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TITOLO DELLA TESI DI DOTTORATO

"Set-up of the resting test as a valid model for the discovery of novel CNS drugs"

S.S.D. FARMACOLOGIA BIO-14

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1 SUMMARY

In the past, several drugs were developed in order to have effect on Central Nervous System (CNS), but only when they were in an advanced development phase it was showed that they could not have any effect on CNS, because they did not pass through the Blood Brain Barrier (BBB). This condition resulted in a waste of time, money and resources for researchers.

With this project we decided to set-up a model which allows to test if a drug can pass through the BBB or not; its application should be feasible by using a non invasive radiological technique and it should be used even during the early development phase of a drug which is being studied to act on CNS.

The model chosen to be developed was the “resting state test”. Resting state corresponds to the condition when the human brain is wakeful and conscious, but relaxed and in absence of any specific stimulus or task.

Several studies have demonstrated that the brain, in its resting state, presents some degree of spontaneous activity measured as low-frequency fluctuations (<0.1 Hz) in the BOLD (Blood oxygen level dependent signal) fMRI signal of magnitude comparable to task-induced signals. Temporal correlations between fMRI signal fluctuations in different brain regions in its resting state have been interpreted in terms of functional connectivity (fc). These connectivity patterns were represented in a natural way as networks with brain regions.

To date, at least five distinct resting states networks were found between different brain areas. Among those networks one has been identified as Default Mode Network (DMN). It can be considered unique in its response to cognitive tasks due to the fact that is characterized through

high activity during rest and deactivation during tasks; it is also easily identifiable by BOLD fMRI technique and its reproducibility is reliable.

Brain regions identified as parts of the resting state DMN include the posterior cingulate cortex, precuneus, medial prefrontal cortex, anterior cingulate cortex, parahippocampus and inferior parietal cortex. A series of studies published in recent years suggest that in subjects with neurological or psychiatric diseases, the DMN works differently than it does in healthy control subjects and the pathological disturbances in intrinsic activity have also been defined as related to the severity of disease.

Significant effects have been also observed with antipsychotic drugs on fc in these brain regions. We hypothesized that these effects may be reflected in an increased fc and that they correlate with levels of receptor occupancy of the drug in the brain.

Moreover, some fMRI studies have shown that the spontaneous fluctuations of resting state are modified during simple movements, like movement of left and/or right hands and it is possible identify a brain activity in motor areas, corresponding to the specific movements.

According to these considerations it was decided to set-up the resting state model by performing a clinical trial in healthy volunteers.

The primary aim of the project was to set-up the resting state test with fMRI and to test its reproducibility in healthy subjects; the secondary aim was to explore the effects of a single oral dose of alprazolam 0,75 mg on brain activity in resting state and in motor task steps.

Alprazolam was chosen as drug due to the fact that, as benzodiazepine (BZD), it affects the CNS after being passed through the BBB with an anxiolitic effect.

It was established to enroll a minimum of 10 healthy male and female subjects with age 18 to 50 years. Main exclusion criteria were diagnosis of mental illness or significant cardiac disease or cardiac conduction disorder and history or presence of neurological or psychiatric conditions.

The radiological technique chosen was BOLD fMRI. Compared to other radiological techniques (i.e. PET) this method is safe and not invasive: it can be applied to young and old subjects and it is based on an endogen contrast. This investigation was undertaken as a double blind, single dose, crossover, randomized study. In two different occasions, each subject received an oral dose of alprazolam or placebo and underwent two fMRI scan sessions: before and 2hrs ± 30 min post dosing (approximately the t-max of alprazolam).

During the first part of the fMRI exam each subject was asked to stay wakeful, motionless and with eyes closed. In the second part each subject performed the motor task which consisted of two steps: during the first one he was asked to stay wakeful, with eyes closed and to open and close the hands alternatively for 10 times; during the second one, he was asked to stay wakeful, motionless and with eyes closed for the same period of the first step. The task was repeated for 5 times.

The clinical phase of the study was carried out as scheduled. The “resting state” parts and the “motor task” steps were performed by applying the model defined and detailed into the protocol. Alprazolam and study procedures were well tolerated.

