# Chemical Mapping of Anxiety in the Brain of Healthy Humans: An in vivo $^{1}$H-MRS Study on the Effects of Sex, Age, and Brain Region

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**Abstract:** We recently presented results in an in vivo study of human brain chemistry in 'physiologic' anxiety, i.e., the anxiety of normal everyday life. Normal subjects with high anxiety demonstrated increased concentration of chemicals in orbital frontal cortex (OFC) as compared to lower anxiety. In a separate study of aging we demonstrated a decrease of total chemical concentration in OFC of middle-aged subjects, as compared with younger age. This brain region also showed gender dependence; men demonstrating decreased chemical concentration compared to women. We hypothesized that these sex- and age-dependent differences in OFC chemistry changes are a result of anxiety effects on this brain region. In the present study we examined these sex- and age-differential regional brain chemistry changes (as identified by localized in vivo proton magnetic resonance spectroscopy [$^{1}$H-MRS]) in relation to the state-trait-anxiety (as measured by the State-Trait Anxiety Inventory) in 35 healthy subjects. The concentrations for all nine chemicals of $^{1}$H-MRS spectra were measured relative to creatine across multiple brain regions, including OFC in the left hemisphere. Analysis of variance showed anxiety-specific effects on chemical concentration changes in OFC, which were different for both sexes and age groups. Male subjects showed larger effect of anxiety on OFC chemistry as compared to females when the same sex high-anxiety subjects were compared to lower anxiety. Similarly, middle-aged subjects showed larger effect of anxiety on OFC chemistry as compared to younger age when the same age subjects with high anxiety were compared to lower anxiety. Largest effect of anxiety on OFC chemistry was due to changes of N-Acetyl aspartate. The results indicate that the state-trait anxiety has sex- and age-differential patterns on OFC chemistry in healthy humans, providing new information about the neurobiological roots of anxiety. *Hum. Brain Mapping 11:261–272, 2000.* © 2000 Wiley-Liss, Inc.

**Key words:** State-Trait Anxiety Inventory; anxiety; orbital frontal cortex; brain chemistry; healthy humans; sex differences; age differences; in vivo proton magnetic resonance spectroscopy; N-Acetyl aspartate; neurotransmitters

## INTRODUCTION

Anxiety disorders are one of the most prevalent psychiatric disorders, which afflict up to a fourth of the general population at some point in their lives [Kessler et al., 1994; Magee et al., 1996; Weissman et al., 1997; Anagnostaras et al., 1999]. Study of neurotransmitter systems and functional neuroanatomy has

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facilitated the progress in understanding anxiety disorders and their neurobiology. First, these studies provided the evidence that pathologic anxiety is associated with alterations of multiple neurotransmitter systems, including glutamate, γ-aminobutyric acid, catecholamines, benzodiazepines, serotonin, cholecystokinin, corticotropin-releasing hormone, and somatostatin [Connor and Davidson, 1998; Coplan and Lydiard, 1998; Barchas and Altemus, 1999; Ninan, 1999]. Second, functional imaging studies of the brain have implicated the involvement of multiple regions in the pathophysiology of anxiety disorders, including anterior limbic/paralimbic regions and orbital frontal cortex (OFC) [Coplan and Lydiard, 1998; Davidson et al., 1999]. Our recent in vivo proton magnetic resonance spectroscopy ($^1$H-MRS) study was the first study of 'physiological' anxiety, i.e., the anxiety of normal everyday life, which demonstrated chemical (we will use the term 'chemical' rather than 'metabolite' since some of the studied chemical compounds are neurotransmitters and some are metabolites) changes in OFC of high-anxiety healthy humans as compared to lower anxiety, and showed relationships between OFC chemical concentrations and the state-trait anxiety behavior [Grachev and Apkarian, 2000a]. Interestingly, the same area of the brain has been repeatedly implicated to be involved in obsessive-compulsive disorder (OCD) [Baxter et al., 1988; Nordahl et al., 1989; Swedo et al., 1989; Rauch et al., 1994; Breiter et al., 1996].

It is well known that anxiety disorders are more common in young adults than in the elderly [reviewed in Flint, 1994, 1997], and women are more likely than men to develop anxiety disorders [reviewed in Pigott, 1999; Weinstock, 1999]. However, little is known about the neurobiology of sex and age differences in anxiety and anxiety disorders. In our recent study of aging we demonstrated a decrease of total chemical concentration in OFC of middle-aged subjects, as compared with younger age [Grachev and Apkarian, 2000]. This brain region also showed gender-dependent effect; men demonstrating decreased chemical concentration compared to women [Grachev and Apkarian, 2000c]. Given that (i) anxiety is associated with OFC chemistry and (ii) there is evidence for sex and age-differential patterns in chemical concentrations for the same brain area, we hypothesized that these sex- and age-dependent differences in OFC chemistry changes are the result of anxiety effects on this brain region. In this study we examine relationships between regional brain chemistry (as identified by localized single-voxel in vivo $^1$H-MRS) and anxiety (as measured by the State-Trait Anxiety Inventory [STAI]) in 35 male and female healthy subjects in the young age (19–31 years) and middle age (40–52 years), across multiple brain regions, including OFC (the main region-of-interest), in the left hemisphere and nine chemicals of $^1$H-MRS spectra (N-Acetyl aspartate [NAA], choline, glutamate, glutamine, γ-aminobutyric acid, inositol, glucose, and lactate as measured relative to creatine). We selected these chemicals because their concentration in the brain has been shown to vary with differences in sex and age in healthy humans. Of these, the concentration of NAA was most changed [Grachev and Apkarian, 2000c]. We expected the largest effect of anxiety on OFC chemistry would be due to changes of NAA (the main chemical-of-interest). This hypothesis was based on our pilot data of anxiety-related changes of NAA in OFC in normals [Grachev and Apkarian, 2000a] and in patients with anxiety disorder due to chronic back pain (unpublished result), as well as previous reports of NAA changes in the brain of patients with OCD [Ebert et al., 1997; Bartha et al., 1998; Fitzgerald et al., 2000].

