Chronic stress is linked to 5-HT$_{1A}$ receptor changes and functional disintegration of the limbic networks

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Abstract

There are increasing reports about stress related cognitive and psychic declines in subjects who have no psychiatric premorbidity, depression, or major life trauma. Yet, little is known about the underlying neurobiology. Based on the typical symptomatology, fMRI data suggesting that stress activates the limbic system, and animal data showing a major involvement of the 5-HT$_{1A}$ receptor in stress regulation, we hypothesized that enduring daily stress causes widespread limbic dysfunctions, and specific changes of the 5-HT$_{1A}$ receptor.

To test these hypotheses combined PET studies were carried out in 16 chronically stressed, and 16 non-stressed subjects. Limbic function was tested by measuring cerebral blood flow during rest, and when using an odor activation paradigm. 5-HT$_{1A}$ receptor binding potential (BP) was assessed with [11C]WAY100635. All subjects went through a battery of neuropsychological tests. Stressed subjects showed a functional disconnection between the amygdala and ACC/medial prefrontal cortex (mPFC), and an impaired odor activation of the ACC. They also displayed a reduced 5-HT$_{1A}$ receptor BP in the anterior cingulate (ACC), the insular-cortex, and the hippocampus. Their performance in attention-, odor discrimination-, and semantic memory tasks was impaired, and correlated with the BP-values in the respective region. The degree of reported stress was inversely correlated with activation of ACC, and the 5-HT$_{1A}$ receptor BP in the amygdala and hippocampus.

Enduring every day psychosocial stress seems to be associated with a limbic reduction of 5-HT$_{1A}$ receptor binding and functional disintegration of ACC/mPFC. These changes support the notion of an impaired top-down regulation of stress stimuli, and identify potential targets for early treatment.

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Introduction

Western societies are facing increasing reports of stress related illness among otherwise healthy, and high performing individuals (Ahola et al., 2006; Copertaro et al., 2007; Fernandez Torres et al., 2006; Rydmark et al., 2006). The symptoms of these persons are generally stereotypical, characterized by memory and concentration problems, sleeplessness, diffuse aches, fatigue, irritability, and anxiety, and often attributed to occupational stress (Maslach et al., 2001). Similar to post traumatic stress disorder (PTSD) the underlying pathophysiology has been described as a disintegration of the hypothalamic–pituitary–adrenal (HPA) axis (McEwen, 2007), based on findings of reduced cortisol- and ACTH-response to the Corticotropin Releasing Hormone after dexametason pretreatment (Rydmark et al., 2006; Wahlberg et al., 2009). The exact mechanisms behind this disintegration are, however, not clarified.

Indications for an involvement of the limbic system in stress

The pathophysiology of cortisol homeostasis is extensively documented in the literature. Abundant experimental data indicates that the regulation of the HPA axis is mediated by certain limbic structures (the hippocampus, the amygdala and the anterior prefrontal cortex), (McEwen, 2007; Pruessner et al., 2009). These data and the specific symptoms (the emotional and cognitive decline) reported among patients suffering from chronic psychosocial stress make it plausible to assume that a prominent and sustained limbic involvement is an important component of the neurobiology of chronic stress in humans. Targeted investigations of limbic function and neurochemistry among the affected individuals are, therefore, warranted. Efforts in this direction have recently been made by fMRI studies of healthy subjects. The generated data show that acute stress affects the anterior cingulate (ACC), the medial prefrontal cortex (mPFC), the amygdala (Dedovic et al., 2009; Goldstein et al., 2010) and the hippocampus (Dedovic et al., 2009), with some regional differences depending of the participant’s sex (Goldstein et al., 2010) and degree of stress response (Pruessner et al., 2008).
Of note are also studies of patients with PTSD, which show a disturbed interaction between the amygdala and mPFC with an exaggerated amygdala response along with a reduced mPFC response to consciously perceived fearful face stimuli in relation to controls (Koenigs and Grafman, 2009; Shin et al., 2005). This may be an effect of dysfunctional or damaged medial prefrontal neurons (Koenigs and Grafman, 2009) which in normal circumstances would modulate amygdala activity by a top-down regulation of neuronal excitability in the basolateral nucleus amygdale via glutaminergic efferents on GABAergic interneurons (Roozendaal et al., 2004; Rosenkranz and Grace, 2002).

The findings from the aforementioned fMRI studies highlight the expected interaction between stress and the limbic networks. However, this interaction was shown in healthy controls or subjects who have been exposed to life threatening trauma, and may therefore not be relevant for the condition developed in response to perceived chronic psychosocial stress without negative major life events. Furthermore, activations in the aforementioned studies were attained using stress-triggering stimuli, which do not probe for the enduring limbic dysfunctions that are likely to underlie the described symptoms, which may remain long after the stress triggers have been removed. If limbic dysfunctions can also be demonstrated with neutral stimuli, important information may be gained for better understanding of the rapidly increasing phenomenon of long-lasting cognitive and psychic decline attributed to stress without life threatening trauma.

