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IMMEDIATE COMMUNICATION

Anxiety in healthy humans is associated with orbital frontal chemistry

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The present study examines relationships between regional brain chemistry (as identified by localized *in vivo* three-dimensional single-voxel proton magnetic resonance spectroscopy (^{1}H-MRS) and anxiety (as measured by the State-Trait Anxiety Inventory) in 16 healthy subjects. The relative concentrations of N-Acetyl aspartate, choline, glutamate, glutamine, γ-aminobutyric acid, inositol, glucose and lactate were measured relative to creatine within six 8-cm^3 brain voxels localized to: thalamus, cingulate, insula, sensorimotor, dorsolateral prefrontal, and orbital frontal cortices (OFC) in the left hemisphere. Analysis of variance, across brain regions, chemicals, and high and low anxiety groups, showed a relationship between anxiety and chemical composition of OFC, with high anxiety subjects demonstrating 32% increase in overall chemical concentrations within OFC, as compared to the lower anxiety group ($F = 60.8$, $P < 10^{-7}$). Other brain regions, including cingulate, showed no detectable anxiety dependence. The combination of the state and trait anxiety was highly correlated with the concentration of OFC chemicals ($r^2 = 0.98$), and N-Acetyl aspartate in OFC was identified as the strongest chemical marker for anxiety (changed by 43.2% between the two anxiety groups, $F = 21.5$, $P = 0.000005$). The results provide direct evidence that the OFC chemistry is associated with anxiety in healthy humans. The method can be used as a neuroimaging/behavioral tool for documentation of OFC chemistry changes in relation to anxiety *per se* and anxiety disorders. The presented relationship between regional brain chemistry and anxiety reflects the functional/behavioral state of the brain, pointing to possible mechanisms of the neurobiology of anxiety. *Molecular Psychiatry* (2000) **5,** 482–488.

Keywords: State-Trait Anxiety Inventory; orbital frontal cortex; brain chemistry; neurotransmitters; N-Acetyl aspartate; *in vivo* proton magnetic resonance spectroscopy; analysis of variance; multiple regression analysis

Introduction

Anxiety can be characterized by harm avoidance behavior such as worrying, irritability, difficulty to relax, etc or as the propensity to interpret ambiguous situations as threatening, and is an adaptive process. Anxiety disorders, which are among the most prevalent psychiatric disorders, afflict up to 25% of the US population at some point during their life and 17% experience an anxiety disorder during a given year.[1–4] Anxiety disorders lead to significant impairment of normal life, and are often associated with great personal distress, treatment resistance and a high level of health care cost.[1,4] The neurobiology of anxiety is better understood in relation to specific diagnostic categories such as anxiety disorders. There is convincing evidence for associations between anxiety disorders and several neurotransmitter systems, including excitatory amino acid glutamate, inhibitory amino acid γ-aminobutyric acid, and other neurotransmitters and neurochemical compounds such as catecholamines, benzodiazepines, serotonin, cholecystokinin, corticotropin-releasing hormone, and somatostatin.[5–8] Functional imaging studies of the brain, including positron emission tomography, single photon emission computed tomography and functional magnetic resonance imaging have identified several brain areas linked to anxiety.[6,9] Most of these studies consistently implicate anterior limbic/paralimbic regions, such as cingulate and orbital frontal cortex, as well as amygdala and thalamus in most types of anxiety disorders. One such disorder, obsessive-compulsive disorder, has been repeatedly implicated in involving the orbital frontal cortex.[10–14] Little is known about the neurobiology of 'physiological' anxiety in normal subjects, ie the anxiety of normal everyday behavior, and there is no consensus about the primary brain regions involved in physiologic anxiety.[15,16] A few *in vivo* magnetic resonance spectroscopy (^{1}H-MRS) studies have examined brain chemistry changes in panic disorder,[17–19] and showed increases in brain lactate concentration during lactate-induced panic. The aim of this study was to

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examine relationships between regional brain chemistry (as identified by localized *in vivo* single-voxel ^{1}H-MRS) and anxiety (as measured by the State-Trait Anxiety Inventory (STAI)). We hypothesized that anxiety in healthy humans is linked with regional chemistry of the brain, specifically with OFC and cingulate cortex. These brain regions were selected based on the results of previous brain imaging studies of anxiety and anxiety disorders.[6,9–14]

