Functional MRI to assess alterations of functional networks in response to pharmacological or genetic manipulations of the serotonergic system in mice

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A B S T R A C T
Imaging methods that enable the investigation of functional networks both in human and animal brain provide important insights into mechanisms underlying pathologies including psychiatric disorders. Since the serotonergic receptor 1A (5-HT1A-R) has been strongly implicated in the pathophysiology of depressive and anxiety disorders, as well as in the action of antidepressants drugs, we investigated brain connectivity related to the 5-HT1A-R system by use of pharmacological functional magnetic resonance imaging in mice. We characterized functional connectivity elicited by activation of 5-HT1A-R and investigated how pharmacological and genetic manipulations of its function may modulate the evoked connectivity. Functional connectivity elicited by administration of the 5-HT1A-R agonist 8-OH-DPAT can be described by networks characterized by small-world attributes with nodes displaying highly concerted response patterns. Circuits identified comprised the brain structures known to be involved in stress-related disorders (e.g. prefrontal cortex, amygdala and hippocampus). The results also highlight the dorsomedial thalamus, a structure associated with fear processing, as a hub of the 5-HT1A-R functional network. Administration of a specific 5-HT1A-R antagonist or use of heterozygous 5-HT1A-R knockout mice significantly reduced functional connectivity elicited by 8-OH-DPAT. Whole brain functional connectivity analysis constitutes an attractive tool to characterize impairments in neurotransmission and the efficacy of pharmacological treatment in a comprehensive manner.

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Introduction
Affective and stress-related disorders (e.g. general anxiety disorders), constitute one of the most proliferating health problems worldwide (Murray and Lopez, 1997). Central to the pathophysiology of stress-related disorders is a dysfunction of serotonin (5-HT) neurotransmission. Yet, current medication (SSRIs are generally recommended as first line agents for anxiety disorders) suffers from weak drug efficacy when compared to placebo, high prevalence of relapse and delayed onset of therapeutic effects (Pyce and Seifritz, 2011). Regarding the last point, it has been hypothesized that negative feedback circuitries, mediated via serotonergic 1A receptors (5-HT1A-R), limit the acute SSRI-induced enhancements in serotonergic neurotransmission. Besides that role in dynamic modulation of serotonergic activity the 5-HT1A-R has been shown to be down-regulated or desensitized by acute stress or administration of corticosteroids and partial or full 5-HT1A-R agonists have shown anxiolytic properties. Subsequent pharmacological, neuroreceptor imaging and genetic studies have further increased evidence of a central role of the 5-HT1A-R in stress-related disorders but on the same time illustrated the complexity of the 5-HT1A-R signaling system. Also the exact interplay between an impaired 5-HT1A-R transmission and its associated neural networks (Cools et al., 2008; Drago et al., 2011; Geyer and Vollenweider, 2008; Puig and Gulledge, 2011) is only fractionally understood: the current limitation to our understanding of the etiology of stress-related disorders and improved treatment lies in the complex dynamics of the numerous reported neurotransmitter systems and circuitries that interact with each other.

By providing non-invasive readouts with spatial and temporal attractive resolutions, functional magnetic resonance imaging (fMRI)
has evolved as a powerful tool for translational research studies (Van der Linden et al., 2009). fMRI may provide important information on aspects underlying the pathophysiology or the pharmacological intervention. For example, compared to the temporal characteristic of a drug induced response, the high tempo-spatial resolution feature of fMRI enables the monitoring of local changes in activity contrast induced at a high rate. The comparison of temporal profiles recorded from different regions of interest might yield information on functional synchronicity of different brain areas in relation to the stimulus (Ramnani et al., 2004; Rogers et al., 2007). Connectivity analyses have been performed in both human and animal studies by use of electroencephalogram (EEG) recordings as well as by positron emission tomography (PET) techniques (Gerstein and Perkel, 1969; Horwitz et al., 1984). However, as compared to fMRI, EEG mapping is compromised by poor spatial resolution. Despite superior sensitivity, functional PET measurements, e.g. assessing glucose utilization are limited by poor temporal resolution preventing the analysis of temporal correlation. Alternatively PET can be used to probe for difference in receptor concentration between healthy volunteers and patients, important information characterizing a disease state. Nevertheless such studies do not provide information on functional networks. Thus, fMRI represents an exciting approach for studying fundamental aspects of psychiatric diseases through investigation of functional networks during resting state or elicited by a pharmacological challenge.

