patients with chronic inflammatory and non-inflammatory visceral pain. PLoS One 9:e84564.

Abstract

Regional cortical thickness alterations have been reported in many chronic inflammatory and painful conditions, including inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS), even though the mechanisms underlying such neuroplastic changes remain poorly understood. In order to better understand the mechanisms contributing to grey matter changes, the current study sought to identify the differences in regional alterations in cortical thickness between healthy controls and two chronic visceral pain syndromes, with and without chronic gut inflammation. 41 healthy controls, 11 IBS subjects with diarrhea, and 16 subjects with ulcerative colitis (UC) underwent high-resolution T1-weighted magnetization-prepared rapid acquisition gradient echo scans. Structural image preprocessing and cortical thickness analysis within the region of interests were performed by using the Laboratory of Neuroimaging Pipeline. Group differences were determined using the general linear model and linear contrast analysis. The two disease groups differed significantly in several cortical regions. UC subjects showed greater cortical thickness in anterior cingulate cortical subregions, and in primary somatosensory cortex compared with both IBS and healthy subjects. Compared with healthy subjects, UC subjects showed lower cortical thickness in orbitofrontal cortex and in mid and posterior insula, while IBS subjects showed lower cortical thickness in the anterior insula. Large effects of correlations between symptom duration and thickness in the orbitofrontal cortex and postcentral gyrus were only observed in UC subjects. The findings suggest that the mechanisms underlying the observed gray matter changes in UC subjects represent a consequence of peripheral inflammation, while in IBS subjects central mechanisms may play a primary role.

Introduction

Inflammatory bowel diseases (IBD) such as ulcerative colitis (UC) are characterized by chronically recurring symptoms of abdominal pain associated with flares of mucosal inflammation. In contrast, in irritable bowel syndrome (IBS), chronically recurring symptoms of abdominal pain and discomfort occur in the absence of mucosal inflammation or other identifiable nociceptive triggers (therefore referred to as "functional" pain syndromes), and symptom flares are often triggered by psychosocial stressors. It is generally assumed that abdominal pain in UC results initially from inflammation induced peripheral and central sensitization of visceral afferent pathways (Bielefeldt et al., 2009), while symptoms in IBS may reflect primarily an alteration in central pain modulation, including alterations in endogenous descending pain modulation mechanisms (Berman et al., 2008). On the other hand, several pieces of evidence support the concept that IBD patients effectively engage endogenous pain inhibition systems (Chang et al., 2000), including greater engagement of a cortico limbic-pontine pain modulation network compared to IBS subjects (Mayer et al., 2005). These differences in the engagement of endogenous pain modulation systems may explain the clinical observation that in uncomplicated UC, abdominal pain is not a prominent symptom even during flares.

Several studies have applied multimodal brain imaging to investigate the presence of grey matter changes in patients with various chronic pain conditions without known nociceptive drive (Schmidt-Wilcke et al., 2007; Davis et al., 2008; Hsu et al., 2009; Seminowicz et al., 2010; Labus et al., 2013a), with presumed nociceptive drive (Apkarian et al., 2004; Schmidt-Wilcke et al., 2006; Seminowicz et al., 2011), and with known inflammatory drive (Gwilym et al., 2010; Farmer et al., 2011; Frokjaer et al., 2012; Jones et al., 2012; Wartolowska et al., 2012; Agostini et al., 2013). Reported abnormalities in these studies suggest some similarities in findings (e.g. gray matter

reduction in insula [INS] and anterior cingulate cortex [ACC] subregions), and increases in CT in

somatosensory regions regardless of pain syndrome, and no clear differences have emerged

between the different pain categories, in particular between chronic visceral pain of "functional"

and of inflammatory origin. The use of different analysis techniques by different groups makes

interpretive comparisons between studies and between different patient populations more difficult.

In the current study, we used the Laboratory of Neuroimaging (LONI) Pipeline (Dinov et al.,

2010; Dinov et al., 2011) for image preprocessing, volumetric analysis and cortical thickness (CT)

analysis. We focused on differences of local morphologic brain alterations between UC and

healthy control subjects (HCs), and compared them to findings in IBS subjects. Specifically, we

aimed to test the following hypotheses: 1) Both IBS and UC patients differ from HCs in terms of

regional CT changes. 2) UC patients show CT changes in brain regions involved in somatosensory

and viscerosensory processing and modulation. 3) IBS patients show CT changes in brain regions

involved in the integration of affective, cognitive and interoceptive signals. 4) In UC patients,

there are correlations between CT changes and duration of gut inflammation, reflecting the chronic

influence of peripheral inflammation on the brain.

Materials and Methods

Subjects.

A total 68 right-handed male and female subjects were recruited through the UCLA Digestive

Disease Clinic and advertisements including HCs (n=41; mean age= 28.2 years old, range=19-48

years; 16 males), IBS with diarrhea (n=11; mean age= 31.6 years old, range=21-47 years; 2 males),

and UC subjects (n=16; mean age=28. 6 years old, range 18-48 years; 10 males). 15 HCs and 5 IBS

subjects of the respective samples have been included in a previously published gray matter