**Indications for an involvement of the serotonin system in stress**

Certain symptoms in subjects suffering from chronic psychosocial stress, like anxiety and irritability, suggest a particular affection of the serotonin system. Numerous animal data show that this system is highly involved in the regulation of the HPA. Of special interest here are the 5-HT1A receptors. These receptors are highly expressed in the human limbic brain (Drevets et al., 1999; Savic et al., 2004), and seem to be an important player in the HPA modulation; Stimulation of hippocampal 5-HT1A receptors is shown to enhance hippocampal expression of the glucocorticoid receptors (McAllister-Williams et al., 1998), whose function is to mediate cortisol feed-back via inhibitory projections to the CRF releasing paraventricular nuclei (Jankord and Herman, 2008). Stimulation of the 5-HT1A receptors in the hypothalamus, on the other hand, triggers an increase in CRF and ACTHrelease. Stress impinges on the interplay between the 5-HT1A receptors and the HPA system by causing a desensitization of the presynaptic 5-HT1A autoreceptors (Lanfumey et al., 1999). A study of male tree shrews has shown that stress also induces widespread reductions of postsynaptic 5-HT1A receptors in the limbic system (Flugge, 1995). Furthermore, hippocampal reductions of postsynaptic 5-HT1A receptors have been detected in response to both prolonged chronic stress, and severe acute stress in rats (Lopez et al., 1999). Also of interest is the effects of single-prolonged stress on glucocorticoid receptors and CRF can be partially inhibited by pretreatment with a 5-HT1A receptor antagonist (Wang et al., 2009). Whether similar changes occur in humans is presently unknown.

Taking into consideration all these data we posited that the limbic networks are dysfunctional in humans who are experiencing long-term psychosocial stress, and that this dysfunction is present and measurable also when no stress stimuli are employed. This hypothesis was tested in three separate types of measurements with positron emission tomography (PET). The studies incorporated 16 patients who reported anxiety, emotional exhaustion, physical fatigue, and cognitive weariness due to prolonged exposure to work-related stressors, and 16 healthy controls, who did not have a history of chronic stress.

Limbic function was probed using odor stimuli during PET measurements of cerebral blood flow with [15O]-H2O; this approach was deemed suitable since odor stimuli specifically activate the limbic structures involved in stress, without being perceived as stressful, (Clumias et al., 2008; Savic et al., 2000). Given that the amygdala is the principle relay for the perception and valence of stress (Koenigs and Grafman, 2009), that stress seems to interfere with the amygdala and the mPFC (Goldstein et al., 2010; Priuressner et al., 2008) and that functional connections between these two structures are important for emotional regulation, we predicted that these particular connections would be disintegrated in the affected patients. The study, therefore, also included an analysis of functional connectivity from the amygdala based on separate measurements of rCBF during rest. Finally, we assumed that major changes would occur in the 5-HT1A receptor binding and carried out PET measurements of the of 5-HT1A receptor binding potential (BP), using the [11C] labeled 5-HT1A receptor antagonist, WAY100635, as PET tracer.

**Materials and methods**

**Subjects**

Sixteen right-handed (Oldfield, 1971) non-smoking patients, (eleven women, mean age 38 ± 5, range 28–47 years, education 15 ± 2 years), who had been diagnosed as having had a ‘reaction to severe stress, and adjustment disorder’ according to the International Classification of Diseases (ICD-10, F43), were recruited from the Stress Research Institute, Stockholm University. In order to compose a study group with homogenous etiology and to reduce variability, we selected only subjects who attributed their illness to prolonged work-related stress, after 60–70 working hours/week continuously over several years before symptom onset. Inclusion criteria consisted of a characteristic symptom course with sleeplessness, diffuse aches, palpitations and fatigue, a subsequent onset of irritability, anxiety, memory and concentration problems, and reduced work capacity (confirmed by the employers) in previously high performing individuals (Rydmark et al., 2006; Sandstrom et al., 2005). All the subjects attributed their symptoms to chronic stress, and had no other known etiology for their distress.

Further requirements consisted of ≥50% sick leave for stress related symptoms during a minimum of 6 months before entering the study, and an average stress-burnout score of ≥3.0 on the Maslach Stress-Burnout Inventory — General Survey (MBI-GS), (Schaufeli and Van Dierendonck, 1995). This 7-point rating scale, ranging from 0 (never) to 6 (daily), consists of three subscales: exhaustion (five items), cynicism (five items) and lack of professional efficacy (six items). The included patients were also assessed with the Shirom-Melamed Burnout Questionnaire (SMBM) (Melamed et al., 1999), since it not only tests exhaustion, but also screens for perceived attention, tension, memory and anxiety problems. MBI-GS and SMBM scorings are highly correlated (Grossi et al., 2003). When rating perceived stress, subjects were asked to take into consideration the last six months, and not only the actual time-point. The average scores for our patients were 4.1 ± 1.0, range 3.0–6.0 for (MBI-GS), and 4.7 ± 1.2, range 3.0–6.3 (SMBM), whereas mean scores found in Scandinavian populations are around 2 for MBI-GS, and 3 for SMBM, (Ahola et al., 2006; Stenlund et al., 2007). The average perceived stress exposure among patients was 4.5 ± 1.2 years.