Materials and methods

Subjects

Sixteen normal subjects (mean age = 46.6 ± 3.4, 12 men and four women) participated in this study. Subjects were recruited by advertisement from the local community. Initial high resolution MRI of all subjects were obtained and examined by a staff neuroradiologist to exclude brain morphologic abnormalities. Subjects with any neurological illness, head trauma, or psychiatric disorder were excluded. Most subjects were professionals within our institution, ie, nurses, doctors, and administrative personnel. 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 in vivo 3D single-voxel ^{1}H-MRS

During brain imaging the subject was placed on the scanner bed, and the whole head gradient coil positioned over the head. The subject's head was immobilized 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. In addition, local shimming was performed for each specific regional volume. All MRI and MRS experiments were performed on a 1.5 Tesla General Electric (Signa, Milwaukee, WI, USA) 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. OFC and cingulate cortex were chosen as our primary regions-of-interest, and four other brain areas (DLPFC, SMC, insula and thalamus) were used as controls. We used 8-cm^3 voxel size for each analyzed volume: OFC (2.0 × 2.0 × 2.0 cm), cingulate (5.0 × 1.6 × 1.0 cm), DLPFC (voxel size 1.7 × 3.0 × 1.6 cm), SMC (2.0 × 1.1 × 3.5 cm), insula (4.0 × 2.0 × 1.0 cm), and thalamus (3.0 × 1.8 × 1.5 cm).[20] These boundaries were first identified on the Talairach atlas[21] using the following sections for regional voxel placement: horizontal −16 mm for OFC, sagittal 5 mm for the cingulate, sagittal 47 mm for DLPFC, horizontal +60 mm for SMC, horizontal +4 mm for the insula, and horizontal +8 mm for the thalamus; and then adjusted to the individual brain's sulcal topography.[22]

Figure 1 shows the position of one regional voxel for OFC and the position of the studied chemicals. Localized proton spectra were collected using a simulated-echo acquisition mode (STEAM) sequence (probe-s PSD, TR = 1500 ms, TE = 30 ms). All spectra were transformed into a standardized scale using the Scion Image analysis package (1998, see Web site http://www.scioncorp.com). Proton spectra were analyzed by measuring heights at specified peaks. These peaks were identified by specific chemical shifts (ppm), with the investigator blinded to brain location and to subject. The relative concentrations of N-Acetyl aspartate (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).

We selected these chemicals because most of them show brain regional variations in healthy humans and these are the only chemicals which can be identified via proton MRS. NAA is the dominant peak in normal adult brain spectra. Cr spectrum is a combination of creatine and phosphocreatine. The proton Cho signal is a combination of Cho and Cho-containing compounds: Cho plasmogen, glycerophosphorylcholine, phosphorylcholine, cytedine-diphosphate-choline, acetylcholine, and phosphatidylcholine.[23] 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.[24] Reliability of the chemical measurements, which was estimated as the coefficient of variation (CV = the standard deviation expressed as a percentage of the mean) for each studied chemical, was conducted using five repeat scans of one brain region on one normal subject, and was generally excellent: NAA/Cr = 4.5%, Cho/Cr = 5.0%, GABA/Cr = 6.8%, Glc/Cr = 4.4%, Ins/Cr = 4.0%, Lac/Cr = 7.5%, Glu/Cr = 7.5%, and Gln/Cr = 5.3%, in the OFC.

Analysis for other brain regions also shows similar excellent reliability (CV ranged from 4–8%). These results were consistent with the data presented by Simmons *et al*,[25] who measured chemical ratios from same-sized voxels, prescribed in the occipital lobe, and repeated five times on eight normal subjects. The reliability study performed on the same subjects over 3 months only slightly worsened the results.[25] We used the ratios method relative to Cr because the effect of subject on total Cr peak and on each regional Cr peak height was non-significant ($F = 0.35$, $P = 0.99$). However, the CV determined for each regional Cr across all studied subjects showed regional variations: Cr in