volume analysis (Labus et al., 2013a). Exclusion criteria for all subjects comprised pregnancy, postpartum or nursing females, current substance abuse or dependence, abdominal surgery, any past or present neurological illness or trauma, claustrophobia or learning disability, and current psychiatric diagnosis. A diagnosis of IBS was made by a gastroenterologist or nurse practitioner with expertise in functional GI disorders based on the ROME II or ROME III symptom criteria during a clinical assessment (Drossman, 2000; Drossman, 2006). The diagnostic criteria include recurrent abdominal pain or discomfort associated with two or more of the following: 1) pain/discomfort is relieved/improved by defecation 2) the onset of pain/discomfort is related to a change in frequency of stool 3) the onset of pain/discomfort is related to a change in the form (appearance) of stool. In order to match the predominant bowel habit of UC patients, only IBS patients with diarrhea were used in this study. In addition, IBS subjects with current regular use of analgesic drugs (including narcotics, opioids and alpha2-delta ligands) were excluded. UC patients were diagnosed by a gastroenterologist, which was supported by biopsies obtained by endoscopy. During screening, all subjects completed the modified Mayo UCDAI (disease activity index for Stool frequency, rectal bleeding and physician rating of disease activity) to assess degree of current disease. A score of 0-1 is considered Remission, score of 2-4 mild disease, and >4 is considered active disease (Lewis et al., 2008a). Exclusion criteria specific to the UC population were corticosteroid use within last 6 months, or use of any psychotropic medications. All procedures were approved by the UCLA Medical Institutional Review Board, and all subjects provided written informed consent. Questionnaires were completed before scanning to determine symptom type, severity, duration of symptoms, and abdominal sensation (UCLA Bowel Symptom Questionnaire, BSQ) (Chang et al., 2001), comorbid affective and mood disorders (Hospital Anxiety Depression Scale, HAD) (Mykletun et al., 2001), and IBS-related fears and anxiety

(Visceral Sensitivity Index, VSI) (Labus et al., 2004; Labus et al., 2007). Details are shown in **Table 1**.

Structural MRI Acquisition.

All high-resolution T1-weighted brain images were collected at the UCLA Brain Mapping Center using a Siemens 3 Tesla Trio with magnetization prepared rapid gradient echo scanning parameters (TR=2200 ms, TE=3.26 ms, flip angle=9°, duration=9:03 mins, FOV=256).

Data analysis.

We employed the LONI pipeline for image preprocessing, cortical surface modeling and gray matter thickness analysis (Dinov et al., 2010; Dinov et al., 2011). Following a de-identification step, the structural neuroimaging data were converted from Digital Imaging and Communications in Medicine to ANALYZE 7.5 format, skull-stripped using the LONI Skull-Stripping Meta Algorithm pipeline workflow (Leung, 2011) and cortical surface models were generated using FreeSurfer 4.0 (Fischl and Dale, 2000) (http://surfer.nmr.mgh.harvard.edu/fswiki and http://ucla.in/xSQPqT). Cortical grey matter thickness was computed at each point of the surface using the distance from the pial surface to the nearest point on the white matter surface. For numerical implementation, we first built the signed distance function (Osher and Sethian, 1988; Mulder et al., 1992) of the white matter surface in 3D space and then computed the CT as the value on the signed distance function at those locations. The cortical surfaces and the corresponding CT maps were registered to the International Consortium for Brain Mapping (ICBM) brain surface (Mazziotta et al., 2001) and then vertex-wise correspondences were established between all cortical surface models using a Conformal Metric Optimization method (Shi et al., 2011). An

experienced human brain researcher rated each brain surface reconstruction by visually inspecting

the surfaces using LONI ShapeViewer (http://www.loni.ucla.edu/Software/ShapeViewer). The

quality of surface reconstruction and accuracy of vertex labeling were assessed on the scale of 0 to

1 (0=completely unacceptable; 1=perfectly reconstructed and labeled). A threshold of 0.7 was

selected as the criterion to reconstruct a subject's surface data to be included in the final analysis.

Region of interest analysis

We examined the CT change in several manually delineated regions of interest (ROIs) in each

hemisphere based on previous studies (Schmidt-Wilcke et al., 2006; May, 2008; Craig, 2009;

Mayer et al., 2009a; Tillisch et al., 2011; Agostini et al., 2012; Ung et al., 2012; Agostini et al.,

2013). These ROIs included insular subregions (anterior INS [aINS], mid INS [mINS], and

posterior INS [pINS]), cingulate subregions (subgenual ACC [sgACC], pregenual ACC [pgACC],

anterior midcingulate cortex [aMCC] and posterior MCC [pMCC]), orbitofrontal gyrus (OFG)

(lateral OFG and medial OFG), pre- and postcentral gyri (Mazziotta et al., 2001). No subregions of

pre- and postcentral gyri were drawn. The subregions of INS and cingulum were manually

delineated on the 3D ICBM brain atlas (Mazziotta et al., 2001) by two well-trained researchers

with good command of neuroanatomical knowledge (**Supplemental Figure 1**). The 3D ROI

masks were transformed back onto the ICBM surface space by resampling the atlas map based on

masks' Euclidean coordinates (Shi et al., 2011).

Statistical analysis

To determine potential protocol differences in ROIs, a general linear model (GLM) was

applied to examine differences in total gray matter volumes within HCs as a function of protocol.

Group differences in CT within ROIs as a function of group, sex and group*sex were determined using the GLM and weighted linear contrast analysis controlling for total gray matter volume, and age in SPSS v19. The contrasts testing the interaction between group and sex were weighted to eliminate any bias caused by unbalanced representation of sexes. Although this was a hypothesis driven study, we implemented a conservative procedure to adjust for multiple comparisons in order to control for type I error. Specifically, the false-discovery rate (FDR) for the 66 contrasts (11 bilateral ROIs [n=22] for each of three independent contrasts) was held at 5% (Benjamini and Hochberg, 2000; Benjamini et al., 2006; Pike, 2011). GLM and linear contrast analysis controlling for sex and age were also applied to examine differences in BSQ between IBS and UC subjects as well as group differences in non-BSQ clinical and behavioral characteristics including VSI and HAD. Significance was determined after controlling FDR at 5% (Benjamini and Hochberg, 2000; Benjamini et al., 2006).

Correlation Analysis of Clinic Variables

Within group exploratory partial correlation analyses controlling for total gray matter volume, sex, and age were performed to characterize the association between subjects' clinic characteristics (BSQ, VSI, HAD and Mayo UCDAI) and regions showing significant group differences in CT. However, for the partial correlation between significant ROIs and symptom duration, we did not include age as a covariate, as these explanatory variables were significantly correlated, for IBS group (duration, r=.39, p=.036), and for UC group (duration, r=.67, p=.001) (Moayedi et al., 2011). Significance was determined after controlling FDR at 5% (Benjamini and Hochberg, 2000; Benjamini et al., 2006).