A total number of 11 subjects were enrolled into the study, because one subject did not complete both study sessions. Only data sets obtained by subjects who completed both sessions were analyzed.

The functional data were analyzed by using software FSL. For resting state analysis the first step was the use of Multi Session Temporal Concatenation in MELODIC FSL component. At this level, it was also applied an F-test. The next step was the dual regression technique. The last step was to collect the different sets of spatial maps across subjects and to analyze them. The results were spatial maps defining the between-subject group-consistency and/or between-subject group-differences. For motor task step analysis, fMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) FSL application.

The reproducibility of DMN across all subjects was demonstrated by the contrast of two baseline conditions: the F-Test (thresholded per $p < 0,05$) showed a statistically non-significant difference. The analysis performed through dual-regression method did not point out a statistically significant difference as well. A very similar condition was showed between two baseline and pre-post placebo conditions.

The main result obtained by the data analysis was a diffuse and increased fc in post alprazolam maps in the posterior component of DMN and also in brain areas out of DMN. This increase of connectivity can be interpreted as a consequence of the administration of a drug with a sedative effect which inhibits tasks, increases activity at rest and makes DMN stronger. The presence of changes in brain areas out of DMN might be due to the binding of alprazolam to BZD receptors in CNS.

The results obtained from motor task steps showed an evident reduction of activity in primary motor areas, bilateral primary somato-sensitive areas and bilateral cerebellar areas. These results are in agreement with characteristics of motor task paradigm as defined into the study protocol.

In summary both primary and secondary aims of the study have been achieved. Resting state test was set up and its reproducibility in healthy volunteers was demonstrated. It was demonstrated that

the effect of a single dose of alprazolam on brain activity in resting state consists in an increased and diffuse connectivity in DMN and in other cerebral areas and alprazolam effects in motor task step consist in a reduction of activity in motor areas activated by motor task.

In conclusion the model can be considered set-up and applicable in humans during future clinical trials. The maps generated during motor task step increase knowledge of human brain topography. In the future we can schedule to carry out a similar clinical trial in patients affected by psychiatric and neurological diseases and to compare results obtained in patients against the ones obtained in healthy volunteers.

2 INTRODUCTION

2.1 Resting state

"Resting state" corresponds to the condition where the human brain is wakeful and conscious, but relaxed and "stimulus-free" i.e. in absence of any specific stimulus or task. It was noted that in this condition the brain appears to be dynamic and not inactive. By using radiological techniques it has been indeed detected the presence of a spontaneous brain activity during resting state.

To date the two radiological techniques mainly applied to investigate this state are the Positron Emission Tomography (PET) and the Functional Magnetic Resonance Imaging (fMRI). fMRI is widely used to detect and delineate regions of the brain that change their level of activation in response to specific stimuli and tasks. However fMRI has become also an important tool to explore the spatio-temporal correlations of spontaneous signal fluctuations in the brain. The most common fMRI technique used for this purpose is BOLD fMRI which uses the blood oxygenation level-dependent (BOLD) contrast. The BOLD signal reflects local variations in the de-oxyhaemoglobin concentration that are determined by a combination of blood flow, blood volume and oxygen metabolism (Fox et al., 2007). A specific study has demonstrated that BOLD fMRI provides the unique contribution of a combined spatial and temporal resolution in a non-invasive technique and it also provides whole-brain coverage at spatial resolutions of millimeters (Rogers et al., 2007). That justifies why this radiological technique has been largely applied to investigate resting state.

By using BOLD fMRI technique several studies demonstrated that brain presents some degree of spontaneous fluctuations of the BOLD fMRI signal during resting state. This spontaneous activity has been studied both in animals and humans across many spatial and temporal scales. Most recent

studies focused specifically on slow (< 0.1 Hz) fluctuations in the BOLD signal. The presence of slow oscillatory BOLD signal changes were first observed by Biswal et al. (1995) and it has been confirmed by further studies. The interest for this range of fluctuations of the fMRI signal arises from the observation that magnitude of these spontaneous fluctuations can be equal to brain responses observed in response to tasks or stimuli (Fox et al., 2007; Damoiseaux et al., 2006; Auer, 2008; De Luca et al., 2006; Gusnard et al., 2001).