Subjects were excluded if they had heredity or previous history of psychosis, personality disorder, major or bipolar depression, alcohol or substance abuse, chronic fatigue, chronic pain, fibromyalgia, neurological, or endocrine disease. Subjects that had experienced prominent stress factors in private life or a major traumatic life event were also excluded. Heredity was defined as having a history of psychiatric illnesses among first and second degree’s blood relatives. Data about family history and psychiatric disorders were based on patients’ self reports. No ongoing daily medication was allowed during two months prior to the study, except contraceptives. There
were no reports of early traumas, or sexual abuse. An additional exclusion criterion was upper respiratory problems.

Sixteen healthy, right-handed, non-smoking, volunteers, (eleven females, age 34±9, range 24–52 years, education 15±1 years), with no history of chronic stress, or heredity for neuropsychiatric disorders were used as a control group. Stress scores were in controls evaluated only with SMBM. The study was approved by the Ethics and Radiosafety Committees at the Karolinska Institute, and written informed consent was received from each participant. All female participants had regular menstruations, and were investigated during the second week of menstrual cycle.

Before the interview, participants completed questionnaires in order to evaluate their stress symptoms and assess their previous life events (Deykin et al., 2001). In addition, the occurrence of major life events among the subjects was assessed through a clinical psychiatric interview based on the non-work related items of the Holmes and Rahe Scale (Holmes and Rahe, 1967). The participants were asked to answer a yes or no to whether they have experienced any non-work related stressful life events (e.g., death of a relative or spouse, recent divorce, and forced family relocation). Subjects were excluded if they answered positively to having experienced such an event in their lives. They also received a medical screening, (physical examination, test of thyroid and liver function). The possible presence of psychiatric disorders or personality disturbances were assessed according to the Diagnostic and Statistical Manual of the American Psychiatric Association, 4th Edition (DSM-IV) by a specially trained psychiatrist (HJ), and included a Structured Questionnaire for DSM-IV® Axes I and II (Structured Clinical Interview for DSM-IV® (SCID-I, and II) (American Psychiatric Publishing Inc, Arlington, 1997), along with a test for depression using the Montgomery–Asberg Depression scale (Montgomery et al., 1978).

MRI

Structural images were acquired according to a previously described protocol (Ciumas and Savic, 2006) with a 1.5 Tesla Sigma S/X scanner, (General Electric, Milwaukee, Wisconsin), and included 3D-weighted T1 SPGR images with 1-mm sections, used for measures of hippocampal volume, and the whole brain volumes [sum of the grey, white matter volume and cerebrospinal fluid, segmented with the SPM2 software package (Wellcome Department of Cognitive Neurology, London, http://www.filion.ucl.ac.uk/spm)].

5-HT<sub>1A</sub> receptor binding potential

The binding potential (BP) to the 5-HT<sub>1A</sub> receptor was investigated using PET images acquired with an ECAT Exact HR 47 scanner (CTI/ Siemens, Knoxville, TN) run in 3D mode (transaxial resolution of 3.8 mm), after bolus injection (190–267 MBq, 800–2000 mCi/cc) of [C]WAY100635. Radioactivity in the brain was measured in a series of 15 consecutive frames for 63 min, of which the nine first frames were acquired over 15 min. Image processing included co-registration of MRI and sum PET images (representing the decay corrected average uptake of [C]WAY100635 during 15 to 63 min after ligand injection), and re-slicing of PET images to avoid spatial mismatch between the two modalities (SPM2). Three-dimensional volumes of interest (VOIs) were then delineated on original individual MR images and transferred to the corresponding individual PET images (first sum image, and dynamic image). The VOI analysis was preferred to an interest (VOIs) were then delineated on original individual MR images of MRI and sum PET images (representing the decay corrected average of 15 consecutive frames for 63 min, of which the nine first frames were calculated with random effect analyses, at T-threshold (AUC)/injected radioactivity, (two-tailed t-test, p<0.05). Because BP varies considerably between various cerebral areas separate analyses of variance (ANOVA) were used for each VOI, factoring for subject group (p<0.005 with Bonferroni correction). Histogram analysis showed that the BP data were distributed normally with short tails.