Subject Characteristics

Subjects' clinical data are summarized in **Table 1**. Compared to the HCs, the two disease groups showed significantly higher measures of symptom related anxiety (VSI scores: IBS>HCs, F=126.28, p<.001; UC>HCs, F=71.97, p<.001; IBS>UC, F=10.61, p=.002), anxiety symptoms (HAD scores: IBS>HCs, F=17.03, p<.001; UC>HCs, F=28.02, p<.001), and depression symptoms (HAD scores: IBS>HCs, F=4.88, p=.031; UC>HCs, F=15.16, p<.001). IBS subjects had significantly higher overall bowel symptoms scores, more abdominal discomfort and pain than UC subjects. Using the Mayo UCDAI (Lewis et al., 2008a), 5 of the UC subjects were in remission, 7 had mild disease and 4 had active disease (mild flare). There were no differences in terms of age of subjects and duration of GI symptoms.

Regional Cortical Thickness Changes Using ROI Analysis

Regional CT differences were observed between UC, IBS and HCs. Mean CT values and statistical significance after FDR correction are shown in **Table 2 and Table S1**. As depicted in **Figure 1a**, compared to both IBS and HCs, UC subjects showed greater CT in left cingulate cortical subregions (aMCC, pMCC, pgACC and sgACC), and in left post central gyrus (statistically significant differences following FDR correction for multiple comparisons are shown in **Figure 1a** and marked with asterisks). As shown in **Figure 1b**, compared to both IBS and HCs, UC subjects had reduced CT in prefrontal regions (left medial and lateral OFG) and in left INS subregions (most significant in pINS). Following FDR correction for multiple comparisons, the results between HCs and UC remained significant even though some differences (OFG and INS) between IBS and UC groups were no longer significant (significant differences are shown in

Figure 1b and marked with asterisks). All results remained significant after controlling for depression. However, after controlling for anxiety, the observed differences between HCs and UC subjects in left pMCC, aINS and mINS were no longer significant after FDR correction (**Table 2**). As shown in **Figure 1c**, compared to HCs, IBS subjects showed significantly reduced CT in right aINS after FDR correction, a difference that was not affected by controlling for anxiety and depression.

Correlation of Cortical Thickness with Behavioral and Clinical Variables

In the UC group, symptom duration was *negatively* correlated with CT in left lateral OFG (r= -.88, p=.0002, q=.0006, **Figure 2a**) and left medial OFG (r= -.78, p=.003, q=.003, **Figure 2b**) and was *positively* correlated with CT in left postcentral gyrus (r=.77, p=.003, q=.003, **Figure 2c**). Adding anxiety and depression scores as covariates did not alter the results. No significant correlations with other clinical parameters (including the Mayo UCDAI) were observed.

Discussion

The primary goal of the current study was to assess regional CT differences between subjects with UC, and two comparison groups: a healthy control group and a disease control group without gut inflammation (IBS).The main findings of the study were: 1) Compared to both IBS and HCs, UC subjects showed *greater* CT in left cingulate cortical subregions, and in left primary somatosensory cortex (SI). 2) Compared with HCs, UC subjects showed *lower* CT in left OFG and in primary viscerosensory cortex (pINS). 3) Compared to HCs, IBS subjects showed *lower* CT in the interoceptive association cortex (aINS) in the right hemisphere. 4) There were large significant correlations of CT reductions in left OFG and CT increases in left SI with symptom duration in UC

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subjects, suggesting a role of chronic inflammation driven afferent input in these changes. The emerging pattern highlights significant differences in CT between patients with chronic gut inflammation, functional GI disorders and HCs, as well as some similarities.

Greater Regional CT in UC Patients

Somatosensory Cortex

In the current study, greater CT in primary somatosensory cortex (SI) was seen in the UC group. SI is part of the central pain processing network and its thickness is positively correlated with individual experimentally induced acute pain sensitivity in healthy subjects (Apkarian et al., 2005; Erpelding et al., 2012). Chronic pain in human patient populations has been shown to be associated with cortical reorganization and changes in SI activity (May, 2008; Gustin et al., 2012; Moseley and Flor, 2012). For example, SI cortical thickening has been reported in patients with migraine (DaSilva et al., 2007) and temporomandibular pain (Moayedi et al., 2011). It has been suggested that the critical factor for S1 to undergo structural reorganization may be the presence of constant sensory input to this brain region (May, 2008; Moayedi et al., 2011; Gustin et al., 2012). Additionally, a voxel based morphometry study showed increased left pre- and postcentral gyri in chronic back pain patients (Ung et al., 2012). In the current study, CT of left postcentral gyrus in UC groups showed a large positive correlation with symptom duration, consistent with a possible etiologic role of chronically enhanced viscerosensory input to the brain due to sensitization of visceral afferent pathways by chronic mucosal inflammation. However, the degree of somatosensory cortex changes did not correspond to the subjective pain reports, as UC subjects had greater CT in somatosensory cortex, but reported lower abdominal pain and discomfort compared to patients with IBS. Even though the reason(s) for these apparent discrepancies

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between CT differences in SI and subjective pain reports are not known, one may speculate that the subjective experience of chronic clinical visceral pain (as opposed to acute experimentally induced pain) is more related to activity and related structural changes in interoceptive association cortex (e.g. the aINS), rather than to primary sensory cortex (Mayer et al., 2005; Blankstein et al., 2010).