The exact nature of these low-frequency (<0.1 Hz) fluctuations is unclear. Some authors hypothesized that spontaneous BOLD fluctuations could be related to cardiac and respiratory rhythms due to low-frequency range. However further studies demonstrated that the frequency contributions to low-frequency BOLD fluctuations from cardiac and respiratory frequencies were less than 10% (Auer, 2008). The conclusion was that spontaneous low frequency fluctuations can not be attributed to cardiac or respiratory factors (Fox & Raichle, 2007; Damoiseaux et al., 2006).

A group of studies concerning resting state were carried out also with the purpose to identify a physiological baseline condition in the "awake" human brain to be used as control (Gusnard et al, 2001; Auer, 2008). The concept which addressed authors in this direction was the definition of physiological baseline of the brain as the absence of activation. During activation there is an increase in blood flow in the brain with a consequent oxygen delivery. This increase is not accompanied by a commensurate oxygen utilization. The relationship between oxygen delivery and utilization decreases during brain activation. In the resting state it was observed that in the brain there is a spatial uniformity of this relationship. This uniformity at rest has been defined as equilibrium reached between the local metabolic requirements that are necessary to sustain a long-term continuing level of neural activity and the level of blood flow in those regions. It was hence proposed that this equilibrium state defines a baseline level of neuronal activity that can be observed when subjects are awake and resting with eyes closed. Furthermore some authors

(Gusnard & Raichle, 2001) suggested that, given that the activity of the areas active during resting state can be considered as arisen from a baseline activity, it is more appropriately defining these areas as functionally “active”, rather than “activated“.

During resting state it also be verified that there is a large energy consumption. The resting human brain represents only 2% of total body mass, but consumes 20% of the body’s energy. Task-related increases in neuronal metabolism is usually small (<5%) when compared with this large resting energy consumption (Fox et al., 2007). For this reason spontaneous brain activity is considered the component that consumes most of the brain’s energy and it was hypothesized that investigation of this component can allows to understand how the brain works.

An other important result confirmed by several studies is that spontaneous BOLD activity is not random noise, but is specifically organized in the resting human brain. Spontaneous BOLD fluctuations measured in the left somatomotor cortex are correlated with spontaneous fluctuations in the right somatomotor cortex and with medial motor areas.

The enormous cost of spontaneous activity in term of energy consumption and its specific organization in the brain suggests that it serves an important role in brain function. Although the exact underlying processes are not known, recent studies suggested that resting state activity reflects a spontaneous neuronal activity that is intrinsically generated by the brain. Furthermore they suggest that it has been implicated in attending to external and internal stimuli (Fox et al., 2007; Raichle et al., 2001), as well as self-referential and reflective activity, including episodic memory retrieval, inner speech, mental images, emotions and planning of future events. (Garrity et al., 2007; Gusnard & Raichle, 2001; Raichle, 2001; Greicius, 2003; Fransson , 2005; Gusnard et al., 2001).

On other suggestion proposed is that spontaneous activity serves as record or memory of previous use or to organize and coordinate neuronal activity (Fox et Raichle, 2007).

To summarize we can say that to date some authors defined resting state as a baseline condition of brain activity while others suggested that it reflects a spontaneous neuronal activity that is intrinsically generated by the brain and it putatively supports self-referential or non directed cognitive processing.

2.1.1 Functional connectivity in resting state and "default mode network"

Several studies showed that there is a synchronization of spontaneous low-frequency (<0.1Hz) BOLD fMRI fluctuations in specific brain areas recorded during resting state; these findings were underlined also by PET studies. The brain areas where the coherence in low-frequency fluctuations has been shown are spatially independent and apparently not correlated. Furthermore it has been demonstrated that this set of brain regions are more active at rest and routinely decrease their activity during task performance. Hence it was hypothesized that the brain remains active in an organized fashion during resting state (Fox & Raichle, 2007; Auer, 2008). The observation that spontaneous BOLD activity is not a random noise, but it is specifically organized in the resting human brain has generated a new avenue of neuroimaging research.