## MATERIALS AND METHODS

### Subjects

Thirty-five healthy right-handed subjects (19 young age [mean age = 22.8 ± 2.9, age range 19–31, 11 men and 8 women] and 16 middle age [mean age = 46.6 ± 3.4, age range 40–52, 12 men and 4 women]) participated in this study. Subjects were recruited by advertisement from the local community (most of them were professionals within our institution, i.e., nurses, doctors, and administrative personnel). Initial high-resolution MRI of all subjects were obtained and examined by a staff neuroradiologist to exclude brain morphologic abnormalities. Subjects with neurological illness, head trauma, or psychiatric disorder (DSM-IV) were excluded. None of our subjects had claustrophobic tendencies (most of them participated in MRI and MRS studies before, and all subjects were instructed to terminate further participation in MRI and MRS experiments if claustrophobic tendencies appeared). The general purpose and the procedures were explained to the subjects. All subjects signed a consent form. The Institutional Review Board at the SUNY Upstate Medical University approved all procedures in this study.

### Localized Single-Voxel in vivo $^1$H-MRS

During brain imaging the subject was placed on the scanner bed and the whole-head gradient coil was positioned over the head. The subject's head was im-

mobilized using a vacuum beanbag. Automated global shimming (part of MRS software package SPECTRO, General Electric) was performed to optimize the magnetic field homogeneity over the entire brain volume. All MRI and MRS experiments were performed on a 1.5 Tesla General Electric (Signa) clinical imaging instrument. High-resolution sagittal and axial views were used for the selection of volumes of interest. T1-weighted multislice spin echo scout images (TR = 500 ms; TE = 12 ms; 2NEX; 256 × 256 matrix; FOV = 24 × 24 cm) of the entire brain were obtained with 6.0 mm slice thickness and a 0.5 mm gap between slices, imaging 20 slice locations. Localized $^1$H-MRS was then performed in two sagittal (cingulate and dorsolateral prefrontal cortex [DLPFC]) and in four axial (thalamus, insula, OFC, and sensorimotor cortex [SMC]) locations in the left hemisphere of right-handed normal volunteers (Fig. 1A,B,C). OFC was chosen as our primary region-of-interest, and five other brain areas (DLPFC, SMC, insula, cingulate, and thalamus) were used as controls. We used 8 cm$^3$ voxel size for each analyzed volume: DLPFC (voxel size 1.7 × 3.0 × 1.6 cm), OFC (2.0 × 2.0 × 2.0 cm), SMC (2.0 × 1.1 × 3.5 cm), thalamus (3.0 × 1.8 × 1.5 cm), cingulate (5.0 × 1.6 × 1.0 cm), and insula (4.0 × 2.0 × 1.0 cm) as described earlier [Grachev and Apkarian, 2000a, 2000c]. Briefly, proton localized spectra were collected using a simulated-echo acquisition mode (STEAM) sequence (probe-s PSD, TR = 1,500 ms, TE = 30 ms, 256 averages). All spectra were transformed into a standardized scale using the Scion Image analysis package and analyzed as described [Grachev and Apkarian, 2000a, 2000c]. These peaks were identified by specific chemical shifts (ppm), with investigators blinded to both the location and the subject. The concentrations (i.e., signal intensities) of NAA (2.02 ppm), choline (Cho, 3.2 ppm), glutamate (Glu, 2.35 ppm), glutamine (Gln, 2.15 ppm), γ-Aminobutyric acid (GABA, 2.25 ppm), myo- and scyllo-inositol complex (Ins, 3.60 ppm), glucose (Glc, 3.43 ppm), and lactate (Lac, 1.3 ppm) were measured relative to concentration for creatine/phosphocreatine complex (Cr, 3.0 ppm) [Fig. 1D]. NAA (our primary chemical-of-interest) is the dominant peak in normal adult brain spectra. Cr spectrum is a combination of creatine and phosphocreatine. Another resonance for Cr, from the $CH_2$ group, can be observed at 3.94 ppm if good suppression of the water peak at 4.7 ppm is achieved; however, only the peak at 3.0 is typically employed in the interpretation of MRS data because it has a larger area [Salibi and Brown, 1998] and was measured in our study. The proton Cho signal is a combination of Cho and Cho-containing compounds: Cho plasmogen, glycerophosphorylcholine, phosphorylcholine, cytedine-diphosphate-choline, acetylcholine, and phosphatidylcholine [Michaelis et al., 1993]. Lac signal, from protons of the $CH_3$ group, is usually a combination of Lac and lipid peaks at 1.3–1.33 ppm (inverted doublet with PRESS, TE = 135 ms; this signal is upright with PRESS, TE = 270 ms and with STEAM at any TE, including 30 ms in our study). Reliability of the chemical measurements, which was estimated as the coefficient of variation (CV) for each studied chemical, was conducted using five repeat scans of one brain region on one normal subject, and was generally excellent: NAA/Cr = 2.5%, Cho/Cr = 4.0%, GABA/Cr = 6.6%, Glc/Cr = 3.4%, Ins/Cr = 2.0%, Lac/Cr = 8.5%, Glu/Cr = 6.5%, and Gln/Cr = 3.3%. We used the peak ratio method as a simple method that provides an internal normalization, which minimizes variations due to technical confounders on the MRS measurements. Only high-quality MRS spectra were selected for data analyses: 33 spectra for thalamus (17 in young age group and 16 in middle-aged group), 27 spectra for DLPFC (16 and 11), 25 spectra for cingulate (14 and 11), 24 spectra for SMC (13 and 11), 27 spectra for insula (17 and 10), and 20 spectra for OFC (11 and 9 accordingly). The mean water line width (i.e., full width at half maximum [FWHM]) from the 8 cm$^3$ voxel was 4.8 Hz (SD = 0.6, maximum FWHM = 6 Hz; all MRS spectra with FWHM above 6 Hz were rejected). In our study Cr spectra were relatively stable across the studied brain regions (CV = 4.9%–8.9%), and used as an internal standard. Although $^1$H-MRS spectra are contaminated by signals from other metabolites and proteins, the prominent signal is from the chemicals with which we identify these peaks [Salibi and Brown, 1998].