Midcingulate Cortex

In the current study, compared to HCs and IBS subjects, the UC subjects had greater CT in subregions of the cingulate cortex, e.g. aMCC and pMCC. MCC is involved in emotion processing, skeletomotor regulation, chronic somatic and visceral pain, and along with the aINS (as part of the "salience network") integrating information to form conceptual pain (Vogt et al., 2003; Vogt, 2005; Taylor et al., 2009; Wiech et al., 2010; Meier et al., 2012). Several studies have reported abnormal MCC activation by acute noxious visceral stimulation in IBS subjects (Bernstein et al., 2002; Verne et al., 2003; Kwan et al., 2005; Labus et al., 2009). Supporting a possible effect of repeated nociceptive stimuli on MCC structure, repeated application of thermal pain stimuli to healthy subjects over a period of 8 days resulted in gray matter increases in both MCC and SI (Teutsch et al., 2008). Together with the observed greater CT in SI, the findings in UC patients are most consistent with the presence of a constant sensory input from the gut, due to sensitization of visceral afferent pathways by chronic mucosal inflammation. This interpretation is also consistent with the fact that in the current study, the IBS group (e.g. without chronically recurring mucosal inflammation) did not show significant CT change compared to HCs. Further support for differential brain mechanisms underlying chronic visceral pain comes from a recent PET ligand study which showed differences in neurokinin-1 receptor (NK-1R) binding potential (e.g. receptor availability) between patients with IBD (including Crohn's disease and UC) and IBS subjects

(Jarcho et al., 2013). Compared to HCs, IBD patients had low NK-1R availability in ACC and MCC, while IBS showed this deficit to a lesser extent. Animal studies have shown that the substance P/NK-1R signaling system is involved in cytogenesis, has neurotrophic and neuroprotective functions and inhibits apoptosis (Lallemend et al., 2003; Tulloch et al., 2011; Wang and Angulo, 2011). This implies a differential involvement of such neuroplastic mechanisms in the two visceral pain syndromes. Our findings differ from those reported in two other chronic inflammatory conditions, e.g. Crohn's disease (Agostini et al., 2013) and osteoarthritis (Rodriguez-Raecke et al., 2009). In both of these studies lower gray matter in the MCC was observed compared to HCs. Differences in patient populations and analysis methodology make it difficult to directly compare these studies with the current report (Rodriguez-Raecke et al., 2009; Agostini et al., 2013).

Reduced Regional CT in IBS and UC Patients

INS Subregions

When compared to HCs, both disease groups showed lower CT in the INS, albeit in different subregions. Several studies in patients with chronic pain including IBS (Schmidt-Wilcke et al., 2005; Kuchinad et al., 2007; Davis et al., 2008; Valfre et al., 2008; Rodriguez-Raecke et al., 2009; Seminowicz et al., 2011; Frokjaer et al., 2012; Jiang et al., 2013; Labus et al., 2013a) compared to HCs, have found lower gray matter volumes and CT in the INS, even though subregions were often not specified. In the current study, UC compared to HCs had significantly reduced CT in the left pINS (observed differences in mINS were no longer seen after controlling for anxiety). It is likely that chronically enhanced afferent input from the gut due to recurrent mucosal inflammation is primarily associated with CT changes in the pINS, which represents the primary interoceptive

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cortex (Craig, 2009; Farb et al., 2012). Even though there was no significant correlation between symptom duration or other behavioral measures with CT changes in the pINS, a chronic low back pain study showed that recovery of CT in pINS (and secondary somatosensory cortex) was correlated with reduction of pain intensity after treatment (Seminowicz et al., 2011), implicating chronic nociceptive and inflammation related signaling as a factor in these CT changes.

In contrast to the UC group, IBS subjects had lower CT in a different subregion of the INS, e.g. the right aINS compared to HCs. The aINS functions as interoceptive association cortex integrates interoceptive input with emotional, salient and cognitive inputs, and provides output to autonomic and pain modulation systems (Paulus and Stein, 2006; Lewis et al., 2008b; Craig, 2009; Wiech et al., 2010; Farb et al., 2012). The aINS also plays a central role in prediction, error processing, and self-awareness of sensations (Paulus and Stein, 2006; Craig, 2009). Even though both patient groups had greater affective scores compared to HCs, IBS subjects reported more abdominal pain and discomfort compared to the UC group. However, there was no significant correlation of the observed changes with affective measures, symptom scores or duration of symptoms in IBS subjects.

Orbitofrontal Gyrus

UC subjects compared to HCs showed lower CT in the bilateral OFG, a brain region which plays an important role in interoception, emotion evaluation and regulation, and in cognitive reappraisal (Ray et al., 2005; Rolls et al., 2008; Moayedi et al., 2011). Decreased gray matter in OFG has also been found in other chronic pain disorders with inflammatory/nociceptive drive including hip osteoarthritis (Rodriguez-Raecke et al., 2009), low back pain (Ung et al., 2012) and migraine (Kim et al., 2008). A negative correlation between left OFG thickness and symptom

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duration was observed in UC subjects, suggesting a role of chronic nociceptive input in the observed CT reductions. In contrast, no such correlation between IBS symptom duration and OFG structure were observed.

Limitations

Limitations of the study include the small sample size of the IBS and UC population, the group differences in level of anxiety and depression symptoms, and the heterogeneity of the groups in terms of sex. However, controlling for anxiety and depression, most of the observed results remained significant. In addition, our GLM and linear contrast were weighted to eliminate any bias caused by unbalanced representation of sexes. Furthermore, the fact that large correlations of some structural changes with disease duration were observed in the UC subjects, and the fact that some of the findings were similar to reports in subjects with other chronic inflammatory conditions (Rodriguez-Raecke et al., 2009; Frokjaer et al., 2012), makes it unlikely that the findings are confounded by these limitations. Future, longitudinal studies in larger patient populations, including the correlation of plasma and mucosal inflammatory disease markers with structural brain changes both during disease flares and remissions are needed in order to better understand the role of colonic inflammation in remodeling of the brain. In such larger studies, presence of comorbidities in the IBS group, as well as differences in the impact of the IBS and UC on daily life activities and social interactions should be taken into account.