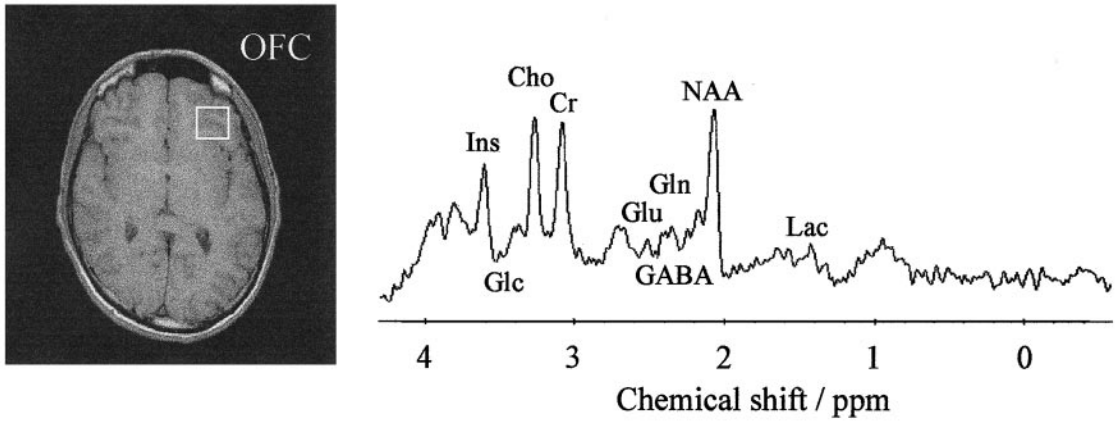

Figure 1 Measurement of chemical concentrations in the left OFC using *in vivo* single-voxel ^{1}H-MRS. This figure illustrates the location of 8-cm^3 voxel in the left OFC of a normal subject. A typical *in vivo* ^{1}H-MRS spectrum obtained from the left OFC showing localization of three major peaks for N-Acetyl aspartate (NAA, 2.02 ppm), creatine/phosphocreatine complex (Cr, 3 ppm), choline (Cho, 3.2 ppm), and smaller peaks for 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). Chemical shifts are indicated in parts per million (ppm).

insula = 4.9%, thalamus = 6.9%, DLPFC = 7.8%, cingulate = 8.1%, SMC = 8.2%, and OFC = 8.9%.

The State-Trait Anxiety Inventory[26]
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 (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 from 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.[26] These anxiety measures were performed just minutes before brain imaging.

Statistical analysis
Distribution of original data for goodness of fit was assessed using Kolmogorov–Smirnov and Chi-Square tests (Statistica, Tulsa, OK, USA). Anxiety group (high anxiety (total STAI score = 70–100) *vs* lower anxiety (total STAI score = 40–69)), 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 3-way ANOVA (2 anxiety groups × 6 brain regions × 9 chemicals), anxiety group, brain region, chemical, and subject were used as explanatory variables. Descriptive planned comparisons were made using F tests to explain observed differences across factors and their interactions. Predictive value of the state-trait anxiety on chemical concentration was analyzed using multiple linear regression (STATISTICA).

Results

The mean STAI scores for normal subjects were as follows: state anxiety = 30.44 ± 8.88, trait-anxiety = 33.63 ± 9.00, and total anxiety = 64.06 ± 17.48. These scores were not different from published results for healthy people and were much lower than published data for anxiety disorders (state anxiety = 49.02 ± 11.62 and trait anxiety = 48.08 ± 10.65).[26] In this study we consider subjects with total STAI score between 40 and 69 as low anxiety (eight subjects) and those with total STAI score above 70 as high anxiety (eight subjects).

The original data for chemical concentrations were distributed normally (Kolmogorov–Smirnov D = 0.07, $P < 0.01$; and Chi-Square $\chi^2 = 60.8$, $P < 10^{-7}$). Differences in concentration of brain chemicals between anxiety groups, across brain regions and chemicals, were quantified using a 3-way ANOVA. A 2 (anxiety group (high *vs* lower anxiety)) × 6 (brain region) × 9 (chemical) measures ANOVA, with relative concentration defined as the dependent variable, revealed multivariate main effects for anxiety group (F (1,504) = 15.6, $P < 0.0001$), brain region (F (5,504) = 42.0, $P < 10^{-7}$), chemical (F (8, 504) = 220.2, $P < 10^{-7}$), the anxiety group × brain region interaction (F (5,504) = 12.1, $P < 10^{-7}$), and the brain region × chemical interaction (F (40,504) = 1.7, $P < 0.01$).

Since the main effects of brain region, chemical and their interaction on chemical concentration have been shown on another group of normal subjects,[20] in this study we describe the effects of anxiety and anxiety × brain region interaction using descriptive F tests. The mean concentration over all studied chemicals and brain regions in high anxiety subjects was 3.7% higher as compared with lower anxiety ($F = 15.6$, $P < 0.0001$). Anxiety-related differences in chemical concentration were found only in one brain region, the OFC ($F = 60.8$, $P < 10^{-7}$). The mean concentration in

OFC for all studied chemicals in high anxiety subjects was 32% higher as compared with lower anxiety (mean ± SEM = 1.18 ± 0.07 for high anxiety *vs* mean = 0.90 ± 0.09 for low anxiety; Figure 2a). Other brain regions, including cingulate cortex, showed no anxiety-dependent significant differences.