There are currently two methodological approaches to study functional connectivity. The first class is data-driven methods and attempts to map connectivity on a voxel-by-voxel basis throughout the brain (Friston et al., 1993). Such methods are bias-free and suited for identification of hitherto unknown networks e.g. when analyzing the effects of novel CNS active compounds. This approach can be for instance illustrated by recent pharmacological fMRI studies performed in rat by Schwarz and co-workers (Schwarz et al., 2007a,b,c, 2008, 2009). By using a functional connectivity method based on seed-voxel correlation mapping and modularity approach, the authors showed that fluoxetine-evoked stimulation of the serotonergic system (through inhibition of serotonin reuptake Stahl, 1998) induced high correlations among well-known structures of serotonergic signaling (Schwarz et al., 2007b). A second group of analysis methods is based on the use of prior knowledge or hypotheses to constrain the analysis to a restricted set of brain regions. This approach is suited to pharmacological fMRI studies (pfMRI). As compared to spontaneous fluctuations of brain activity at resting state, acute pharmacological challenge can elicit robust and reliable activations making possible the correlation between tempo-spatial profiles observed in distinct areas of the brain (Bifone et al., 2010). To date, a few pfMRI studies in rats regarding connectivity related to global dopaminergic (Schwarz et al., 2007a,b) and serotonergic (Schwarz et al., 2007b) systems, as well as specifically to dopaminergic D3 receptor (Schwarz et al., 2007c) mediated signaling have been published.

In order to better understand the complex functional interactions of the 5-HT1A-R with other brain systems implicated in serotonin-related psychopathologies, we investigated brain functional connectivity related to this receptor system by use of pfMRI in normal C57Bl6 mice and in a 5-HT1A-R knock-out (KO) mouse strain which exhibits an anxiety-related phenotype (Toth, 2003). Most preclinical pfMRI studies targeting the 5-HT system have been carried out in the rat focusing on the 5-HT2C receptor using the 5-HT2C receptor agonist metoclopramide (m-CPP) or the 5-HT1A-R agonist 8-OH-DPAT SB269970, respectively (Canese et al., 2011; Gozzi et al., 2010a).

In a previous pfMRI study we have demonstrated the feasibility of mapping region-specific and dose-dependent changes in serotonergic neurotransmission in the mouse following stimulation with the 5-HT1A-R agonist 8-OH-DPAT (Mueggler et al., 2011); the response pattern observed after injection of a receptor agonist represents a combination of hemodynamic changes at the primary site of action of the drug and downstream effects in other parts of the circuit. In this study we used cross-correlation analysis among a predefined set of regions throughout the brain to elucidate the functional connectivities within the circuitry elicited by activation of 5-HT1A receptors. Combining 8-OH-DPAT induced activation with a second 5-HT1A-R specific readout would significantly enhance the specificity of the approach. Therefore, in a second step, the effects of modulation of 5-HT1A-R signaling were evaluated by analyzing the 8-OH-DPAT response in normal mice that were co-injected with the selective 5-HT1A-R inhibitor WAY-100635 (Fletcher et al., 1996). This drug has been shown to lead to a significant modulation of the CBV response to 8-OH-DPAT: the CBV decrease elicited by 8-OH-DPAT was reversed by subsequent or co-administration of the 5-HT antagonist WAY100635. (Mueggler et al., 2011; Scanley et al., 2001). Also the extension of the paradigm to the 5-HT1A-R (heterozygous KO) line allows testing for specificity.

Materials and methods

Animals

All experiments were carried out in strict adherence to the Swiss Law for Animal Protection, approved by the veterinary office of the canton of Zurich, Switzerland. Male C57/Bl6 mice (3–4 months of age) of 22–35 g body weight have been used. Animals were housed under a normal light/dark cycle (12:12 h) with standard rodent chow and tap water ad libitum. Heterozygous 5-HT1A-R knockout mice lacking the 5-HT1A-R and corresponding wild-type littermates (wt) were obtained from EMBL, Monterotondo, Italy (courtesy C. Gross, Gross et al., 2002; Ramboz et al., 1998). Genotyping was performed using standard protocol and primers for 5HT1A-R (XZAL 5′–CAG TCT CTA GAT CCC CTC CCT T (common primer), XZTL 5′–AAG GGC AAA AGT GAC GAT TAT CTT G, HTR1A 5′–GGG CTT CTT GTT CAT CAC GTA G).

Animal preparation and imaging

The experimental procedures have been described earlier (Mueggler et al., 2011). In brief, animals were anesthetized with isoflurane® (Abbott, Cham, Switzerland), endotracheally intubated, placed on a cradle made from Plexiglas comprising warm water circulation and artificially ventilated according to body temperature was kept constant at 36.5 ± 0.5 °C. Ear bars secured reproducible positioning and reduced motion artifacts. The tail vein was cannulated with a 30 G needle (0.3 mm × 13 mm, BD Microlance, Drogheda, Ireland) for drug and contrast agent administration. Animals received a single i.v. injection of gallamine triethiodide (15 mg/kg) to avoid motion artifacts.