Conclusions

To our knowledge, this study represents the first comparison of brain structure between UC patients and both HCs and IBS subjects. The findings demonstrate significant differences in CT

between UC and HC subjects, and differences between the two disease groups. Based on the

correlation of structural changes with symptom duration in IBD, one may speculate that the

observed gray matter reorganization of IBD subjects represents a consequence of chronic

viscerosensory input to the brain due to sensitization of visceral afferent pathways by recurrent gut

inflammation. The mechanisms by which such increased viscerosensory to the brain input can

produce both increases and decreases of grey matter in different brain regions remains to be

determined.

Tables

Table 1. Clinical and behavioral characteristics

	HCs			IBS subjects			UC subjects			F	Sig.
	N	Mean	SD	N	Mean	SD	N	Mean	SD		
Sex (Male/Female)	16/25			2/9			10/6				
Age	41	28.17	8.43	11	31.55	9.49	16	28.56	8.95	.66	.52
Anxiety symptoms[1]	41	2.8	2.33	11	6.91	3.67	16	7.31	3.55	18.29	<.01
Depression symptoms[1]	41	.85	1.22	11	2.64	2.73	16	3.44	3.54	7.77	<.01
Visceral Sensitivity Index[2]	40	2.83	5.17	11	37.45	12.94	15	26.13	14.09	61.68	<.01
Overall Bowel Symptoms[3]	-	-	-	11	11.91	2.74	13	4.69	2.46	54.12	<.01
Abdominal Pain[4]	-	-	-	11	10.55	4.3	14	4.14	3.61	18.01	<.01
Abdominal Discomfort[5]	-	-	-	11	11.91	4.21	16	4.06	3.86	49.63	<.01
Duration of symptoms[6]	-	-	-	10	8.5	6.13	16	9.81	9.35	1.67	.69

F= main effect of group from ANOVA and t-tests for four and two group comparisons, respectively.

1. HAD: Hospital Anxiety and Depression (Mykletun et al., 2001);

2. VSI: Visceral Sensitivity Index (Labus et al., 2004; Labus et al., 2007);

BSQ: Bowel Symptom Questionnaire (Chang et al., 2001)

3. BSQ Overall Symptoms in the Past week (0-20)

4. BSQ Abdominal Pain in the Past week (0-20)

5. BSQ Discomfort in the Past week (0-20)

6. BSQ Duration in years, derived from onset of symptom

Statistically significant p<0.05

Table 2. Significant cortical thickness differences in the ROIs between HCs, IBS and UC subjects with and without controlling for anxiety and depression scores

ROI		Difference	F	P value	q value	F(A)	q(A)	F(D)	q(D)
lOFG	L	HC>UC	23.736	<.00001	.00023	15.898	.00411	23.592	.00020
lOFG	R	HC>UC	15.778	.00019	.00160	9.971	.01654	14.000	.00275
		HC>IBS	17.591	.00009	.00086	12.647	.00925	17.184	.00102
mOFG	L	HC>UC	23.124	.00001	.00023	13.904	.00715	26.905	.00009
mOFG	R	HC>IBS	9.052	.00383	.01686	8.744	.02156	9.802	.01304
Post	L	UC>HC	9.895	.00258	.01216	9.397	.01966	17.446	.00102
		UC>IBS	11.701	.00113	.00678	11.859	.01001	15.595	.00155
aINS	L	HC>UC	6.701	.01207	.04193	2.234	.26245	7.698	.02568
aINS	R	HC>IBS	18.098	.00007	.00082	12.384	.00925	16.893	.00102
mINS	L	HC>UC	10.304	.00213	.01173	3.859	.14306	12.453	.00489
pINS	L	HC>UC	30.701	<.00001	.00005	19.658	.00150	29.416	.00008
aMCC	L	UC>HC	18.231	.00007	.00008	11.277	.01137	18.651	.00080
		UC>IBS	19.862	.00004	.00006	19.387	.00150	21.006	.00040
pMCC	L	UC>HC	8.870	.00042	.01724	2.278	.26245	11.277	.00758
		UC>IBS	7.831	.00690	.02677	7.666	.03185	9.082	.01568
pgACC	L	UC>HC	11.713	.00112	.00678	8.839	.02156	8.901	.01607
		UC>IBS	10.127	.00232	.01175	10.014	.01654	9.690	.01304
sgACC	L	UC>HC	11.754	.00110	.00678	8.692	.02156	9.611	.01304
		UC>IBS	7.704	.00734	.02690	7.609	.03185	7.559	.02611

R: right; L: left; lOFG: lateral orbitofrontal gyrus; mOFG: medial orbitofrontal gyrus; Post: postcentral gyrus; aINS: aINSula; mINS: mid insula; pINS: posterior insula; aMCC: anterior mid cingulate cortex; pMCC: posterior mid cingulate cortex; pgACC: pregenual anterior cingulate cortex; sgACC: subgenual anterior cingulate cortex; q value: p value after FDR corrected at 5%, q value <.05 was considered significant; F(A): F score after controlling for anxiety; q(A): corrected p value after controlling for anxiety; F(D): F score after controlling for depression; q(D): corrected p value after controlling for depression.