Odor activations

The procedure was as described in several of our previous studies (Savic, 2002; Savic et al., 2000). In summary, regional cerebral blood flow (rCBF) was measured using PET and [15O]H<sub>2</sub>O during baseline (denoted BL), consisting of rest with closed eyes, plugged ears, and passive breathing of the unscented environmental air, and activation (denoted AO) characterized by passive, birhinal, smelling of four odors, [butanol (10% dilution in distilled water), and undiluted cedar oil, lavender oil and eugenol). According to our previous studies these odors are perceived as rather neutral with respect to pleasantness, irritability, intensity and familiarity (Savic et al., 2000), and yield reproducible activations of the amygdala, piriform cortex, and portions of the anterior insular and cingulate cortices (Savic, 2002; Savic et al., 2001).

During odor smelling, each of the four odorants was presented consecutively, during 15 s and with an interval of 5 s of breathing the air in the scanner room. There were six 60 s PET scans per person, (three scans/condition, balanced and randomly interleaved). Subjects were informed that they would smell either odor or unscented air while an open and empty, or odor containing jar, was presented 10 mm under the nose. They were instructed to relax, with eyes closed and ears plugged, and breathe without sniffing or hyperventilating (which they practiced twice before the PET sessions), and refrain from active thinking or judging the odorants. Respiratory movements were recorded continuously during each scan, by using a strain gauge around the lower thorax connected to a graph (Comair, Stockholm). After the scanning sessions, odors were presented again for ratings of subjective perception of odor characteristics (Supplemental information).

Within group activations (OO–BL), and deactivations (BL–OO) and between group differences (controls–patients and patients–controls) were calculated with random effect analyses, at T-threshold corresponding to P=0.01, minimum cluster size 0.8 cm<sup>2</sup>, at a corrected P<0.05. With this method the material of the present size is regarded sufficient to generate inference at group level, implying that each individual was representative for his/her designated group (Mechelli et al., 2002).

Functional connectivity

As opposed to analyses of odor activation and BP, which included both men and women, the calculation of connectivity was restricted...
to heterosexual females (eleven patients, age 20–42 years; eleven controls, age 20–39 years). The underlying rationale was the previously demonstrated sex difference among healthy controls (Kilpatrick et al., 2006; Savic and Lindstrom, 2008). Functional connectivity was calculated only during baseline, and defined operationally as the extent to which normalized rCBF in selected seed volumes of interest (VOIs) co-varied with voxel-based rCBF values across the investigated subjects using SPM 2 as described previously (Ciumas et al., 2008; Savic and Lindstrom, 2008). The seed regions, delineated at T1 images of the SPM2 program, consisted of the right and left amygdala, (6 mm sphere, with the centre 16 –4 –16, and –16, –4 –16 expressed in Talairach’s co-ordinates). We predicted that the connections detected previously in healthy females, (to the contra lateral amygdala, and ACC), would be impaired in patients. Significant covariations were calculated for each group in a first level fixed-effects analysis (the height threshold was P = 0.01 uncorrected, P<0.05 corrected), using the entire brain as search space. Group differences in the predicted regions were calculated at T-threshold for the peak activation corresponding to P = 0.01, P<0.05 uncorrected, (multi subject condition and covariate analysis within SPM2); P<0.05 corrected was used for other regions, and for analyses of within group co-variations.

Evaluation of cognitive functions and odor psychophysics

A condensed battery of neuropsychological tests was employed to investigate if the patients’ subjective cognitive impairments were objectively verifiable. The battery took into account major cognitive domains (attention, working memory and verbal memory), and consisted of Trail Making Test, part B (TMT-B); digits span tests, a three-back verbal working memory test, and a test of verbal encoding and retrieval. We also assessed odor threshold to make sure that possible abnormalities in odor activation were not caused by impaired odor detection. Finally, odor quality discrimination was evaluated because this test has been shown to reveal limbic dysfunction (Jones-Gotman and Zatorre, 1988; Savic et al., 1997). The neuropsychological test battery is described in the Supplemental web information. Significant group differences in performance were tested using repeated measures analysis of variance (ANOVA), with study groups as the independent variable. This was followed by separate one-way ANOVAs for the specific tasks (p<0.05). Simple regression (p>0.05) was used to test the relation between regional BP, and performance in those tests where there was a difference between patients and controls.

Results

The average SMBM stress score was 4.7 ± 1.2, (range 3.0–6.3) among the patients, and 1.8 ± 0.6 (range 1.0–2.6) among controls. None of the subjects showed Axis I- or II-disorders; two patients were among the patients, and 1.8±0.6 (range 1.0–2.6) among controls.

Table 1

Demographics and neuropsychological test data.