Experiments were performed using a Pharmascan MR system 47/16 (Bruker BioSpin GmbH, Ettlingen, Germany) equipped with a 200 MHz cryogenic transceiver RF probe. Prior to fMRI, anatomical images were recorded using a multi-slice RARE spin echo sequence (8 slices). The fMRI protocol consisted of a RARE sequence with a field-of-view FOV = 20 × 13 mm2, matrix dimension MD = 200 × 130, slice thickness STH = 0.7 mm, and inter-slice distance ISD = 1.2 mm, resulting in nominal voxel dimensions of 100 × 100 × 700μm3, repetition time TR = 900 ms, echo spacing TE = 10.4 ms, effective echo time TE_eff = 22 ms, and RARE factor 5. The fMRI protocol consisted of a RARE sequence with a spatial resolution of 156 × 156 × 700μm3. Eight slices were recorded to cover the forebrain of the mouse. Images were T2-weighted with a repetition delay of TR = 2500 ms and an echo delay of TE_eff = 81.2 ms, and a RARE factor of 8, resulting in a temporal resolution of 40 ms/multislice data set. At first, 8 pre-contrast images $S_{pre}^i$ were acquired prior to the i.v. administration of the contrast agent (Endorem®, 55 mg Fe/kg). After 15 min, to allow the contrast...
agent to reach steady-state concentration in plasma, 86 sequential post-contrast images were acquired with drug administration following image 35. The average of images 5 to 35 yielded the baseline image \( \overline{S}_0 \) with the subsequent images \( S(t) \) reflecting the effect of the drug at time \( t \).

Functional connectivity analysis were performed in five groups of mice: wt mice receiving saline injection \((n = 4)\), wt mice receiving an administration of the 5-HT\(_{1A}\)-R agonist 8-OH-DPAT \((n = 6)\), wt mice receiving an administration of 8-OH-DPAT immediately followed by administration of the potent 5-HT\(_{1A}\)-R antagonist WAY-100635 at a dose of 0.54 mg/kg \((n = 7)\) (Mueggler et al., 2011; Scalley et al., 2001), and for analysis of the effects of receptor knock-out heterozygous 5-HT\(_{1A}\)-R KO mice receiving 8-OH-DPAT \((N = 9)\) and the corresponding wt animals receiving 8-OH-DPAT \((n = 10)\). 8-OH-DPAT was administered at a dose of 0.1 mg/kg and WAY-100635 at a dose of 0.54 mg/kg, respectively.

**CBVrel changes analysis**

Quantitative analysis of drug induced cerebral blood volume (CBV) changes was carried out using Biomap (Novartis Institute for Biomedical Research, M. Rausch). Prior to the analysis of the CBV response due to drug administration, data were detrended to account for signal changes due to the washout of the contrast agent. This was carried out for each region-of-interest (ROI) individually as baseline CBV values are region-specific. Data were detrended to account for the clearance of the contrast agent. For detrending the last intensity of the 5 data points prior to drug administration and the last 5 data points of the serial data acquisition (31 to 33 min following drug administration) was set to 0 ± 5%. This is justified given duration of 8-OH-DPAT \((0.1 \text{ mg/kg i.v.) elicited effects of typically 15 to 20 min} \) (Mueggler et al., 2011; Scalley et al., 2001). Following detrending changes in CBV relative to baseline values \( \Delta \text{CBV}_{\text{rel}} \) were computed for selected ROIs according to:

\[
\Delta \text{CBV}_{\text{rel}}(t) = \frac{\ln(S(t)/\overline{S}_0)}{\ln(\overline{S}_0/\overline{S}_{\text{pre}})} \cdot 100
\]

In total 31 ROIs were defined across the brain including structures a priori based on their involvement in the serotonergic systems but also control regions expressing only a low density of 5-HT\(_{1A}\)-R. All ROIs were manually drawn for each mouse and are listed in Fig. 1. Since the analysis did not reveal any evident difference of effect between right and left sides of the bilateral ROIs, both sides were merged.

**Functional connectivity analysis and matrices generation**

Functional connectivity (FC) patterns derived from neuroimaging data may be represented as networks, with anatomically-defined structures representing the nodes, and a measure of correlation between the responses in each pair of nodes determining the edges.