Figures
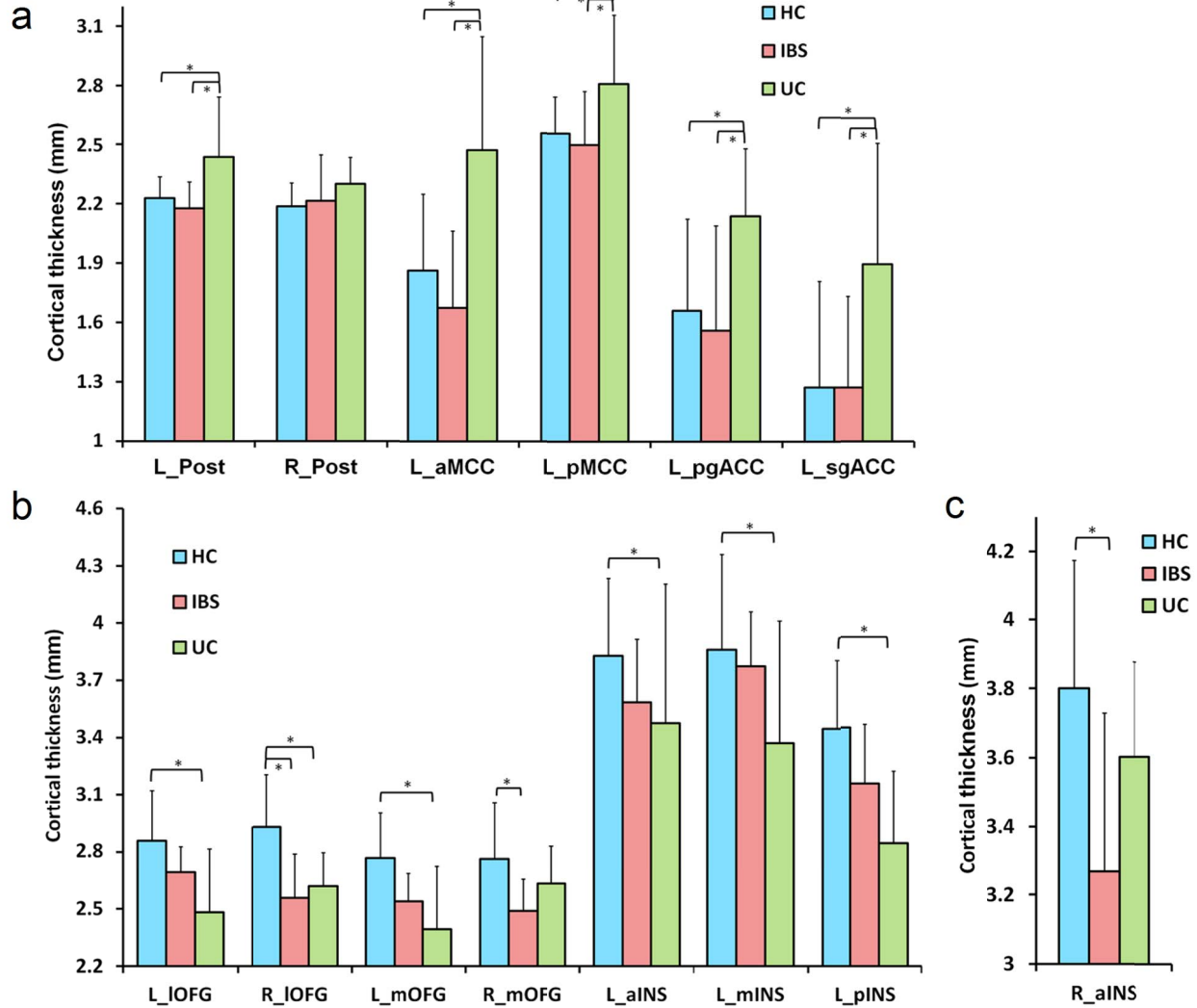
Figure 1. Mean cortical thickness of the ROIs showing significant group differences. a) UC subjects showed the greatest CT in the regions of somatosensory and cingulate cortex. b) In the subregions of OFG and INS, UCs had lower CT compared with HCs. c) IBS subjects had lower CT in right aINS compared to HCs. Error bars reflect standard deviation. Asterisk indicates significant differences between groups (q<0.05) after controlling for sex, age, total gray matter volume and FDR correction.
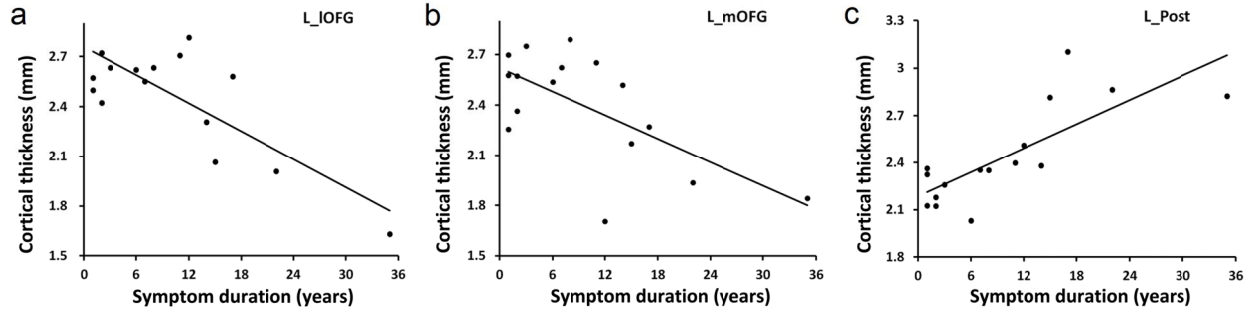
Figure 2. Correlation between cortical thickness and UC symptom duration. a) Cortical thickness in left lateral orbitofrontal gyrus (L_lOFG) and b) left medial orbitofrontal gyrus (L_mOFG) were negatively correlated with symptom duration in UC group. c) Cortical thickness in left postcentral gyrus (L_Post) showed large positive correlation with UC symptom duration.
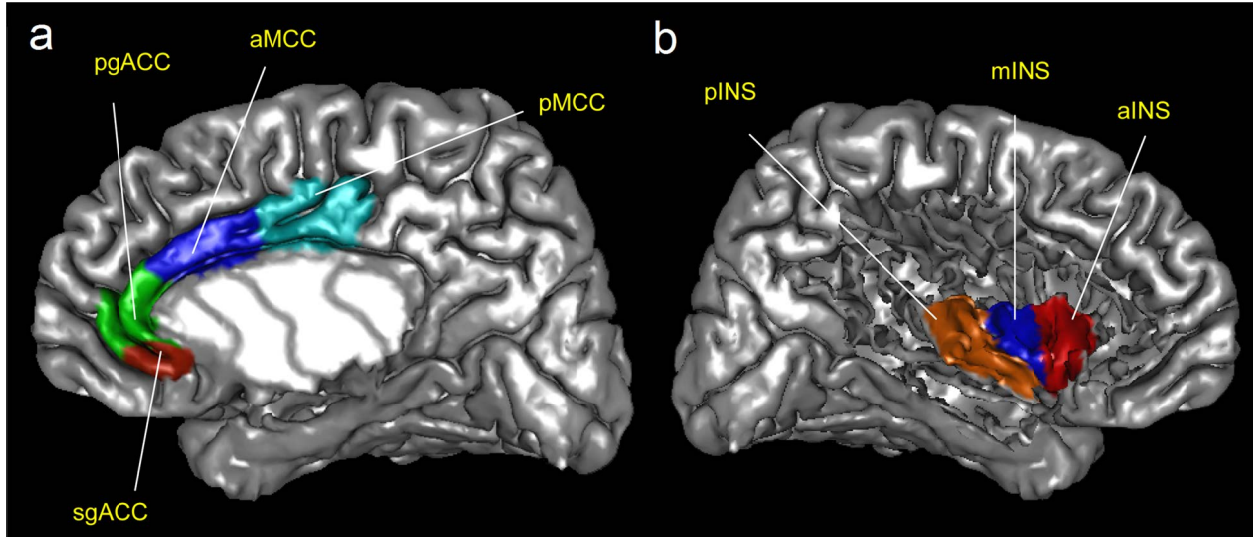
Supplemental Figure 1. Manually delineated subregions of interest on the 3D International Consortium for Brain Mapping brain atlas. a) Subregions of cingulate cortex: anterior mid cingulate cortex (aMCC), posterior mid cingulate cortex (pMCC), pregenual anterior cingulate cortex (pgACC) and subgenual anterior cingulate cortex (sgACC). b) Subregions of insula: anterior insula (aINS), mid insula (mINS) and posterior insula (pINS).