<table>
<thead>
<tr>
<th>Region</th>
<th>Patients (n = 16)</th>
<th>Controls (n = 16)</th>
<th>p-value</th>
<th>F-value, df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>38 ± 5</td>
<td>34 ± 9</td>
<td>0.06</td>
<td>F = 4.00, 1</td>
</tr>
<tr>
<td>Education</td>
<td>15.4 ± 1.5</td>
<td>15.3 ± 1.4</td>
<td>0.42</td>
<td>F = 0.03, 1</td>
</tr>
<tr>
<td>MADRAS</td>
<td>11.3 ± 6.3 a</td>
<td>4.1 ± 3.9</td>
<td>0.0006</td>
<td>F = 14.6, 1</td>
</tr>
<tr>
<td>MBI-GS</td>
<td>4.5 ± 1.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SMBM</td>
<td>4.7 ± 1.2</td>
<td>1.8 ± 0.6</td>
<td>&lt;0.0001</td>
<td>F = 37.4, 1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>281 ± 110</td>
<td>150 ± 90</td>
<td>0.0015</td>
<td>F = 12.7, 1</td>
</tr>
<tr>
<td>TMT-B (s)</td>
<td>43.6 ± 16.2</td>
<td>40.0 ± 18.5</td>
<td>0.03</td>
<td>F = 0.23, 1</td>
</tr>
<tr>
<td>Three-back</td>
<td>132.5 ± 5.9</td>
<td>132.2 ± 5.4</td>
<td>0.09</td>
<td>F = 0.02, 1</td>
</tr>
<tr>
<td>Digits span</td>
<td>120.0 ± 17.1</td>
<td>170.0 ± 31.1</td>
<td>0.0001</td>
<td>F = 24.5, 1</td>
</tr>
<tr>
<td>Claeson Dahl (CDSS)</td>
<td>65.8 ± 37.2</td>
<td>27.0 ± 22.0</td>
<td>0.0014</td>
<td>F = 12.7, 1</td>
</tr>
<tr>
<td>OD threshold</td>
<td>6.8 ± 1.6</td>
<td>7.4 ± 2.3</td>
<td>0.014</td>
<td>F = 0.9, 1</td>
</tr>
<tr>
<td>OD discrimination</td>
<td>10.2 ± 2.0</td>
<td>13.5 ± 0.8</td>
<td>0.0001</td>
<td>F = 24.4, 1</td>
</tr>
</tbody>
</table>

Table 2

5-HT1A receptor binding potential.

<table>
<thead>
<tr>
<th>Region</th>
<th>Patients (n = 16)</th>
<th>Controls (n = 16)</th>
<th>Values are given as mean ± SD.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdala</td>
<td>4.5 ± 1.2</td>
<td>5.6 ± 1.5</td>
<td>BA 10 = Brodmann Area 10.</td>
</tr>
<tr>
<td>Anterior Cingulate</td>
<td>3.8 ± 0.8</td>
<td>5.1 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Dorsolateral prefrontal cortex</td>
<td>3.3 ± 0.7</td>
<td>3.9 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>5.3 ± 1.2 b</td>
<td>7.3 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Insular cortex</td>
<td>4.8 ± 0.9 c</td>
<td>6.3 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>3.5 ± 0.5</td>
<td>4.2 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Temporal cortex</td>
<td>4.2 ± 0.6</td>
<td>4.9 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Orbitofrontal cortex</td>
<td>2.9 ± 0.7</td>
<td>3.8 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>Raphe nuclei</td>
<td>3.1 ± 1.3</td>
<td>3.6 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>BA10</td>
<td>3.4 ± 0.9</td>
<td>4.3 ± 1.1</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as mean ± SD. BA 10 = Brodmann Area 10. a, b, and c indicate significant differences (after Z normalization). a: p = 0.0004, F = 16.0, df (1) = 0.0008, F = 14.2. b: p = 0.0015, F = 12.7, df (1) = 0.0022, F = 11.6. c: p = 0.0009, F = 3.7, df (1) = 0.0022, F = 11.5. The corresponding p-values when using MADRAS as covariate were: p = 0.0006 (anterior cingulate), p = 0.0013 (hippocampus), and p = 0.0013 (insula cortex). No significant interaction with MADRAS was observed.
deactivations (BL–OO) of the parieto-occipital cortex (patients–controls). Group comparisons revealed significant clusters in the ACC when using the control–patient contrast, and in the occipito-parietal cortex when reversing the contrast (patient–control). The two groups had similar odor ratings (p=0.3, F=1.4, df=1), and showed similar respiratory amplitude X frequency index both during odor activation and the baseline condition (p=0.22, F=1.22, df=1), (Supplementary web information, Tables S2 and S3). Odor thresholds were within the normal range in both groups (Table 1).

**Functional connectivity during the baseline condition**

Only female controls and female patients were included in this analysis. In female controls, the baseline rCBF in each seed ROI (right and left amygdala) co-varied with the contra lateral amygdala, the ACC, parts of Brodmann Area (BA)10, and the middle temporal gyrus. Female patients, on the other hand, showed co-variation with the contra lateral amygdala, but not with the frontal lobe structures, and direct group comparisons revealed significant difference in a cluster covering the ACC and BA10 (Table 4, Fig. 2).

**Neuropsychological tests**

Neuropsychological tests showed an overall group interaction (p<0.027, F=5.6) where patients had lower performance in three tests: verbal memory (p=0.0014, F=12.7, df=1), digits span (p<0.0001, F=24.5, df=1), and odor discrimination (p<0.0001,
The indicated regions describe coverage of the respective cluster. R = right; L = left; NS = not significant. *Covers the anterior cingulate cortex. Inf = infinite.