Cross-correlation (CC) is a measure of similarity of two waveforms, in our case the hemodynamic responses to drug stimulation within and between structures were depicted for the full post-injection interval of the temporal profiles (i.e. 0–33 min, \( t = 0 \) being the time point of drug administration) using one-way ANOVA (Statview5, SAS Institute Inc., Cary, NC). Drug treatment and genotype effects on connectivity within and between structures were tested by comparing CC coefficients for the full post-injection interval of the temporal profiles using one-way ANOVA. Time effect on connectivity was tested by comparing CC coefficients for the selected intervals using one-way ANOVA. To improve results readability, when subregions of anatomical structures displayed homogenous responses/connectivities, they were regrouped together (e.g. cortical areas ROIs-1-8). For grouped ROIs, comparisons were performed on coefficients average. Group ‘zero value’ consisted in 1 sample \((n = 1)\). Results are expressed as mean ± SEM. Detailed \( F \) and \( p \) values are presented in supplementary results (Supplementary Tables 1, 2 and 3).

**Results**

**Serotonergic challenge**

Administration of 0.1 mg/kg of the 5-HT\(_{1A}\)-R agonist 8-OH-DPAT in wt mice induced signal changes in brain areas known to express a high level of 5-HT\(_{1A}\) receptors such as cortex (CX), hippocampus (HC) and amygdala (AMG). Analysis of the functional connectivity \((r_{ij})\) revealed functional community structures, i.e. strong correlations within defined anatomical structures (hubs) as well as connection between these hubs (Figs. 2a, d). The connectivity diagram obtained using \( r_{ij} \geq 0.70 \) revealed a network comprising the key structures involved in serotonergic signaling (Fig. 3b). Hubs of the evoked network included CX, AMG, HC, dorsomedial thalamus (dMT) and periaqueductal gray (PAG). Activation of the serotonergic signaling evolves over time associated to the local concentration

\[
\Delta \text{CBV}_{\text{rel}}(t) = \frac{\ln(S(t)/\overline{S}_0)}{\ln(\overline{S}_0/\overline{S}_{\text{pre}})} \cdot 100
\]

\[r_{ij} = \frac{\sum_{k=0}^{N-1} (\Delta \text{CBV}_{\text{rel}}(k \cdot \Delta t) - \Delta \text{CBV}_{\text{rel}}(z_j)) \cdot (\Delta \text{CBV}_{\text{rel}}(k \cdot \Delta t) - \Delta \text{CBV}_{\text{rel}}(z_j))}{\sum_{k=0}^{N-1} (\Delta \text{CBV}_{\text{rel}}(k \cdot \Delta t) - \Delta \text{CBV}_{\text{rel}}(z_j))^2}
\]

using Matlab (The Mathworks Inc, Natick, USA). \( \Delta \text{CBV}_{\text{rel}}(k \cdot \Delta t) \) represents the relative CBV change in the ROIs \( i \) and \( j \), respectively, at sampling point \( k \) of a data set comprising \( N \) sampling points,
of the 5-HT_{1A}-R agonist 8-OH-DPAT. Correspondingly, the amplitude of the ΔCBV_{ij}(t) response changed over time (Figs. 1, 2). In accordance we also observed a temporal evolution of serotonergic evoked functional connectivity (Fig. 4): following agonist administration, r_{ij} values increased until reaching a maximum response at a delay of approximately 10 min after injection followed by a decrease. While the r_{ij} values changed over time, the general appearance of the correlation matrix, i.e. the functional community structures, remained unchanged. The FC matrix prior to 8-OH-DPAT administration and at the end of the experiment (20-24 min) looked remarkably similar, displaying high r_{ij} values only in among cortical structures.

Overall, administration of saline solution did not significantly change ΔCBV_{ij}(t) values (Figs. 2b, d, Supplementary Table 1). The correlation analysis revealed a community structure comprising the cortical areas as well as a weak connectivity pattern across other brain structures similar to the pattern at baseline (Figs. 2a, 4a). When compared to the response to 8-OH-DPAT, the average strength of the FC induced by saline administration was significantly weaker (Figs. 2d, 3a, Supplementary Table 1). Analysis of FC within ROIs of the somatosensory CX (ROIs 3–5) revealed a strong saline evoked FC response, similar to the one evoked by 8-OH-DPAT (Figs. 2a, d).

Pharmacological vs. genetic manipulation of 5-HT_{1A}R function

Modulation of the agonist evoked fMRI response to 5HT_{1A}R mediated activity was studied following the simultaneous administration of a selective receptor antagonist or by using heterozygous KO mice. Co-injection of the 5HT_{1A}-R antagonist WAY-100635 led to reduced ΔCBV_{ij}(t) amplitudes and to significantly smaller r_{ij} values as compared to administration of the agonist alone (Figs. 5a-c, 6, Supplementary Table 3). Analysis of the FC matrix revealed that intracortical connectivities were largely preserved, while the connectivity to other serotonergic hubs was compromised. Except for a more pronounced functional disconnection between cortical areas, similar changes have been observed in the response of heterozygous 5-HT_{1A}-R KO mice to administration of 8-OH-DPAT (Figs. 5d-f).