### State-Trait Anxiety Inventory

State anxiety refers to anxiety felt at a particular moment, while trait anxiety refers to a habitual tendency to be anxious over a long period of time. In responding to the STAI State-Anxiety Scale [Spielberger et al., 1983] (STAI form Y-1), examinees indicate the number that best describes the intensity of their feeling. In responding to the STAI Trait-Anxiety Scale (STAI form Y-2), examinees are instructed to indicate how they generally feel by rating the frequency of their feelings of anxiety on a four-point scale. To obtain scores for the S-Anxiety and T-Anxiety scales, the sum of the weighted scores for the 20 items that make up each scale was calculated, taking into account the reversed scores [Spielberger et al.,

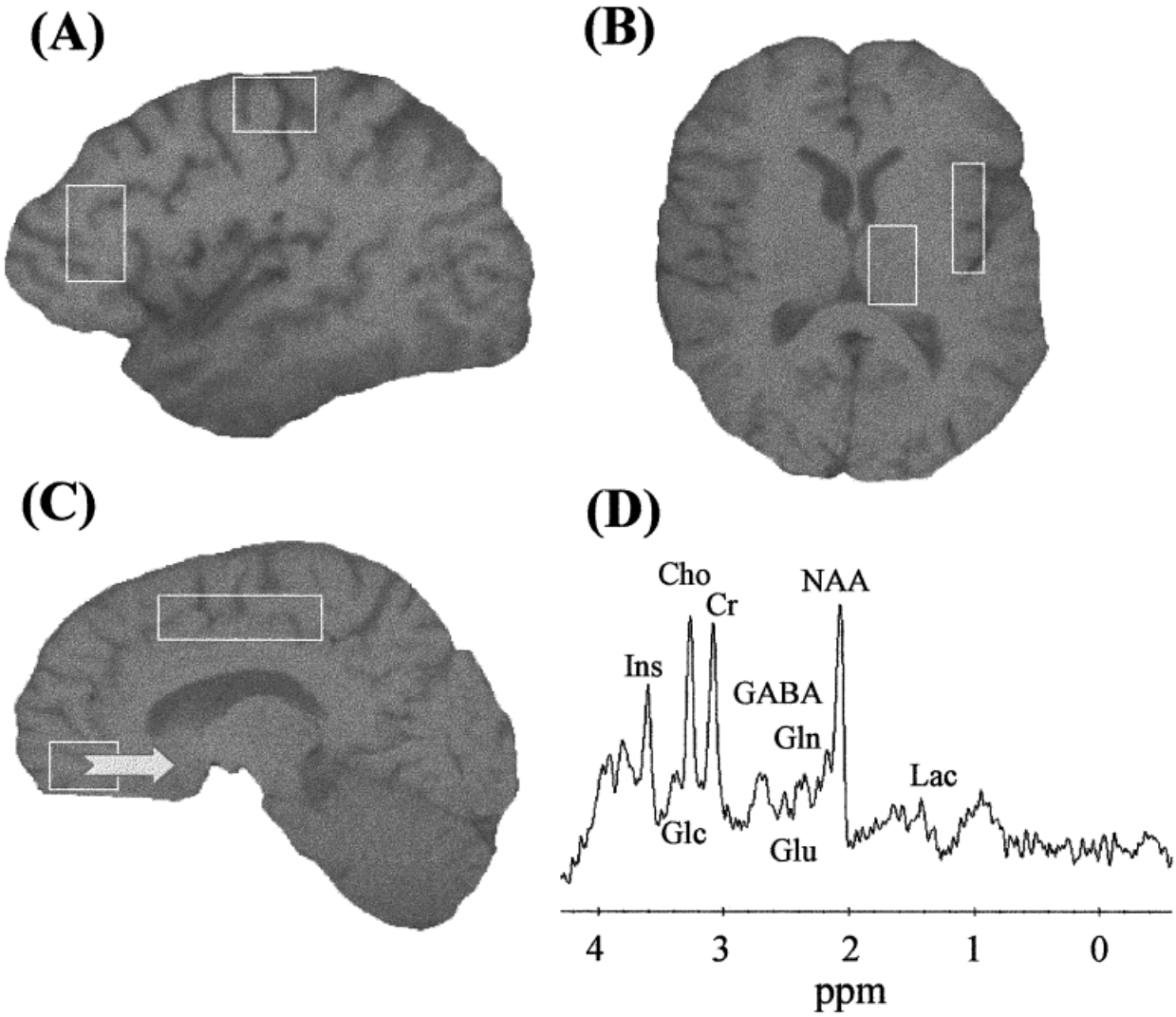


**Figure 1.**

An in vivo localized single-voxel proton MR brain spectroscopy. T1-weighted spin echo high-resolution MR images showing the position of six brain voxels in the left hemisphere. The size and positioning of regional voxels for DLPFC and SMC (**A**), thalamus and insula (**B**), and OFC (arrow) and cingulate (**C**). Typical in vivo proton MR spectra obtained from OFC (**D**) showing position of large peaks for NAA, Cr, Cho, and smaller peaks for Glu, Gln, GABA, Ins, Glc, and Lac.

1983]. These anxiety measures were performed just minutes before brain imaging in the environment of MRI and MRS experiments.

### Statistical Analysis

Distribution of original data for goodness of fit was assessed using Kolmogorov-Smirnov and Chi-Square tests (STATISTICA, Tulsa, OK). Differences in sex, age (young vs. middle age), anxiety (high anxiety [total STAI score above 67, see results] vs. lower anxiety [total STAI score below 67]), brain region, and chemical effects on concentration were analyzed with analysis of variance (ANOVA), using the general linear model (STATISTICA). The outcome variable was taken to be chemical concentration relative to the Cr peak. In two four-way ANOVAs, sex and age effects were analyzed separately. Descriptive planned comparisons were made using F tests to explain observed differences across factors and their interactions (STATISTICA). We used correlation analysis to test empirical relationships between anxiety perception (total STAI score) and concentration of regional chemical, which mainly changed in ANOVAs (NAA in OFC), across the studied sex and age groups (STATISTICA).

**TABLE I. Means, standard deviations (SD), and number of subjects (N) for the STAI scales, across sex and age groups (19–31 vs. 40–52 years) in normal subjects (N = 35)**

| | 19–31 | | 40–52 | | 19–31 | 40–52 | All ages | | |
|---|---|---|---|---|---|---|---|---|---|
| | Males | Females | Males | Females | All sexes | All sexes | Males | Females | All sexes |
| S-Anxiety | | | | | | | | | |
| Mean | 36.11 | 30.71 | 31.83 | 26.25 | 33.75 | 30.44 | 36.11 | 30.71 | 32.09 |
| SD | 7.03 | 8.16 | 9.59 | 5.06 | 7.78 | 8.88 | 7.03 | 8.16 | 8.38 |
| N | 11 | 8 | 12 | 4 | 19 | 16 | 23 | 12 | 35 |
| T-Anxiety | | | | | | | | | |
| Mean | 36.25 | 37.33 | 34.00 | 32.50 | 36.71 | 33.63 | 36.75 | 37.33 | 35.07 |
| SD | 6.54 | 8.41 | 9.96 | 6.24 | 7.11 | 9.00 | 6.54 | 8.41 | 8.19 |
| N | 11 | 8 | 12 | 4 | 19 | 16 | 23 | 12 | 35 |
| Total-Anxiety | | | | | | | | | |
| Mean | 71.00 | 68.17 | 65.83 | 58.75 | 69.79 | 64.06 | 71.00 | 68.17 | 66.73 |
| SD | 12.27 | 15.14 | 19.20 | 11.24 | 13.09 | 17.48 | 12.27 | 15.14 | 15.60 |
| N | 11 | 8 | 12 | 4 | 19 | 16 | 23 | 12 | 35 |