Chapter 6

Conclusions and Future Works

The current work demonstrates that patients with irritable bowel syndrome (IBS) exhibit

structural and functional alterations in the default mode network, and in brain networks related to

salience detection, sensorimotor function, executive control, and emotional arousal. These

network alterations are observed in evoked and non-evoked brain imaging studies. Based on these

data, the salience network and the anterior insula have been suggested to play a key role in the

pathophysiology of IBS and in the observed sex-related differences. The following results

supporting sex-related differences and the role of anterior insula in IBS are obtained: 1)

Oscillations of blood oxygen level-dependent signals in the anterior insula had higher power in the

high frequency domain compared to the low frequency domain. This frequency power distribution

in the anterior insula was strongest in female IBS patients compared to female healthy controls

(HCs) and male IBS patients. Supporting the relevance of these brain alterations for clinical

symptoms, abdominal discomfort level was positively correlated with the frequency power

distribution (Hong et al., 2013). 2) Female IBS patients had greater negative connectivity of

anterior insula with the precuneus (one of the key regions in the default mode network) compared

to female HCs and male IBS patients. Again, the connectivity between anterior insula and

precuneus was positively correlated with symptom intensity in the past 24 hours. In addition,

negative insular connectivity with medial prefrontal cortex (another key region in the default mode

network) was observed in female IBS patients. In contrast, male HCs and male IBS patients

showed positive insular connectivity with medial prefrontal cortex (Hong et al., 2014a). 3) In the

study described in Chapter 4, I have demonstrated that female IBS patients had greater ventral

anterior insula and precuneus activation in response to an ambiguous contextual threat compared

to female HCs, suggesting altered brain responses associated with hypervigilance. On the other hand, this altered brain response to uncertainty of threat was not observed in male IBS patients. 4) IBS patients (most were female) showed structural alterations in the anterior insula in the form of decreased cortical thickness compared to HCs (Hong et al., 2014b).

A large amount of internal and external information is received by our brain every moment. Thus, the detection, appraisal and processing of important information necessary for making appropriate responses are critical for health and well-being. The relative salience of given information is determined by contrasting other incoming information, background or previous experiences (Naatanen et al., 2007; Legrain et al., 2011). Therefore, the novelty of the information, the intensity of a given stimulus and the variation of a signal can influence the magnitude of salience (Legrain et al., 2011). Similarly, the concept of salience has been suggested in an interoception model (Mayer et al., 2009a). In this model, the salience of an interoceptive stimulus will be computed based on the mismatch between an actual interoceptive input and a previous interoceptive memory. This computed salience will result in proper adjustment and feedback in the brain and body (Mayer, 2011). Salience detection is associated with appropriate motor responses (e.g. fight-or-flight), cognitive function (e.g. attention) and autonomic or homeostatic responses (Legrain et al., 2011; Uddin, 2014). With advanced neuroimaging technologies, the salience network has been identified in the human brain (Seeley et al., 2007). In addition, the salience network has been suggested to coordinate the switching between the central executive network and the default mode network (Menon and Uddin, 2010; Gao and Lin, 2012; Goulden et al., 2014; Hong et al., 2014a; Di and Biswal, 2015).