Nevertheless, comparing the effects of genetic down-regulation of the 5-HT_{1A}-R with those of pharmacological receptor deactivation, dissimilarity has been found. Both pharmacological and genetic inhibition of 5-HT_{1A}-R function induced a major reduction of serotonergic-evoked FCs between CX areas (including prefrontal, retrosplenial and entorhinal cortices) and HC (mainly CA1 and dentate gyrus, DG). Similarly, we found reduced r_{ij} values between dmTH and both CX and HC.
characteristic features are a high level of local clustering among nodes of the networks combined with relatively short paths linking the nodes of the network. Characteristic features are a high level of local clustering among nodes of the networks combined with relatively short paths linking the nodes of the network.

Discussion

Characterization of functional networks evoked by 5HT1A-R mediated activity

In agreement with a recent report on the characterization of brain connectivity in mice at resting state (Jonckers et al., 2011), a connectivity pattern involving cortical areas has been observed both during baseline and after injection of saline, which might reflect the resting state of the mouse brain during isoflurane anesthesia. Obviously, we cannot exclude effects from auditory inputs elicited by periodic gradients switching on the FC observed in cerebral CX. In addition to cortical structures, low frequency BOLD fluctuations have also been reported for thalamus (TH) and hippocampus (HC) in a resting state fMRI study performed in rats anesthetized with isoflurane (Kannurpatti et al., 2008). Yet, FCs within and in-between those regions and CX were weak and negligible compared to those elicited by the administration of the serotonergic drug.

Administration of 5-HT1A-R agonist exhibited a functional network characterized by high clustering with nodes such as CX, HC, AMG, and PAG, possibly reflecting the underlying structural organization of anatomical connections (Chuang et al., 2011). The network identified in mice revealed “small-world” network attributes that characterize neural networks and are associated with high local efficiency of information transfer and robustness (Bullmore and Sporns, 2009): characteristic features are a high level of local clustering among nodes of the networks combined with relatively short paths linking the nodes of the network.

CX, HC, AMG and PAG are brain regions known to express high levels of 5-HT1A-R (Bockaert et al., 2006; Hoyer et al., 1986; Laporte (CA2 and CA3) as well as between HC and hypothalamus (HT). In addition, pharmacological receptor inhibition specifically affected the functional interactions of AMG with CX and HC, as well as between HC and HT. In contrast, genetic manipulation induced a specific reduction of the FC between CX and HT. Significant common changes to both manipulations are summarized in Fig. 6a while specific changes are presented in Fig. 6b.
(a) Connectivity elicited by administration of 5-HT1A-R agonist 8-OH-DPAT. All changes in connectivity values indicated were statistically significant. Gray areas represent nodes which correspond to anatomical structures. Gross structures are indicated in blue text, subareas in black text. Color-coded arrows indicate edges with individual r values mentioned next to the arrows, with r representing absolute CC coefficients and Δr = r(5-HT1A-R agonist) − r(saline). Connectivities are displayed for r > 0.7. Brain structures displaying only weaker correlation but were found to be involved in connectivity changes induced by antagonist treatment or in heterogeneous 5-HT1A-R KO animals are indicated in light gray. (a) Connectivity elicited by administration of 5-HT1A-R agonist 8-OH-DPAT. All changes in connectivity values indicated were statistically significantly different from the respective saline values (p < 0.001, except ***p < 0.0001). Only values r exceeding a threshold of r = 0.7 are displayed. (b) For saline, the threshold criteria r > 0.7 was only fulfilled for intra-cortical connectivities (see Fig. 2). For comparison values for connectivities identified after 8-OH-DPAT administration are given if the exceed a threshold of r = 0.2. Apart from intracortical ones, none of the connectivities observed after saline administration was found to be statistically significantly different from zero (n.s. p < 0.5).