## RESULTS

The results of anxiety measures in the studied subjects are shown in Table I. The total STAI scores and scores on two subscales (S-Anxiety and T-Anxiety) were not different between sex and age groups. These scores were similar to published data for normals in these age groups (mean ± SD = 36.8 ± 9.6 for S-Anxiety and 36.6 ± 10.3 for T-Anxiety in young adults, and slightly lower scores on both subscales in middle-aged subjects) [Spielberger et al., 1983] and were much lower than those for anxiety disorders (S-Anxiety = 49.02 ± 11.62 and T-Anxiety = 48.08 ± 10.65) [Spielberger et al., 1983]. We consider subjects with total STAI scores below 67 as low anxiety (mean = 55.12 ± 7.51, 18 subjects) and subjects with total STAI scores above 67 as high anxiety (mean = 81.55 ± 6.77, 17 subjects). This arbitrary subdivision was made by splitting all data for the total STAI score on two extremes (50% of the data above median and 50% below median) and was used for analyses of anxiety effects on chemical concentration differences across sex and age groups.

The original data for chemical concentrations were distributed normally (Kolmogorov-Smirnov D = 0.06, $P < 0.01$; and $X^2 = 163.4$, $P < 10^{-7}$), which is consistent with our previous report [Grachev and Apkarian, 2000b]. First we quantified sex differences in concentration of brain chemicals between anxiety groups, across brain regions and chemicals using a four-way ANOVA. A 2 (Sex [males vs. females]) × 2 (Anxiety [high vs. lower anxiety]) × 6 (Brain Region) × 9 (Chemical) measures ANOVA, with relative concentration defined as the dependent variable, revealed multivariate main effects for Sex ($F(1, 981) = 4.22$, $P < 0.04$), Brain Region ($F(5, 981) = 55.17$, $P < 10^{-7}$), Chemical ($F(1, 981) = 417.81$, $P < 10^{-7}$), Anxiety ($F(1, 981) = 3.38$, $P < 0.06$), the Sex × Brain Region interaction ($F(5, 981) = 5.12$, $P < 0.0001$), the Sex × Anxiety interaction ($F(1, 981) = 3.20$, $P < 0.07$), the Brain Region × Chemical interaction ($F(40, 981) = 3.65$, $P < 10^{-7}$), the Brain Region × Anxiety interaction ($F(5, 981) = 6.68$, $P < 0.000004$), and the interaction between the Sex, Brain Region, and Anxiety ($F(5, 981) = 2.44$, $P < 0.03$).

Effects of sex, brain region, chemical, and their interaction on chemical concentration [Grachev and Apkarian, 2000c] as well as effect of anxiety [Grachev and Apkarian, 2000a] have been presented. Because these data are replicated here on larger cohort of subjects, in this study we describe only the effects of the sex in relation to anxiety and brain region using descriptive F tests. Since anxiety-related differences in chemical concentration were found only in OFC [Grachev and Apkarian, 2000a], we describe the effects of sex on the Anxiety × Brain Region (OFC) interaction for males and females separately. The mean concentration in OFC for all studied chemicals in high-anxiety males was 22.4% higher as compared with lower anxiety males ($F(1, 981) = 50.69$, $P < 10^{-7}$) (Fig. 2). In female subjects the anxiety effect was smaller showing only 8.2% increase for the mean concentration of OFC chemicals in high anxiety females as compared with lower anxiety females ($F(1, 981) = 3.28$, $P < 0.07$). Other brain regions showed no sex-dependent differences in chemical concentrations in relation to anxiety level in the studied healthy subjects.

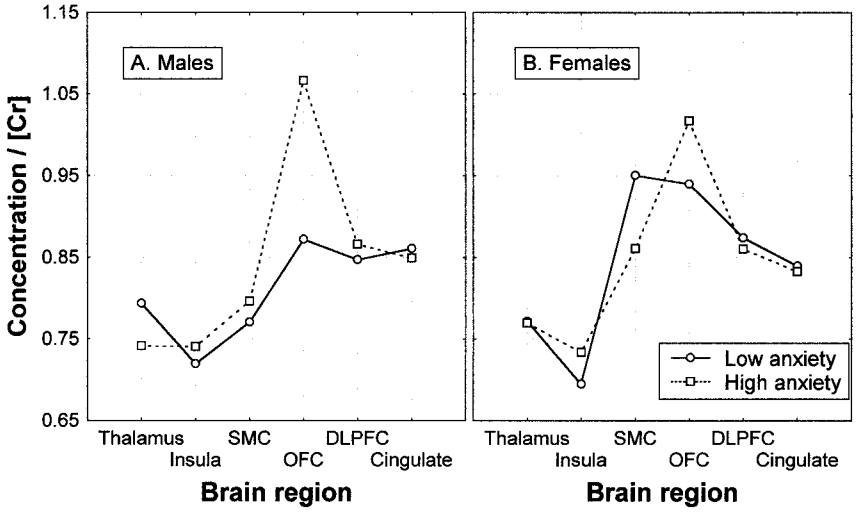


**Figure 2.**
Effect of the Sex × Anxiety × Brain Region interaction ($F = 2.44$, $P < 0.03$) on the total chemical concentration across brain regions in healthy males (**A**) and females (**B**). Male subjects showed larger effect of anxiety on OFC chemistry as compared to females, when the same sex high-anxiety subjects were compared to lower anxiety.

Because for both sexes the strongest regional effect of anxiety was detected only in the OFC with larger effect in males vs. females, we used descriptive F tests to examine which chemicals are responsible for these differences. In the OFC of high-anxiety males, increased chemical concentrations were observed for NAA by 28.0% ($F = 16.67$, $P < 0.00005$), GABA by 28.7% ($F = 5.15$, $P < 0.02$), Glu by 26.4% ($F = 3.64$, $P < 0.05$), GABA + Glu by 27.6% ($F = 17.44$, $P < 0.00003$), Gln by 37.5% ($F = 12.12$, $P < 0.0005$), Ins by 20.6% ($F = 5.04$, $P < 0.02$), and Lac by 44.7% ($F = 11.79$, $P < 0.0006$) as compared to lower-anxiety males (Fig. 3). Contrary to what was found for the male subjects, in the OFC of high-anxiety females only one chemical showed significant increase of concentration by 26.7%, the Ins ($F = 5.22$, $P < 0.02$), as compared to lower-anxiety females (Fig. 4).