Because the strongest regional effect of anxiety was detected in the OFC, we used post-hoc analysis to examine which chemical concentration changes can explain observed differences in the anxiety group × brain region interaction. In the OFC of high anxiety normal subjects, increased chemical concentrations were observed for NAA by 43.2% (mean = 1.69 ± 0.04 for high anxiety *vs* mean = 1.18 ± 0.05 for low anxiety, $F = 21.5$, $P = 0.000005$), GABA by 33.8% (mean = 0.87 ± 0.03 for high anxiety *vs* mean = 0.65 ± 0.03 for low anxiety, $F = 3.8$, $P < 0.05$), GABA + Glu by 26% (mean = 1.60 ± 0.06 for high anxiety *vs* mean = 1.27 ± 0.06 for low anxiety, $F = 9.0$, $P < 0.003$), Gln by 47.4% (mean = 1.12 ± 0.03 for high anxiety *vs* 0.76 ± 0.03 for low anxiety, $F = 10.5$, $P = 0.001$), Glc by 34.8% (mean = 1.20 ± 0.13 for high anxiety *vs* mean = 0.89 ± 0.06 for low anxiety, $F = 7.8$, $P = 0.005$), and Ins by 44.8% (mean = 1.39 ± 0.01 for high anxiety *vs* mean = 0.96 ± 0.06 for low anxiety, $F = 15.4$, $P = 0.0001$), as compared to lower anxiety subjects (Figure 2b).

We used multiple linear regression analysis to predict relationships between concentration of OFC chemicals and the behavioral measurements for anxiety (STAI forms Y-1 and Y-2). Highly significant empirical relationships were seen between two anxiety predictors and total concentration of OFC chemicals. The combination of the state and trait anxiety predicts the concentration of OFC chemicals ($r^2 = 0.98$, $F(2,78) = 5.4$, $P < 0.006$). We used the combination of both types of anxiety as one parameter since the state anxiety highly correlated with trait anxiety ($r = 0.90$; Figure 3). Post-hoc analysis revealed that two OFC chemicals: NAA and Gln, which showed the largest differences in concentration between high *vs* lower anxiety groups, were strongly correlated with total anxiety score ($r = 0.64$ for NAA and $r = 0.62$ for Gln, $P < 0.05$; Figure 4). Overall, these results suggest the presence of specific relationships between the regional chemicals in OFC and anxiety behavior in healthy humans.

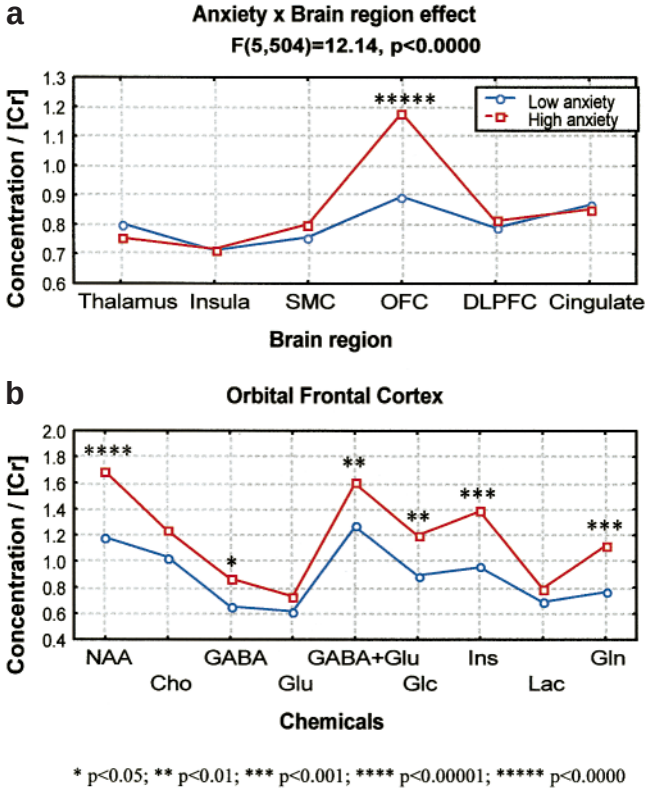

Figure 2 (a) Effects of anxiety × brain region interaction on the total chemical concentration across brain regions in healthy subjects. Total chemical concentration is increased in OFC of high anxiety subjects, as compared to lower anxiety subjects. (b) Chemical concentration changes in the OFC of high anxiety *vs* low anxiety subjects, across the studied chemicals. Increased chemical concentrations are observed in OFC for most chemicals in high anxiety subjects, as compared to lower anxiety subjects.