![Graphical representation of functional connectivity in brain of wt mice elicited by administration of saline or 5-HT1A-R agonist 8-OH-DPAT. Gray areas represent nodes which correspond to anatomical structures. Gross structures are indicated in blue text, subareas in black text. Color-coded arrows indicate edges with individual r values mentioned next to the arrows, with r representing absolute CC coefficients and Δr = r(5-HT1A-R agonist) − r(saline). Connectivities are displayed for r > 0.7. Brain structures displaying only weaker correlation but were found to be involved in connectivity changes induced by antagonist treatment or in heterogeneous 5-HT1A-R KO animals are indicated in light gray. (a) Connectivity elicited by administration of 5-HT1A-R agonist 8-OH-DPAT. All changes in connectivity values indicated were statistically significantly different from the respective saline values (p < 0.001, except ***p < 0.0001). Only values r exceeding a threshold of r = 0.7 are displayed. (b) For saline, the threshold criteria r > 0.7 was only fulfilled for intra-cortical connectivities (see Fig. 2). For comparison values for connectivities identified after 8-OH-DPAT administration are given if the exceed a threshold of r = 0.2. Apart from intracortical ones, none of the connectivities observed after saline administration was found to be statistically significantly different from zero (n.s. p < 0.5).](image)

Fig. 3. Graphical representation of functional connectivity in brain of wt mice elicited by administration of saline or 5-HT1A-R agonist 8-OH-DPAT. Gray areas represent nodes which correspond to anatomical structures. Gross structures are indicated in blue text, subareas in black text. Color-coded arrows indicate edges with individual r values mentioned next to the arrows, with r representing absolute CC coefficients and Δr = r(5-HT1A-R agonist) − r(saline). Connectivities are displayed for r > 0.7. Brain structures displaying only weaker correlation but were found to be involved in connectivity changes induced by antagonist treatment or in heterogeneous 5-HT1A-R KO animals are indicated in light gray. (a) Connectivity elicited by administration of 5-HT1A-R agonist 8-OH-DPAT. All changes in connectivity values indicated were statistically significantly different from the respective saline values (p < 0.001, except ***p < 0.0001). Only values r exceeding a threshold of r = 0.7 are displayed. (b) For saline, the threshold criteria r > 0.7 was only fulfilled for intra-cortical connectivities (see Fig. 2). For comparison values for connectivities identified after 8-OH-DPAT administration are given if the exceed a threshold of r = 0.2. Apart from intracortical ones, none of the connectivities observed after saline administration was found to be statistically significantly different from zero (n.s. p < 0.5).

![Evolution of CC matrix as function of time. CC matrices for various time windows following injection of 8-OH-DPAT: 4 min baseline and 6 blocks post-injection from 0–4, 4–8, 8–12, 12–16, 16–20 and 20–24 min. Selected r(t) values are given revealing correlations within (left) and in between (right) cortical areas, amygdala and thalamus. Values were statistically significantly different from baseline values as indicated by *p < 0.05, **p < 0.01, ***p < 0.001 for CC(t) vs. CC(baseline).](image)

Fig. 4. Evolution of CC matrix as function of time. CC matrices for various time windows following injection of 8-OH-DPAT: 4 min baseline and 6 blocks post-injection from 0–4, 4–8, 8–12, 12–16, 16–20 and 20–24 min. Selected r(t) values are given revealing correlations within (left) and in between (right) cortical areas, amygdala and thalamus. Values were statistically significantly different from baseline values as indicated by *p < 0.05, **p < 0.01, ***p < 0.001 for CC(t) vs. CC(baseline).
et al., 1994). Thus, the strong connectivity observed within and between these structures following a challenge with a 5-HT1A-R agonist is not surprising. However, it is interesting to note the similar connectivity strength that links a brain region with lower receptor expression levels such as dmTH (Hamon and Hoyer, 2008) to CX and HC. This pattern is likely to reflect major projections between CX and dmTH (Haque et al., 2010), but we cannot exclude a higher sensitivity of the thalamic 5-HT1A-R, or an eventual interaction of the 5-HT1A-R with other systems (e.g. 5-HT2A-R expressed by GABAergic interneurons (Geyer and Vollenweider, 2008; Salmi and Ahlenius, 1998). The relevance of the FC network identified is supported a) by its gradual manifestation during drug infusion (Fig. 4), i.e. with increasing occupancy of the SHT1A-R and b) by its reproducibility across individual mice (Fig. 2c).

An asset of mapping the connectivity across the brain is its holistic nature that may reveal implications both locally and globally that would otherwise have remained unidentified (Borsook et al., 2006). Binding studies assessing the involvement of the 5-HT1A-R in animal models of psychiatric disorders have mainly focused on the regions that are known to express high levels of the receptor: reductions in cortical, hippocampal and amygdalar 5-HT1A-R binding levels have been reported in various anxiety disorders (Lanzenberger et al., 2010).
2007; Nash et al., 2008; Neumeister et al., 2004; Nikolaus et al., 2010), as well as in preclinical models of anxiety-related behaviors (Hodges et al., 1987; Overstreet et al., 2003; Solati et al., 2011). Recently, a decrease of 5-HT7-R binding levels has been reported in the dmTH of adult mice presenting anxious-like behaviors (Franklin et al., 2011). Since it has previously been suggested that functional circuitry related to the 5-HT1A-R may involve different brain structures than the ones directly affected by a change in receptor binding (Sarnyai et al., 2000), hubs such as dmTH and PAG that may play a crucial role in the serotonin-evoked circuitry related to stress response (Bandler et al., 2000; Beracochea and Krazem, 1991; Chauveau et al., 2005) represent promising targets in the functional evaluation of stress-related psychopathologies (Borsook et al., 2006).