Then, we used a similar statistical approach (i.e., four-way ANOVA) to quantify age differences in concentration of brain chemicals across anxiety groups, brain regions, and chemicals. A 2 (Age [young vs. middle]) × 2 (Anxiety [high vs. lower anxiety]) × 6 (Brain Region) × 9 (Chemical) measures ANOVA, with relative concentration defined as the dependent variable, revealed multivariate main effects for Brain Region ($F(5, 981) = 71.92$, $P < 10^{-7}$), Chemical ($F(8, 981) = 499.27$, $P < 10^{-7}$), Anxiety ($F(1, 981) = 12.05$, $P < 0.0005$), the Brain Region × Chemical interaction ($F(40, 981) = 3.92$, $P < 10^{-7}$), the Age × Brain Region interaction ($F(5, 981) = 15.59$, $P < 10^{-7}$), the Age × Chemical interaction ($F(8, 981) = 2.04$, $P < 0.04$), the Brain Region × Anxiety interaction ($F(5, 981) = 14.07$, $P < 10^{-7}$), the Age × Anxiety interaction ($F(1, 981) = 6.30$, $P < 0.01$), the interaction between the Age, Brain Region, and Chemical ($F(40, 981) = 1.41$, $P < 0.05$), and the interaction between the Age, Brain Region, and Anxiety ($F(5, 981) = 4.20$, $P < 0.001$).

Because we already presented the effects of age, brain region, chemical and their interaction on chemical concentration [Grachev and Apakarian, 200b], in this study we describe only the effects of the age in relation to anxiety and brain region. Similar to the approach that we described above to study effects of sex on the Anxiety × Brain Region interaction, we pursue descriptive F tests to study effects of age on the Anxiety × Brain Region (OFC) interaction for young

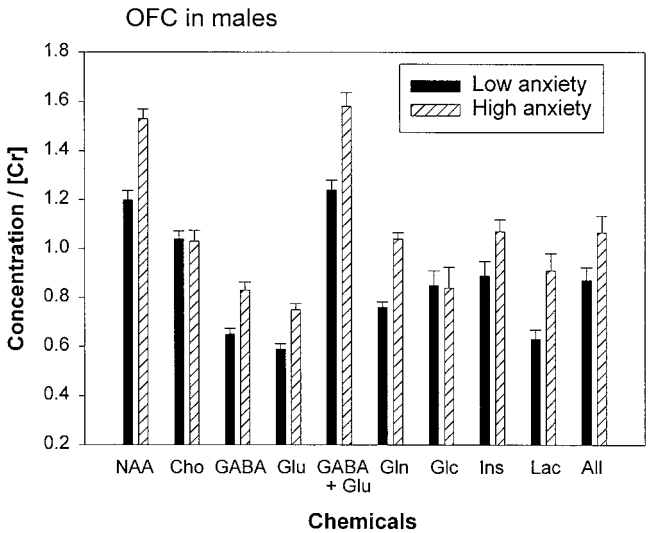


**Figure 3.**
Effect of Anxiety × Chemical type interaction on chemical concentration for males in OFC, low anxiety vs. high anxiety. Increased chemical concentrations are seen for most chemicals (NAA, GABA, Glu, GABA + Glu, Gln, Ins, and Lac) in OFC of high-anxiety males (Mean ± SEM).

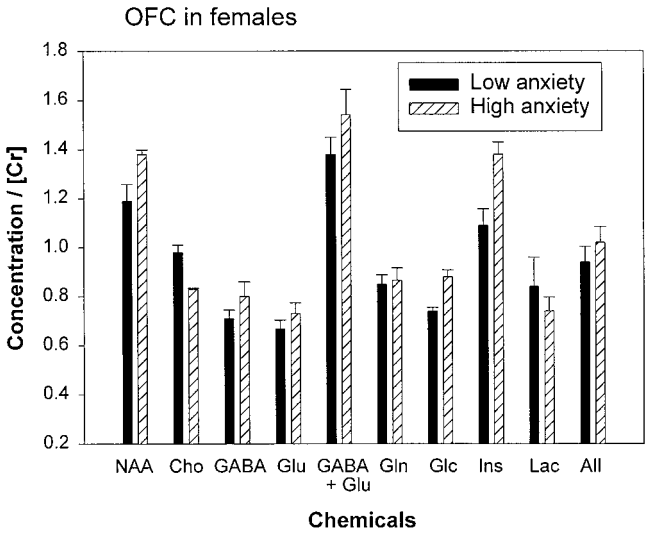


**Figure 4.**
Effect of Anxiety × Chemical type interaction on chemical concentration for females in OFC, low anxiety vs. high anxiety. Increased chemical concentrations are seen only for Ins (Mean ± SEM) in OFC of high-anxiety females.

and middle-aged subjects separately. The mean concentration in OFC for all studied chemicals in middle-aged subjects with high anxiety was 32.0% higher as compared with the same age subjects who had lower anxiety ($F(1, 981) = 65.44$, $P < 10^{-7}$) (Fig. 5). In young-aged subjects the anxiety effect was smaller, showing only 13.0% increase for the mean concentration of OFC chemicals in high-anxiety subjects as compared with the same age subjects who experienced lower anxiety ($F(1, 981) = 12.87$, $P < 0.0003$). Other areas of the brain showed no age-dependent differences in chemical concentrations in relation to the state-trait anxiety level.

Given that the strongest regional effect of age on anxiety-related neurochemistry was detected only in the OFC with larger effect in middle vs. young age, we used post-hoc F tests to describe effects of each chemical in OFC on the age-dependent anxiety differences. In the OFC of high-anxiety middle-aged subjects, increased chemical concentrations were observed for NAA by 43.3% ($F = 23.09$, $P < 0.000002$), GABA by 32.9% ($F = 4.10$, $P < 0.04$), GABA + Glu by 26.0% ($F = 9.71$, $P < 0.002$), Gln by 46.6% ($F = 11.30$, $P < 0.0008$), Ins by 45.2% ($F = 16.54$, $P < 0.00005$), Glc by 34.4% ($F = 8.42$, $P < 0.004$), and Cho by 19.7% ($F = 3.66$, $P < 0.06$) as compared to the same age subjects with lower anxiety (Fig. 6). In contrast to our findings for the middle-aged subjects, in the OFC of high-anxiety young-aged subjects, only a few chemicals showed increase of concentration: NAA by 14.6% ($F = 3.26$, $P < 0.07$), GABA + Glu by 18.9% ($F = 6.53$, $P < 0.01$), and Lac by 27.6% ($F = 3.93$, $P < 0.05$), as compared to the same age subjects with lower anxiety (Fig. 7).