Several fMRI and PET studies were addressed to investigate the organization of the human brain. However it was found that these techniques did not readily account for the brain's network organization. To address the latter, it was applied an other analysis method, the functional connectivity (fc) analysis, i.e. interaction between spatially disparate brain responses (Cordes et al., 2000; Fox et al., 2007). This analysis may refer to any study examining inter-regional correlations in neuronal variability (Fox et al., 2007). At the beginning it was applied to PET studies and later to fMRI studies. It can be used in both resting state and task state studies.

By applying fc analysis the interregional correlations between fluctuations of BOLD fMRI signals were interpreted as functionally correlated. These connectivity patterns were represented in a natural way as networks with brain regions, with individual image voxels or anatomically-defined structures representing the nodes and a measure of correlation between each pair determining the edges (Gusnard et al., 2001). Consistent patterns of correlated fMRI activity have been reported by several authors and related to specific electrophysiological signatures (Mantini et al., 2007). A correlation between regions of the motor network, both within and across hemispheres of the brain at rest was first demonstrated by Biswal (1995). Recent studies have established that interregional correlations between different components of circuits in each of the visual, language, motor and working memory systems can be detected in resting state. For example De Luca et al. (2006) found at least five distinct resting states networks. There is no unified terminology hence to describe the fc maps derived from spontaneous brain activity, but they are commonly referred to as "resting state networks".

Among those networks one has been identified as Default Mode Network (DMN) or Default Network. This term was first coined by Raichle et al (2001) and DMN presence has been validated in several further studies. It is also easily identifiable by BOLD fMRI technique and it was also demonstrated that the DMN is characterized through high activity during rest and deactivation during tasks (Auer, 2008; Damoiseaux et al., 2006); for this reason it is also called "task-negative network". The DMN is therefore not unique in showing resting state activity, but can be considered unique in its response to cognitive tasks (Fox et al., 2007). During last ten years, in fact, several studies were focused to understand exactly what functionality is mediated by this network and why it is suppressed by cognitive tasks and, although much is known, more has yet to be discovered.

2.1.2 Brain areas in DMN

Brain regions identified as parts of the resting state DMN include the posterior cingulate cortex, precuneus, medial prefrontal cortex, anterior cingulate cortex, parahippocampus and inferior parietal cortex, among others (Gusnard et al., 2001; Rombouts et al., 2003). This hypothesis is supported by fMRI studies of conscious rest (Beckmann et al.,2005; Fox et al., 2005; Fransson, 2005; Greicius et al., 2003; Laufs et al., 2003) and cognitively undemanding tasks (Greicius and Menon, 2004; Greicius et al.,2004) showing strong temporal coherence of low-frequency BOLD signal oscillations among brain regions implicated in the network. DMN areas were investigated by using different techniques, but they converge on a similar finding: neuro-anatomical systems in the human brain can be identified on the basis of correlation patterns in spontaneous BOLD activity. Hence correlation patterns of spontaneous activity reflect functional topography. The figure 1 shows the core regions identified as part of DMN.