Specificity of the pharmacological challenge for 5HT1AR mediated activity

The modulation of the 5-HT1A-R evoked connectivity pattern under application of a specific receptor antagonist or by genetic down-regulation of the receptor supports the notion that the FC observed are clearly associated with 5HT1A-R mediated serotonergic neurotransmission. We observed residual connectivity following co-injection of serotonergic agonist and antagonist. This might be explained by an incomplete inhibitory efficacy of the antagonist as for incomplete blockade of receptor residual 5HT1A-R associated functional connectivity should occur. It is unclear to what degree this can be attributed to the simultaneous administration of agonist and antagonist used in our protocol in comparison to a study design with a pretreatment time of the antagonist of e.g. 15 min (Yelleswarapu et al., 2012) as we did not assess pharmacokinetic parameters. In a previous fMRI study the decrease in CBV elicited by 8-OH DPAT was reversed when WAY100635 was given immediately following the agonist administration (Scanley et al., 2001). This indicates that the antagonist reached the 5-HT1A receptors rapidly and displaced the agonist from the receptor binding site, as expected for compounds interacting in a competitive manner with the receptor (Zuiderveld et al., 2002). Alternatively, potential interaction of 8-OH-DPAT with other receptor systems due to lack of specificity may account for this effect. Indeed, a potential limitation of pharmacological challenges might be insufficient specificity for the target receptor: for example, 8-OH-DPAT displays a high affinity for the 5-HT1A-R with pKi = 9.33 (rat, Lejeune et al., 1997) but is also known to bind the 5-HT7 receptor, yet with a lower affinity (rodent, pKi = 5.53–7.28, Eriksson et al., 2012; Ruat et al., 1993). However, our data do not support a major interaction between 8-OH-DPAT and 5-HT7-R. Indeed, upon activation of the 5-HT7 receptor system an increase in CBV is expected because the 5-HT7 receptor has the opposite action on adenylate cyclase (AC) than the 5-HT1A receptor, which is negatively coupled to AC, the 5-HT7 receptor (Hannon and Hoyer, 2008). Though this does not rule out a certain contribution to the observed response due to interaction with the 5-HT7 receptor we assume this is rather of modulatory nature and the observed pattern is primarily induced via 5-HT1A-R engagement. Besides, we did not observe significant correlations within and between structures known to express the 5-HT7 receptors, in particular between centromedial AMG and anterior HC, two structure known to express high levels of 5-HT7-R (Gustafson et al., 1996). In contrast, WAY-106635 has been reported to interact agonistically with dopaminergic receptors D4 (Chemel et al., 2006; Marona-Lewicka and Nichols, 2009; Martel et al., 2007) though with lower affinity than for 5HT1A-R. Since these receptors are mainly expressed in the HC, caudate putamen (CPU) and nucleus accumbens (NAC) of the mouse brain (Defagot et al., 2000), the connectivity observed between these structures following the administration by the antagonist may reflect a minor intrinsic interaction of WAY-106635 with dopaminergic receptors D4. Finally, the results from a recent CBV fMRI study support the specificity of the response we observed after 8-OH-DPAT: the authors did not observe any significant fMRI
response across the brain of 5-HT1A KO mice following intra-arterial injection of 0.5 mg/kg 8-OH-DPAT (Gozzi et al., 2010b).

Compensatory mechanisms occurring in heterozygous 5-HT1A-R KO mice

Altersations of functional connectivity specific to constitutive genetic manipulation do not necessarily reflect the diminished distribution of receptors but is likely to be also affected by compensatory changes or neurodevelopmental adaptation subsequent to life-long suppression of 5-HT1A-R gene expression, in particular since the 5-HT1A-R is known to play a crucial role in many neurodevelopmental processes (Whitaker-Azmitia et al., 1996). Thus, early down-regulation of 5-HT1A-R expression may induce important alterations during brain maturation that may translate into changes at the level of brain functional networks and behavior. For instance, alteration of intra-cortical connectivity and between cortical and hippocampal areas might be related to the learning deficits reported for 5-HT1A-R KO mice (Meneses and Hong, 1999). Yet, alterations of brain connectivity observed in heterozygous animals were in general weaker than those induced by acute pharmacological treatment, which may indicate that adaptive mechanisms have in fact occurred during brain development in order to compensate the functional alterations induced by the reduced expression levels of the 5-HT1A-R. In fact, adaptive mechanisms have been reported in KO animals, including 5-HT1A-R KO (Compan, 2007). Thus, the connectivity changes involving the CPU that were specifically observed in KO animals may reflect the reduced levels of serotonin transporter that were found in the striatum of 5-HT1A-R KO mice as compared with wt animals (Ase et al., 2001).