Because the main anxiety-related differences were in concentration of NAA (this chemical changed the most in males and in middle-aged subjects, the two groups with largest effect of anxiety on OFC chemistry). We used correlation analysis to test whether empirical relationships are seen between total anxiety (the state anxiety highly correlated with the trait anxiety, $r = 0.90$) and concentration of NAA in OFC across the studied groups. Post-hoc analysis revealed that NAA in OFC strongly correlated with total anxiety score in three of four studied groups: $r = 0.85$ for males, $r = 0.64$ for middle-aged subjects, $r = 0.63$ for young-aged subjects, $r = -0.23$ for females, and $r = 0.58$ for all groups, $P < 0.05$). This suggests the pres-

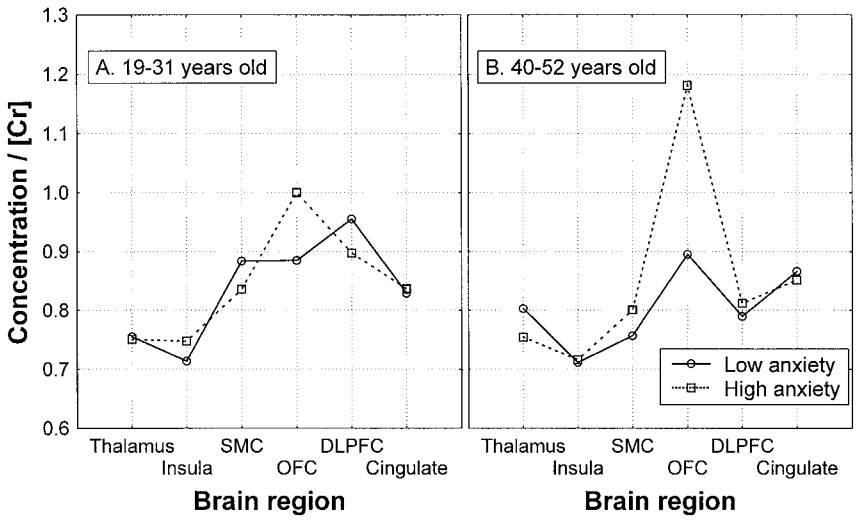


**Figure 5.**
Effects of the Age × Anxiety × Brain Region interaction ($F = 4.20$, $P < 0.0009$) on the total chemical concentration across brain regions in healthy young (**A**) and middle-aged subjects (**B**). Middle-aged subjects showed larger effect of anxiety on OFC chemistry as compared to younger age, when the same age subjects with high anxiety were compared to lower anxiety.

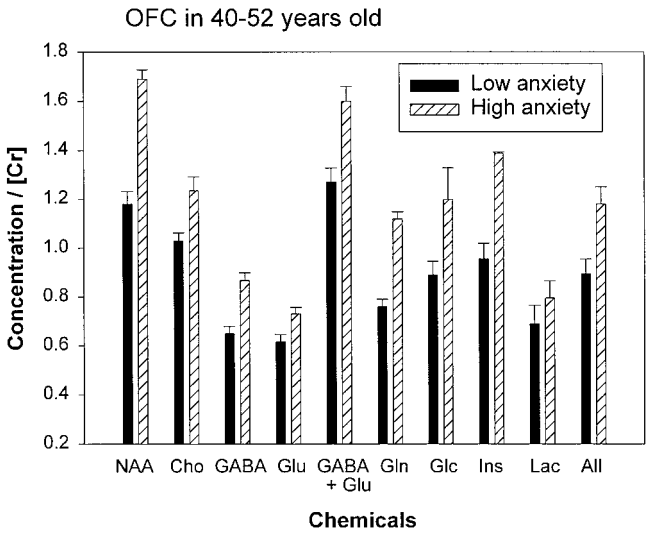


**Figure 6.**

Effect of Anxiety × Chemical type interaction on chemical concentration for middle-aged subjects in OFC, low anxiety vs. high anxiety. Increased chemical concentrations are seen for most chemicals (NAA, Cho, GABA, GABA+Glu, Gln, Ins, and Glc) in OFC of high-anxiety middle-aged subjects (Mean ± SEM).

ence of specific relationships between NAA in OFC and anxiety perception in healthy humans.

Because sex and age are two major neurobiological confounders on the $^1$H-MRS measurement of chemical concentrations, and a sample size for each sex and age group was different (which might impact on the observed differences), we tested the interaction between sex and age factors. The effect of the Sex × Age group interaction was not significant ($F(1, 1296) = 0.69$, $P = 0.4$, four-way ANOVA). The result indicates that at least for the current analysis, sex and age differences are not due to unequal sample size and/or effects of one factor (e.g., sex) when we analyze another one (e.g., age).

## DISCUSSION

The results showed anxiety-specific effects on chemical concentration changes in OFC, which were different for both sexes and age groups. Male subjects showed larger effect of anxiety on OFC chemistry as compared to females, when the same sex high-anxiety subjects were compared to lower anxiety. Similarly, middle-aged subjects showed larger effect of anxiety on OFC chemistry as compared to younger age, when the same age subjects with high anxiety were compared to lower anxiety. We identified NAA as the chemical with largest effect of anxiety on OFC chemistry. Thus, the data indicate that the state-trait anxiety has sex- and age-differential patterns on OFC chemistry in healthy humans, which provide new information about neurobiological roots of anxiety.

In our previous $^1$H MRS study of anxiety we demonstrated a chemical-behavioral network in the brain of healthy subjects as one possible mechanism for development of physiologic anxiety [Grachev and Apkarian, 2000a]. Specifically, the combination of the state and trait anxiety predicted the concentration of OFC chemicals, and chemicals with highest correlation value were identified. Because of the sex- and age-differential patterns of anxiety demonstrated here, it would be worthwhile to perform multiple linear regression analyses for each sex and age groups separately on a larger cohort of subjects, which was not performed here due to limitations of the sample size. Also, in case of replication on patients with anxiety disorders, the results might suggest using sex and age-corrected doses for anxiolytic drugs, which may be different for different types of anxiety disorders.