Discussion

In this report we provide direct evidence for regional brain chemical changes in OFC between high and low anxiety healthy humans, and demonstrate significant empirical relationships between OFC chemical concentration and the state-trait anxiety. This is a first report in the field of anxiety neuroscience that demonstrates a chemical-behavioral network in the brain, specifically in OFC, as a possible mechanism for development of anxiety. This study is of interest not only in relation to the issue of where anxiety is represented in the brain, but also in relation to what neurotransmitters and other chemicals in what brain regions are associated with anxiety. These findings need to be tested on different types of anxiety disorders, and for the right hemispheric regions as well. In case of replication, the results may be useful in the documentation of anxiety level, and possibly for diagnosis of anxiety disorders. Future development of more effective pharmacological

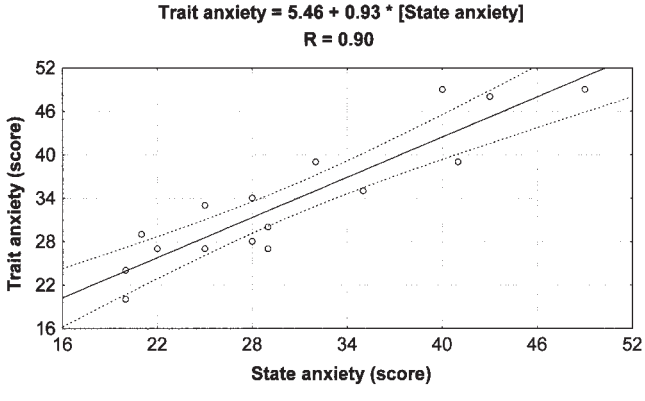

Figure 3 Correlation between state (S-STAI) and trait anxiety (T-STAI) scores. The two measures of anxiety are highly interrelated in normal subjects.

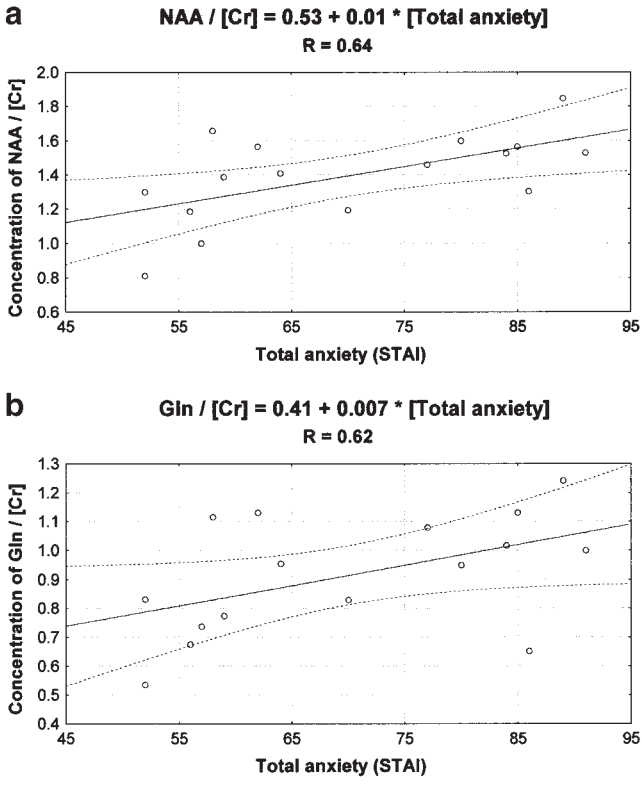

Figure 4 Regression plots between levels of NAA/Cr in OFC and total anxiety scores (a), and between levels of Gln/Cr in OFC and total anxiety scores (b) in healthy subjects. These two regional chemicals strongly correlated with total anxiety score as compared to the other chemicals studied in the same region ($r = 0.64$ for NAA and $r = 0.62$ for Gln, $P < 0.05$).

interventions for anxiety relief may be another valuable application of these findings.