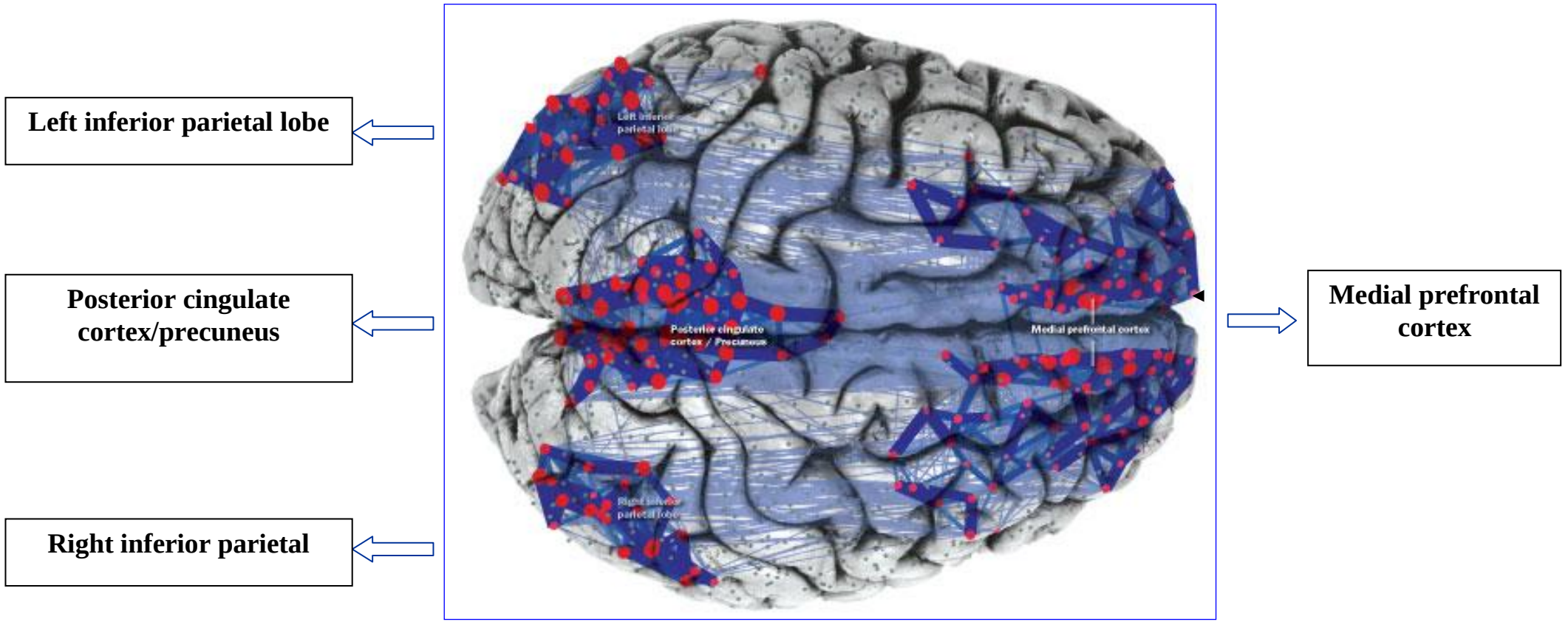

Figure 1. Default Mode Network core regions. Modified from http://www.sciencenews.org/view/access/id/45263/title/Wandering_and_wondering

A quite recent study carried out by Grecius et al (2008) was performed by combining two techniques in order to demonstrate that resting-state fc reflects structural connectivity: diffusion tensor imaging (DTI) tractography with resting-state fcMRI. Those techniques allowed to investigate connectivity within the set of regions which represent DMN (see figure 2).