Our previous study of aging demonstrated a decrease of total chemical concentration in OFC of middle-aged subjects, as compared with younger age [Grachev and Apkarian, 2000b]. This brain region also showed gender-dependent effect; men demonstrating decreased chemical concentration compared to women [Grachev and Apkarian, 2000c]. Our current results suggest that these sex- and age-dependent differences in OFC chemistry changes are a reflection of

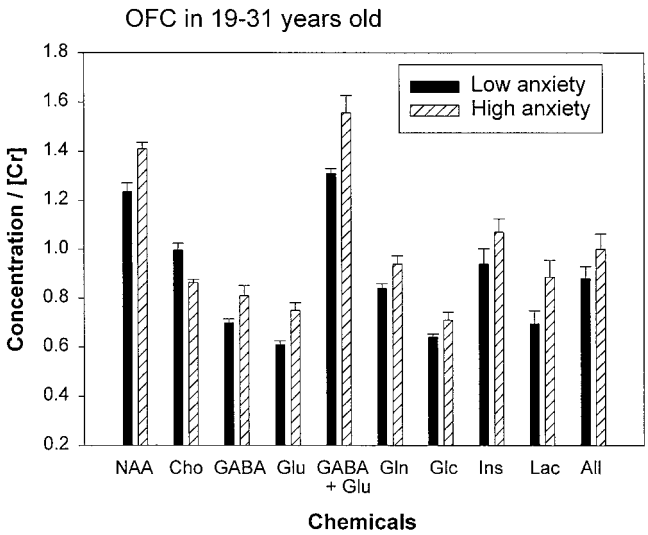


**Figure 7.**

Effect of Anxiety × Chemical type interaction on chemical concentration for young-aged subjects in OFC, low anxiety vs. high anxiety. Increased chemical concentrations are seen for NAA, GABA + Glu and Lac (Mean ± SEM) in OFC of high-anxiety young-aged subjects.

their specific relationships and may be a result of anxiety effects on this brain region. Although numerous functional imaging studies of distinct anxiety disorders consistently implicate anterior limbic/paralimbic regions, including OFC [Rauch et al., 1994, 1995, 1996; Benkelfat et al., 1995; Breiter et al., 1996], and several biochemical studies confirm the relationships between anxiety disorders and multiple neurotransmitter systems [Connor and Davidson, 1998; Coplan and Lydiard, 1998; Barchas and Altemus, 1999; Ninan, 1999], we do not find any evidence in the literature of sex and age effects on anxiety-related differences in neurobiology, including brain chemistry. Epidemiological studies of anxiety disorders showed that these types of psychiatric disorders are less common in the elderly than in younger adults [reviewed in Flint, 1994, 1997] and women are more likely than men to develop anxiety disorders [reviewed in Pigott, 1999; Weinstock, 1999]. This is consistent with our previous reports for sex and age-differential patterns of chemical concentrations in OFC. Specifically, the higher concentration of chemicals in healthy females [Grachev and Apkarian, 2000c] and in healthy younger subjects [Grachev and Apkarian, 2000b] corresponds to enhanced vulnerability to anxiety disorders for these sex and age groups. However, observed sex- and age-differential patterns on OFC chemistry changes in high vs. low anxiety normal subjects are in opposite direction to what is known about epidemiology of anxiety disorders.

Several questions remain to be answered: (1) Why do we observe larger effect of physiologic anxiety on regional brain chemistry (i.e., OFC) in middle-aged subjects as compared to younger age, when pathologic anxiety (i.e., anxiety disorders) become less common with advancing age? (2) Why do we see larger effect of physiologic anxiety on chemical changes at the OFC in males as compared to females when two age groups were pooled together, when pathologic anxiety are less common in males? These differences might be due to (1) the difference between physiologic and pathologic types of anxiety (all previous epidemiological studies were focused on anxiety disorders), (2) the difference between neurobiological and epidemiological methods, and (3) the difference between elderly and middle-aged groups in effects of anxiety on OFC chemistry changes (we did not study the elderly age group). It may also be the case that males show greater anxiety-induced arousal (as physiologic and adaptive stress-related response, which evolution designed to avoid any situations of personal threat) than do females; and middle-aged subjects show greater anxiety-induced arousal than do younger age at the same level of anxiety. This mechanism may explain the differences that we observe across the studied sex and age groups. This explanation is also consistent with the multiple demonstrations of the plasticity of the adult cortex. Thus, when males and middle-aged subjects have larger chemical changes in OFC (compensatory or adaptive response) as a result of anxiety effect on this brain region, they may become less vulnerable (more adaptive) to anxiety disorders in their lives as compared to females and younger age groups (less chemical/adaptive changes corresponds to increased vulnerability). Similar ideas for age differences in depression were suggested [Christensen et al., 1999]. The precise causes of the enhanced vulnerability to anxiety disorders for females and younger adults remain undetermined.

Sex and age differences in effects of endocrine factors as a possible mechanism, which might explain these effects on regional brain chemistry, have been discussed in our previous study [Grachev and Apkarian, 2000c]. Several studies suggest that vulnerability to anxiety disorders may be determined by genetic factors [Kendler et al., 1992a, 1992b, 1995]. Potential role of female gonadal hormones as possible mediators of increased susceptibility to the development of pathologic anxiety have been shown [reviewed in Pigott, 1999]. It was shown that progesterone is associated with dysphoric and some anxiolytic effects in women [Sherwin, 1991; Halbreich, 1997]. The latter may be due to its effects on GABA-benzodiazepine receptors [Majewska, 1992; Kroboth and McAuley, 1997]. Also, deficit of GABA receptors in some types of anxiety disorders have been shown in several brain regions, including OFC [Malizia et al., 1998]. Animal models suggest decreased GABA-receptor clustering results in enhanced anxiety [Crestani et al., 1999]. In this study we examined what OFC chemicals were mostly changed in males vs. females and in middle vs. young-aged subjects in relation to anxiety. The main anxiety-related differences were in concentration of NAA, which is a precursor of a neurotransmitter N-acetyl-aspartyl-glutamate and neuronal/axonal marker. This chemical changed most in males and in middle-aged subjects (these two groups had largest effect of anxiety on OFC chemistry). The results of correlation analysis demonstrated specific relationships between concentration of NAA in OFC and anxiety perception in most studied groups (males, middle-aged, and young-aged subjects), suggesting that our results provide evidence for a link between this chemical marker and anxiety. Also, we identified other neurotransmitters and chemicals in OFC, which show some anxiety effects in the brain across the studied sex and age groups, although

these chemical compounds seems to have less specific relationships with anxiety. Thus, in males, chemicals related to anxiety are: (1) NAA, (2) inhibitory neurotransmitter GABA, (3) excitatory neurotransmitter Glu, (4) precursor of the excitatory neurotransmitter glutamate (Gln), (5) energy substrate Lac, and (6) second messenger Ins. In females, only Ins in OFC was related to anxiety. Neurotransmitters and other chemicals in OFC, which are involved in anxiety of middle-aged subjects, were more similar to those described for males than for females. This cannot be explained simply by predominance of males in this age group (the interaction between sex and age were not significant); Glc instead of Lac was used as a main energy substrate. In the young-aged group, only NAA, GABA, and Glu (sum of two) and Lac in OFC were related to anxiety.