$F=24.4$ df = 1). (Table 1). The three-back working memory test showed ceiling effects in both groups (other tests did not).

**Post hoc analyses**

To investigate whether the degree of perceived stress was related to the detected changes, linear regression analysis was carried out between the summed scores on the (SMBM) and the 5-HT receptor BP in the affected regions. Even though the 5-HT$_{1A}$ receptor BP was not significantly reduced in the amygdala, the correlation analysis also included this region, because of its key role in stress perception. The odor activation study showed impaired activation in the ACC in patients. To test whether this impairment was related to stress an odor activation study showed impaired activation in the ACC in patients. To test whether this impairment was related to stress a linear correlation analysis was carried out between the SMBM scores and the individual beta scores in the ACC cluster generated by the control−patient contrast. This calculation was restricted to control−patient contrast in OO−BL; these scores reflected the individual degree of activation. This calculation was restricted to patients in order to avoid the risk of creating a false correlation by entering a healthy population clustering at one end of the spectrum and a patient population at the other end. SMBM scores were in patients inversely correlated with the 5-HT$_{1A}$ receptor BP in the amygdala ($r=-0.77$, $p=0.0001$) and hippocampus ($r=-0.59$, $p=0.049$), and with the ACC activation ($r=-0.54$, $p=0.046$), Fig. 3. The inverse correlation between SMBM and BP was restricted to these two regions.

Next, we tested whether the observed limbic changes were functionally relevant. Since the choice of limbic region and behavioral test in our post hoc analysis was based on the difference between stressed and non-stressed individuals we carried out hypothesis based tests. Data exclusively from the patient group were included in a simple regression model. Based on previous information we assumed that hippocampal 5-HT$_{1A}$ receptor BP would be related to semantic memory (van der Veen et al., 2006), that insular BP would be related to odor discrimination performance (Savic, 2002), and that both 5-HT$_{1A}$ receptor BP and the degree of odor activation in ACC would be related to attention performance. The weighted score from the verbal memory test (higher score more impairment) was negatively correlated with the hippocampal BP ($r=-0.57$, $p=0.02$); odor discrimination scores were positively correlated with insular BP ($r=0.61$, $p=0.016$), whereas scores in digits span test showed a positive correlation with the 5-HT$_{1A}$ receptor BP in the ACC ($r=0.68$, $p=0.004$); (Table S4, Fig. 3).

Finally, since 5-HT$_{1A}$ receptor BP, as well as in odor activation was reduced in ACC we wondered whether these changes were interrelated. To investigate this, the patients’ beta scores from the ACC cluster generated by the control−patient contrast with regard to OO−BL were plotted against the corresponding BP-values. A positive correlation ($r=0.60$, $p=0.027$) was found.

The significance level for all the regression analyses was 0.05.

**Discussion**

To the best of our knowledge, this is the first brain imaging study of subjects reporting enduring daily psychosocial stress that cannot be attributed to psychiatric premorbidity, a major psychic trauma or a major negative life event. The results from three separate types of PET measurements coalesced to show that changes specifically occur in the limbic networks that process memory, attention, and emotion. These changes were, in contrast to many other studies of chronic stress, detected during the processing of stimuli not designed to be stressful. The changes seem to reflect a consistent malfunction. Their functional relevance was indicated by the correlation between 5-HT$_{1A}$ receptor BP, and the three neuropsychological tests in which patients underperformed, (Fig. 3). Furthermore, the 5-HT$_{1A}$ receptor BP in the amygdala and hippocampus as well as the degree of activation in ACC was found to be inversely correlated with the degree of perceived stress (Fig. 3). These findings are consistent with the symptoms described in patients suffering from chronic psychosocial stress, they suggest an interaction between stress and the 5-HT$_{1A}$ receptor, as reported in several animal experiments, and expand the information about the psychopathology of chronic stress in humans. The major findings in the present study are illustrated in Fig. 4.
**Potential underlying mechanisms**

The reductions in receptor binding measured with PET may, theoretically, reflect changes in receptor density or affinity, or receptor down regulation, internalization or destruction, or blockage by the endogenous ligand [even though $^{11}$C WAY100635 seems insensitive to endogenous levels of serotonin (Rabiner et al., 2002)]. Therefore, any statement concerning the precise mechanisms underlying the present 5-HT$_{1A}$ receptor BP reductions must be speculative. The pattern of these reductions was remarkably similar to the pattern of previously reported stress-induced reductions of 5-HT$_{1A}$ receptor binding sites in male shrews (detected in the hippocampus, cingulate, and insular cortex, but not in the amygdala and the raphe nuclei) (Flugge, 1995). One possibility is that these changes, in both animals and humans, could be an effect of high cortisol levels (Magarinos and McEwen, 1995; McEwen, 1997). More extensive cortisol measurements were beyond the scope of this study, which focused on limbic functions and the integrity of 5-HT$_{1A}$ receptors, and not on the HPA. It should be mentioned, in any case, that the existence of a strong connection between cortisol levels and serotonin receptors is not undisputable. Isolated hypercortisolaemia without stress is found to be accompanied by decreased as well as normal 5-HT$_{1A}$ receptor binding (Gemert, 2006; Montgomery et al., 2001; Porter et al., 1998). Moreover, corticosteroid administration to controls and subjects...