The anterior insula, one of the key nodes within the salience network, is involved in sensory, visceral, emotional and pain processing (i.e. salience processing), feeling generation,

prediction/anticipation during uncertainty, attention allocation, and perceptual decision making (Seeley et al., 2007; Craig, 2009; Singer et al., 2009; Wiech et al., 2010; Tillisch et al., 2011; Uddin, 2014; Lamichhane and Dhamala, 2015). Von Economo neurons (large, spindle-shaped projection neurons) are highly populated in layer V of the anterior insula and the adjacent prefrontal cortex (Butti et al., 2013). With their large size, simple dendritic architecture and location, von Economo neurons have been implied to play roles in interoception, salience detection, cognition and social awareness by connecting with other parts of the brain and rapidly relaying information (Allman et al., 2011; Cauda et al., 2014). The characteristics and roles of von Economo neurons may be related to the findings in Baria et al. (2011) that high frequency blood oxygen level-dependent oscillations were dominated in the multimodal brain areas or more complex brain areas (e.g. insula). These observations were also in line with Hong et al. (2013) that insula and subcortical areas had frequency power distribution skewing toward the high frequency band. Moreover, the reduction of von Economo neurons has also been observed in several neuropsychiatric disorders (Allman et al., 2011). With all above, I may speculate that the decreased anterior insular cortical thickness in IBS patients (possibly female-predominant) is associated with degeneration or loss of von Economo neurons (Butti et al., 2013; Hong et al., 2014b). The structural reduction of anterior insula might result in overcompensation of anterior insula activity in which abnormally high degree of frequency oscillation power was seen in female IBS patients (Hong et al., 2013). Consistently, patients with chronic limb pain and multi-somatoform disorder showed unusual increase of power spectral density in high frequency domain within the insula (Malinen et al., 2010; Otti et al., 2013).

As previously discussed, the salience network dynamically mediates the switching between the default mode network and the central executive network (Uddin et al., 2011). The disruption or

dysfunction of this dynamic regulation mechanism has been suggested in patients with traumatic brain injury (Jilka et al., 2014), posttraumatic stress disorder (Sripada et al., 2012), schizophrenia (Manoliu et al., 2013b; Moran et al., 2013) and complex regional pain syndrome (Becerra et al., 2014). Patients with IBS also showed altered anterior insular connectivity with key regions of the default mode network, as well as central executive and sensorimotor networks (Hong et al., 2014a). In addition, these alterations were sex-related in which female and male IBS patients had different anterior insular connectivity patterns. Given the literature regarding the role of the salience network, I may speculate that the findings in Chapter 4 where regional hyperactivity (anterior insula, amygdala and precuneus) found in female IBS patients in response to a contextual/uncued threat were related to the disrupted balance between the salience network and other brain regions (Legrain et al., 2011). It is conceivable that the disrupted brain network dynamics prevents the ability to generate appropriate responses and provide feedbacks in the motor, homeostatic, interoceptive, attentional, executive control, antinociceptive, emotional, or autonomic systems (Seeley et al., 2007; Sridharan et al., 2008; Menon and Uddin, 2010; Mayer, 2011; Gao and Lin, 2012; Kullmann et al., 2012; Beissner et al., 2013; Manoliu et al., 2013b; Otti et al., 2013; Uddin et al., 2013; Kucyi and Davis, 2015). However, further research to better understand and distinguish the dynamic mechanism in different disease models including chronic pain disorders is still needed.

Several issues need to be considered in future studies. 1) Sex hormone effects on pain in healthy individuals and chronic pain populations have been suggested in some studies (Choi et al., 2006; Kilpatrick et al., 2010a). In my dissertation, I did not collect blood samples to formally evaluate or control for the menstrual cycle in my analyses. However, I have tried to recruit female subjects during their premenopausal state as much as possible, and to balance the groups based on

phases. Although menstrual cycle effects on brain imaging studies are still inconclusive, future

sex-related studies should cautiously consider natural and chemically induced variation of sex

hormones to identify their modulatory effects on the observed brain alterations. 2) Differences

between subgroups of IBS patients were not addressed in my analyses, as sample sizes were not

sufficiently large to address both sex and other subgroupings. 3) For a long time, global signal

regression (Desjardins et al., 2001) has been widely used to remove uninteresting physiological

noises such as changes of arterial carbon dioxide level and cardiac pulsation rate (Wise et al.,

2004; Shmueli et al., 2007; Murphy et al., 2009). However, there are controversies about whether

global signal regression method should or should not be applied. Murphy et al. suggested the

global signal correction technique would introduce artifacts of anti-correlated brain networks

while Fox et al. believed this method could facilitate examination of anatomical and physiological

correlations (Fox et al., 2009; Murphy et al., 2009). To avoid the issues of global signal regression

methods, a component based noise correction method (Whitfield-Gabrieli and Nieto-Castanon,

2012) was implemented in my resting-state functional connectivity analysis. However, the

accuracy and performance of this method may be compromised depending on the analysis criteria

and dataset (Behzadi et al., 2007). It will therefore be important to determine the appropriate noise

correction approach for different study designs and to improve signal-to-noise ratio for future

brain imaging studies. 4) In this dissertation, I have only focused on the macroscopic structural and

functional brain regions. However, questions about how the blood oxygenation level-dependent

signal fluctuations are related to neural activities and how the neurophysiologic plasticity affects

structural alterations at cellular level are still unclear. Until the two questions are answered, one

can only speculate about the possible mechanisms connecting the structural and functional

findings. 5) The results summarized in my dissertation contain only the spatial information. Since

the brain activities are non-stationary and dynamically associated, it will be important to examine the temporal information to determine the time-dependent interactions or coupling between regions or networks. Applications of time-dependent techniques include Granger causality analysis (Roebroeck et al., 2005), dynamic causal modeling (Stephan et al., 2010) or group independent component analysis combined with sliding time-window correlation analysis and graph theory-based analysis (Allen et al., 2012; Yu et al., 2015). Finally, comprehensive behavioral and symptom measures need to be collected together with the brain data. Questionnaires such as the Pain Catastrophizing Scale (Sullivan et al., 1995) and Irritable Bowel Syndrome Severity Scale (Francis et al., 1997) can be used to correlate with imaging findings in the future studies. Along with such clinical measurements, future longitudinal studies will be able to better explain whether the brain alterations in IBS patients are consequences or causes of the disorder.

In conclusion, I have identified several brain signature changes in patients with IBS. With limited number of sex-related studies in current IBS research field, not only does this work provide better understanding of sex-related differences underlying central mechanisms of IBS, but it also emphasizes the importance of differentiating or controlling sex for future studies. The structural and functional alterations identified in this dissertation may lead to the identification of biomarkers for IBS and related chronic visceral pain disorders, and assist in the development of more effective therapies.

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