Although the evidence for OFC as a primary area of anxiety neurochemistry has been shown in this report, numerous previous functional imaging studies of distinct anxiety disorders in conjunction with symptom provocation paradigms consistently implicate anterior limbic/paralimbic regions, including both OFC and cingulate.[13,14,27–29] We had hypothesized that the cingulate should be linked with anxiety. It is not clear why we do not observe this region's involvement in anxiety. A number of technical reasons may explain the negative result. It might be due to: (1) the low sensitivity of ^{1}H-MRS for this region due proximity to the corpus callosum and the interhemispheric sulcus; resonance effects of fiber tracts and CSF, these effects can be reduced by using higher magnetic field scanners; (2) studying only left hemispheric regions; right cingulate chemicals might be more related to anxiety; (3) studying only the middle portion of cingulate area; the more anterior portion is considered an affective area and implied in a variety of emotional tests; (4) studying only healthy subjects; different types of anxiety disorders, mostly studied using functional imaging methods, show a more complex neural circuitry in the brain; (5) the limitations of Spielberger STAI. It is also possible that the cingulate is not a specific area for anxiety since multiple cognitive tasks activate this region, including tasks targeting attention, affect and pain.[30–33] The cingulate cortex may be involved in more general non-specific attentional states, including a readiness to escape from a threatening situation. The role of OFC may be more specifically linked to anxiety since this area of the brain is involved in decision-making behavior, including ambiguous situations of personal threat. The evidence for involvement of OFC in decision-making processes has been presented.[34–37]

It is noteworthy that the spontaneous experience of physiologic anxiety (as measured by the Spielberger STAI) in a natural environment is different from test-induced anxiety in experimental settings, and anxiety-provoking environments might induce much higher inter-individual variability as compared to normal life. Also, it is still not clear whether the induced anxiety states have validity for the actual everyday life anxiety since different induction paradigms might activate multiple cognitive-affective brain areas, evidence of which we observe in the literature.

The growing biochemical literature supports the idea that pathologic anxiety states are associated with alterations of multiple neurotransmitter systems,[5–8] and, similarly, numerous functional imaging studies of the brain have implicated the involvement of multiple regions in the pathophysiology of anxiety disorders.[6,9] We do not discuss these studies here in detail as they have been well reviewed recently. It should be pointed out here that OFC is one of the anxiety-related regions, which has been demonstrated fairly consistently in obsessive-compulsive disorder.[10–14] In patients with panic disorder a deficit of GABA receptors has been shown in several brain areas, including OFC.[38] Decreased GABA-receptor clustering resulted in enhanced anxiety, which was demonstrated in an animal model.[39] Our post-hoc analysis examined what chemicals in OFC were mostly changed in relation to anxiety, and identified multiple neurotransmitters and other chemicals: NAA (as a precursor of a neurotransmitter N-acetyl-aspartyl-glutamate, which after breakdown produces excitatory neurotransmitter aspartate; this chemical was changed the most); GABA (as an inhibitory neurotransmitter); Gln (as a precursor of the excitatory neurotransmitter glutamate); Glc (as an energy substrate); and Ins (as a second messenger that liberates Ca^{2+} from the endoplasmic reticulum and is involved in recognition of chemical signals). Most of these chemicals are localized within axonal and neuronal compartments reflecting neuronal functional-synaptic properties,[40–44] and are involved in the interrelated cascade of metabolic reactions that give rise to specific brain functions. Anxiety 'stresses' this region with neurotransmitters and other chemical increases. From this viewpoint, the OFC chemical changes most likely reflect the changes in the chemical network related to anxiety behavior due to neuronal reorganization. When the number of axons and synaptic connections across anxiety-related regions increase (ie, sprouting), the concentration of NAA and other chemicals also might be increased. The conclusion that anxi-

ety may be associated with neuronal reorganization needs to be re-examined with advanced morphometric analysis (concentration of NAA and other chemicals might be directly related to the neuronal/axonal number and cortical volume). Using morphometric analysis, we found enlarged prefrontal cortical volume in one type of anxiety disorder, OCD,[45] which is consistent with the proposed mechanism.

In summary, N-Acetyl aspartate in OFC was identified as a main chemical marker for anxiety (this chemical showed highest specificity). A correspondence between OFC neurochemistry and the state-trait anxiety has not been previously reported in healthy subjects and in anxiety disorders, which open a new avenue in psychiatry research. The approach described here underlines the neurobiology of anxiety and can be used as a neuroimaging/behavioral tool for documentation of OFC chemistry changes in relation to physiologic and pathologic anxiety.

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