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**Fig. 2.** Connectivity pattern from the left (a) and right (b) amygdala VOI in patients and controls. Co-variation clusters are superimposed on the MRI of a standard brain. The figure illustrates functional disconnection between the amygdala and anterior cingulate/BA10 in patients. Co-ordinates for the images: coronal Y = −7; horizontal Z = −16; sagittal x = −4. Sokoloff’s color scale illustrates T values, reflecting the degree of co-variation. The cluster in the left amygdala in patients (Fig. 3b) was not significant. As same brain sections are shown in both groups the figures do not always illustrate maximal activation.
remitted from depression showed no effect on $5\text{-HT}_{1A}$ receptor binding, nor did antiglucocorticoid treatment change the $5\text{-HT}_{1A}$ function in depressed patients and healthy subjects (Montgomery et al., 2001; Price et al., 1997). Also noteworthy is that patients with chronic stress symptoms frequently display low or normal cortisol levels (Gill et al., 2008; Rydmark et al., 2006), whereas reports of high concentrations are more unusual among these patients (Melamed et al., 1999). Consequently, it is just as likely or perhaps more likely that the observed receptor changes are indicative of an ongoing epiphenomenon involving an extensive chain of events related to chronic stress rather than being a simple function of cortisol levels and HPA.

Support for this view is given by the observed functional disintegration between the amygdala and prefrontal cortex, and the correlation between odor activation and $5\text{-HT}_{1A}$ BP in the ACC. During normal conditions, GABAergic and serotonergic inhibitory inputs via the mPFC counteract excitation of the amygdala. Absence of prefrontal inhibition causes amygdala hyperactivity (Roozendaal et al., 2004), and provides a context for an imbalance between excitation and inhibition of the limbic networks. Interestingly, several animal studies of chronic stress show an elevation of extracellular glutamate levels in the hippocampus and the medial prefrontal cortex, as well as a retraction of dendritic spines (Hunter et al., 2009; Iijima

Fig. 3. Correlation analyses. Upper raw: Correlation between cognitive performance (horizontal axis) and $5\text{-HT}_{1A}$ BP (vertical axis). Cognitive performance is expressed in scores for the respective test. Some subjects had overlapping values. Lower raw: Correlation between perceived stress (expressed in SMBM scores), $5\text{-HT}_{1A}$ BP in the hippocampus, and amygdala, and between SMBM and degree of activation (expressed in beta-values) in the ACC.

Fig. 4. Limbic dysfunctions associated with chronic psychosocial stress. Schematic presentation of the present findings in subjects reporting enduring daily psychosocial stress; results from three separate types of PET measurements (odor activation, functional connectivity and receptor binding). Neuropsychological tests with impaired performance are shown and the observed relationships between individual regional $5\text{-HT}_{1A}$ binding potential (BP) and the score in the functionally corresponding test are also presented. Together, these results show that changes in this patient group specifically occur in the limbic networks that process memory, attention, and emotion.
et al., 2007; Lowy et al., 1993; Magarinos and McEwen, 1995; McEwen et al., 1997). One possibility is, therefore, that excitoexcitotoxic mechanisms may be involved. Repeated stress stimuli could, thus, trigger a vicious cycle in which neuronal hyperexcitation is propagated from the amygdala to its primary limbic connections where it causes excitotoxic lesions and genomic changes. Such a chain of events is compatible with the observed reductions in 5-HT₁A receptor binding, the reduced baseline connectivity between the amygdala and the ACC and mPFC, and the inability of our patients to activate the ACC during odor perception. It is also congruent with the observed correlation between regional 5-HT₁A receptor BP and cognitive functions, and the inverse BP correlation with the perceived stress load. That the 5-HT₁A receptor BP and degree of activation were positively correlated in ACC further supports the notion that an excitotoxic lesion may be involved (since the 5-HT₁A receptor mediates inhibition, a reduction in 5-HT₁A receptor BP, occurring in conjunction with otherwise normal ACC functioning, would facilitate rather than hamper ACC activation). This line of thought is also supported by the observed functional disintegration between the amygdala and the prefrontal cortex.