We propose several mechanisms of the association of studied regional chemicals and anxiety. There are two possibilities: First, anxiety "stresses" the human brain (OFC the most) with neurotransmitters and other chemicals released (most of these chemicals are involved in the interrelated cascade of biochemical reactions). Another possible mechanism is a predisposition to anxiety and different forms of anxiety disorders due to genetically predetermined control of neurotransmitter receptors and as a result chemical variations in the brain. Association between multiple receptors (mostly studied for GABA, glutamate, benzodiazepines, and serotonin receptors) and anxiety is a current biochemical hypothesis of anxiety disorders [Connor and Davidson, 1998; Coplan and Lydiard, 1998; Barchas and Altemus, 1999; Ninan, 1999]. It is still less clear why the levels of NAA and other chemicals should increase in anxiety. What are the causal relationships between them? It seems the genetic predisposition for anxiety may be linked with a control for number of neurons and their receptors in the brain. When the number of neurons and synaptic connections across anxiety-related regions increases, the concentration of NAA and other chemicals also should increase (NAA is a well-defined marker for the neuronal number). Correspondence between these processes was discussed in our previous report [Grachev and Apkarian, 2000a]. Closely associated with this issue is the question as to what brain mechanisms of anxiety are different under physiological and pathological conditions? It appears that in physiological anxiety the most triggered region is OFC, which sends chemical messages to (i) additional stress-related systems in the brain (cortical and thalamic circuits that process different aspects of sensation, cognition, emotion, and motor outputs, including limbic/paralimbic areas) that participate in complex decision-making behavior to escape from situations of personal threat (OFC as a main region involved in decision-making behavior was demonstrated [Bechara et al., 1994, 1997, 1998; Damasio, 1998]) and (ii) to neuronal networks to inhibit excessive anxiety, perhaps via connections between OFC and other brain regions (involvement of OFC in inhibitory control mechanisms was suggested [Fuster, 1997]). In the condition of physiological anxiety these latter mechanisms are strong enough to work almost immediately and anxiety-related changes are seen mainly in OFC (other regions already become adapted to anxiety response). In the condition of pathological anxiety, these inhibitory processes may not be efficient enough to block excessive anxiety and as a result of these mechanisms we might expect to see a multiregional brain involvement in their pathophysiology. This idea is supported by numerous imaging data published on the subject, which showed multiregional brain changes in pathological anxiety, and less evidence for that in physiological condition [Coplan and Lydiard, 1998; Davidson et al., 1999]. It is noteworthy that OFC is a main anxiety-related region in healthy humans, which seems to be involved in pathologic anxiety as well. The existing literature [Reiman, 1997] and our current findings suggest different mechanisms for pathological and physiological anxiety. Also, our findings should be interpreted cautiously because the imaging ($^1$H-MRS)/behavioral approach, proposed here, has not been used for anxiety disorders, which we hope to achieve in our future research. These are only the first steps in the field of anxiety neuroscience to understand the difference between normal and pathological forms of anxiety.

Because the combination of OFC chemicals involved in physiologic anxiety were different across the studied age and sex groups, this knowledge may be used for objective assessment of anxiety effects on OFC chemistry, which seems to be dependent upon sex and age effects. However, it has to be kept in mind that small peaked chemicals (Glu, Gln, GABA, Glc, Lac) are contaminated by signals from other metabolites and protein compounds, while the chemicals with large distinct peaks (NAA, Cr, Cho, Ins) are minimally contaminated. Thus, results need to be tested on higher magnetic field scanners and/or on multiple element phased array receivers that might optimize signal to noise ratio for chemical concentration measurements. The role of the studied chemicals in neuronal and glial compartments has been identified [Miller, 1991; Gruetter et al., 1996; Shulman and Rothman, 1998; Sibson et al., 1998; Faull et al., 1999; Magistretti et al., 1999]. Some of them (e.g., NAA, GABA,

and Glu) are localized primarily in cortical neurons and synapses, and some of the detected compounds are of glial origin (e.g., Ins and Cho). Sex and age-effects on OFC neurochemistry in relation to the state-trait anxiety have not been previously reported. Documentation of these chemical changes in relation to anxiety behavior in anxiety disorders might be interesting to explore in the future research. Previous $^1$H-MRS studies of OCD found abnormal changes of NAA in the cingulate cortex [Ebert et al., 1997], striatum [Ebert et al., 1997; Bartha et al., 1998], and medial thalamus [Fitzgerald et al., 2000]; it is well known that these regions have dense connections with OFC [Fuster, 1997; Pandya and Yeterian, 1998; Rolls, 1998]. Because NAA was found to be the strongest chemical marker of anxiety effects on OFC chemistry in our current report, future studies also need to focus on morphometric evaluation of this brain region in anxiety disorders. First steps in this direction using advanced cortical parcellation techniques have already detected structural abnormalities in some types of anxiety disorders. Specifically, we found enlarged prefrontal cortical volume in OCD patients as compared with controls [Grachev et al., 1998].

## CONCLUSIONS

The $^1$H-MRS method was shown to be sensitive to detect sex- and age-differential regional brain chemistry changes in relation to the state-trait-anxiety. The strong regional correlations between brain chemistry (NAA) and perceptual measures of anxiety provide convincing evidence that the chemistry is related to the specifics of the anxiety state. The localized $^1$H-MRS study of the brain in conjunction with the Spielberger State-Trait Anxiety Inventory can be used as a neuroimaging/behavioral tool for objective measurement of anxiety. Male subjects showed larger effect of anxiety on OFC chemistry as compared to females. Middle-aged subjects showed larger effect of anxiety on OFC chemistry as compared to younger age. In males and in middle-aged subjects, the main anxiety-related differences were in concentration of NAA (this chemical is a more specific marker for anxiety than other studied chemical compounds). The results indicate that the state-trait anxiety has sex- and age-differential patterns on OFC chemistry in healthy humans.

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