The 5-HT1A-R full KO mouse has been proposed as a model of vulnerability to develop anxious disorders (Toth, 2003). Yet, heterozygous 5-HT1A-R KO mice, that exhibit an intermediate behavioral phenotype (Ramboz et al., 1998) and alterations of connectivity in brain regions known to be involved in the circuitry of anxiety, may reflect more closely the genetic alterations which might occur in the human population. Brain regions reported to be involved in the ‘circuitry of anxiety’ show remarkable similarities between mice and men (Leonardo and Hen, 2006). If translatable to humans, connectivity impairments identified in 5-HT1A-R KO mice might provide a ‘fingerprint’ biomarker of genetic vulnerability to the development of anxiety-related disorders, that might enable the early and non-invasive detection of patients at risk and consequently, increasing the chance of beneficial preventive or therapeutic outcomes. An important issue when studying constitutive KO animals is the existence of possible compensatory changes that have taken place in the animal over its lifetime in response to its altered genetic background (Gross et al., 2000). To account for such potential long-term adaptive changes in KO mice, we analyzed and compared common and divergent alterations in functional connectivity elicited by pharmacological and genetic inhibition.

Methodological aspects

Functional connectivity analysis may offer higher sensitivity than the classical ROI based fMRI methods but some limitations have to be considered. Artificial ventilation, moderate level of isoflurane and control of body temperature were applied to ensure stable physiological parameters and hemodynamic conditions throughout the entire fMRI experiment [see also (Mueggler et al., 2011)]. This of course does not exclude a drug induced change in blood pressure (BP). Yet, several lines of evidence allow eliminating major contribution of systemic blood pressure changes. Firstly, significant changes in BP would cause global and not region-specific changes in CBV following the injection of 8-OH-DPAT. Secondly, it was shown that 8-OH-DPAT produces a short-lasting increase in diastolic BP in the rat at doses of 0.3 mg/kg and higher (Centurion et al., 2006) potentially via vascular α1-adrenoceptors. At the dose of 8-OH-DPAT used (0.1 mg/kg i.v.) a significant change in BP is therefore unlikely. This is confirmed by BP data obtained in the rat after i.v. infusion of 0.1 mg/kg 8-OH-DPAT (Scanley et al., 2001) showing a transient decrease in BP of short duration (1 min). Neither the temporal pattern nor magnitude of BP change correlated with the temporal CBV profile assessed by fMRI. Effects of isoflurane per se on brain connectivity can be largely disregarded, but we cannot exclude an eventual interference of the anesthetic on brain physiology and pharmacological challenge that may compromise the translational aspect of the method (Ramani and Wardhan, 2008), though this notion holds for any type of fMRI studies in anesthetized animals. While fMRI methods measuring CBV changes as surrogate of neuronal activity have been shown to be more sensitive than blood-oxygenation-level dependent contrast (BOLD) based fMRI (Zaharchuk, 2007), signal to noise ratio will be limited in small and deep nuclei, in particular when using surface coil receivers or transceivers. This will compromise the quality of the FC analysis. Furthermore, small structures will be subject to partial volume effects, which may constitute a significant limitation of the approach. Finally, fMRI is an indirect readout of neuronal activity that is based on the integrity of the neurovascular coupling, which might be affected by pharmacological interventions or under pathological conditions. On the other hand, brain activation patterns/networks detected by fMRI have been reported to be more sensitive than behavioral essays; hence functional networks analysis represents a valuable tool to evaluate the validity of animal models, develop circuit-specific novel drugs and evaluate their potential neurobiological side effects (Borsok et al., 2006).

Conclusion

Our results demonstrate the feasibility of characterizing functional networks elicited by serotonergic stimulation and of assessing their modulation by genetic or pharmacological manipulations. The consistency between the structures reported to be involved in anxiety-related disorders and the impairments of functional connectivity that we identified in heterozygous 5-HT1A-R KO mice supports the relevance of this analysis.

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Disclosure/conflict of interest

The authors have no disclosure or conflict of interest to report.

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