The question of whether the present findings simply reflect depression is worth discussing. We find this unlikely, not only since the results did not change when the calculations were restricted to those subjects with normal MADRAS scores, but also because the BP was similar among patients with normal and elevated scores, and no interaction with MADRAS was found. According to a recent meta analysis, normal, reduced, and elevated 5-HT₁A receptor bindings have all been found in depressed subjects (Smith and Jakobsen, 2009). Furthermore, when detected, the changes do not seem to be restricted to the limbic structures (Dreyvet al., 1999; Sargent et al., 2000). Emotional reactions to chronic stress and major depression may, thus, represent separate constructs. They share, however, certain symptoms, perhaps due to the affection of similar limbic networks, and the higher MADRAS scores among the four patients who were not diagnosed as depressed could be an effect of this co-morbidity. Given that depression can be triggered by stress, it is also possible that these subjects were developing depression, which was at a subclinical level at the time.

Patients showed more pronounced deactivations than controls. Preussner et al. made a similar observation in fMRI/PET studies of provoked stress, albeit in different brain regions and during different experimental conditions (Preussner et al., 2008). Whether this indicates that the default brain state is altered during stress, as indicated by one study of PTSD (Lanius et al., 2010), is currently unknown.

Several methodological issues deserve comment. One concerns the fact that amygdala connectivity was only analyzed in women. As a consequence, the present data on functional integrity are not directly applicable to male patients. Amygdala connections have been previously found to be sex differentiated (Kilpatrick et al., 2006; Savic and Lindstrom, 2008), which precluded investigations of gender-diminished populations. To investigate male and female patients separately, was not feasible, as only five male patients participated in the study. Based on previous observations (Savic and Lindstrom, 2008; Wang et al., 2007), we assumed that women were particularly prone to developing limbic changes in response to chronic stress, and therefore decided to limit this first analysis of functional connectivity to females. Whether the previously reported sex differences in respect to the functional connections emanating from the amygdala are reflected by sex differences in respect to the effects of chronic psychosocial stress on the brain is an important issue which should be addressed separately in future studies. The study groups for measurements of 5-HT₁A receptor BP and odor activation were gender matched.

Under baseline conditions, the participants were cued to breathe unscented air from a glass jar. This has the advantage of minimizing variations caused by spontaneous reflections, but may itself constitute a task. However, our purpose was not to evaluate the true “resting” state condition, but to compare the functional connectivity of patients and controls when no stressful, emotional, or cognitive tasks were involved. With this design, the present data add to the recent observations of task-related prefrontal disconnections after stress exposure (Liston et al., 2009), (Liston et al., 2009), and supports the possibility that disturbances in the medial prefrontal cortex may be a more general and enduring feature of stress.

The mean values of 5-HT₁A receptor BP were lower in several regions, but significant reduction was detected only in the hippocampus, the insular cortex and the ACC. These limbic regions have inherently the highest 5-HT₁A receptor density in the brain. To exclude a possible statistical flaw implying a better chance to reach statistical significance in a high-density region, we carried out a post hoc test, in which the measured BP-values were separately z-normalized for each included brain region. Entering the z-transformed values into the model resulted in slightly altered F and p-values (Table 2), but without changing the fact that significant reductions in the 5-HT₁A receptor BP were detected only in the hippocampus, the insular and anterior cingulate cortex. The reasons underlying the generally slightly lower BP found among the patients can only be speculated upon. The AUC in the reference region were similar among the two groups, and therefore does not help to explain the situation. Similar findings have been reported in partial epilepsy – another condition that exhibits pronounced affection in certain target regions (Savic et al., 2004; Giovacchini et al., 2005; Didelot et al., 2008). One possibility is that the homeostasis of the serotonin system may be disturbed, which may be secondary to the regional changes in 5-HT₁A receptor binding.

Finally, the absence of a group-difference in amygdala 5-HT₁A receptor BP was unexpected. One tentative explanation is that it may be due to the high variation in BP-values. This is a problem that is inherent to the PET method, as the scanner resolution does not allow the analysis of separate amygdala nuclei, which is needed when studying the 5-HT₁A receptors that are normally expressed only in the magnocellular basal nucleus amygdalae (Flugge, 1995). It is equally possible, however, that stress affects the amygdala differently than it does other limbic structures, as suggested by Flugge et al. (Flugge, 1995) and by the finding that stress shortens spine synapses in the hippocampal neurons, but leads to their increases in the amygdala (Rainnie et al., 2004).

Due to its cross-sectional design, the study is not able to distinguish between cause and effect. The congruence with animal data which shows that chronic stress causes 5-HT₁A receptor reduction (Flugge, 1995), and the correlation found in the present study between the degree of perceived stress and reductions in BP and ACC activation favor, however, the possibility of stress effects. By investigating the 5-HT system of subjects who are experiencing chronic psychosocial stress, but who do not have a particular psychosocial trauma in their history, the data of the present study expands upon the data from previous animal studies as well as human PTSD data, and synthesizes the available knowledge into an expanded model of how chronic psychosocial stress may affect our brains. It implies that special attention should pay to limbic protection and to the reestablishment of top-down regulation in order to prevent future stress hazards.

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