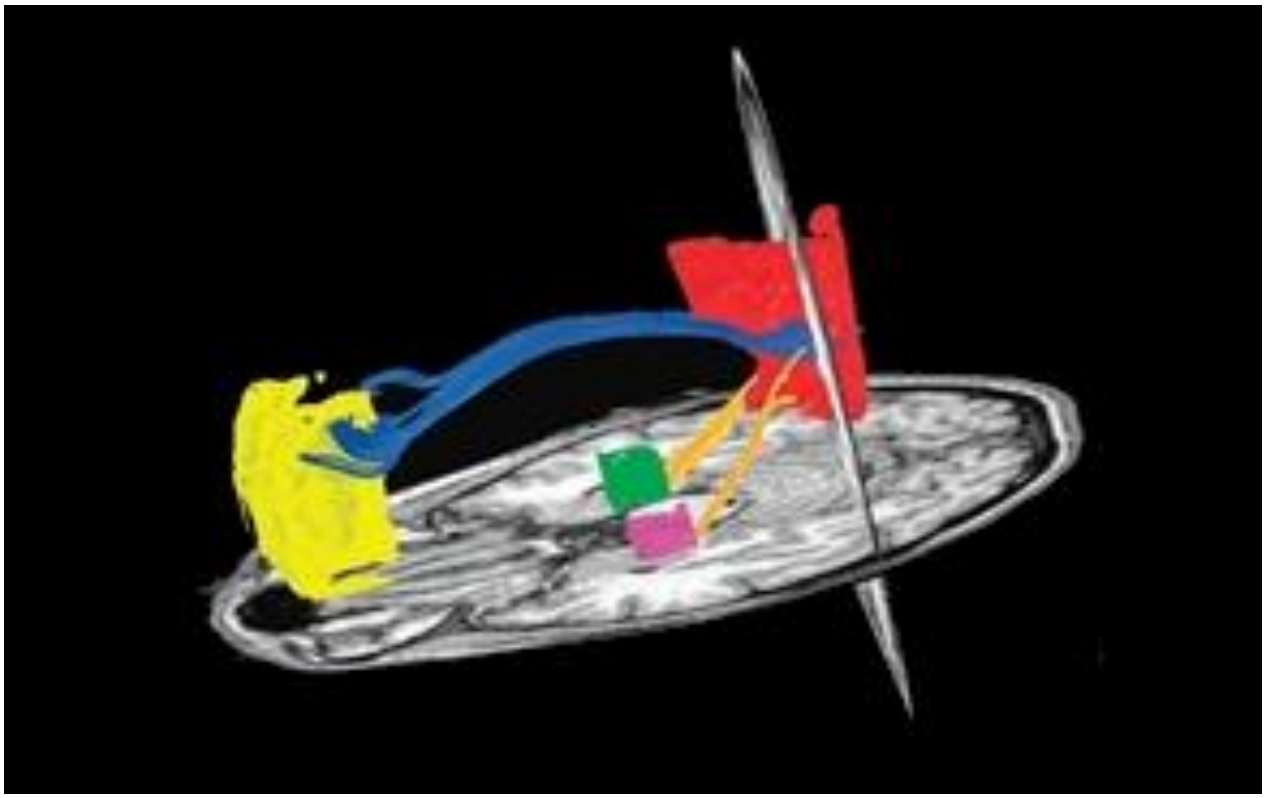

Figure 2. Functional connectivity reflects structural connectivity in the DMN. The figure 2 can show the activation of DMN and the fc. It displays the set of DMN brain areas and their connectivity: cingulum bundle (blue tracts) connecting the posterior cingulate cortex (PCC) (red) to the medial prefrontal cortex (MPFC) (yellow). The yellow tracts connect the bilateral medial temporal lobes (MTL) (purple/green) to the PCC. Note that generally the tracts from the MPFC enter the more rostral aspect of the PCC/Retro splenial cortex (RSC) regions of interest corresponding to the PCC proper, whereas the tracts from MTL enter the more caudal aspect of the PCC/RSC regions of interest corresponding to the RSC proper. Taken from Grecius et al, 2009.

2.1.3 Changes in patterns of DMN

The DMN can be defined as a set of functionally and anatomically organized neural regions that are active during a behavioral resting state and deactivated or suppressed during task performance. In literature it was reported that the functional organization of the DMN is stable across species (Auer, 2008; Damoiseaux et al., 2006). Furthermore this connectivity, or coactivation, pattern may be intrinsic to the primate brain (Glahn et al., 2010; Smith et al., 2009; Seeley et al., 2009), because it is present in sleeping infants (Fransson et al., 2007) and anesthetized nonhuman primates (Vincent et al., 2007).

A series of studies published in recent years suggest that, in subjects with neurological or psychiatric disease, the DMN works differently than it does in healthy control subjects. Disturbances in the correlation structure of spontaneous activity have been reported for a number of pathological states as Alzheimer's disease, multiple sclerosis, depression, schizophrenia, attention deficit hyperactivity disorder, autism, epilepsy. (Fox & Raichle, 2007; Auer, 2008). In Alzheimer's disease reduced connectivity of the hippocampus as well as within the DMN was noted (Auer, 2008). In the schizophrenia, some researchers noted that the DMN is overactive and faultily wired and it was observed an altered temporal frequency of the BOLD fluctuations, sparser connectivity, and different spatial distribution of the DMN (Garrity et al, 2007). In the Figure 3 it is shown as the network, as measured by its blood flow with fMRI, resonates slowly and regularly in healthy subjects, while, in patient affected by schizophrenia the activity is increased and more irregular.

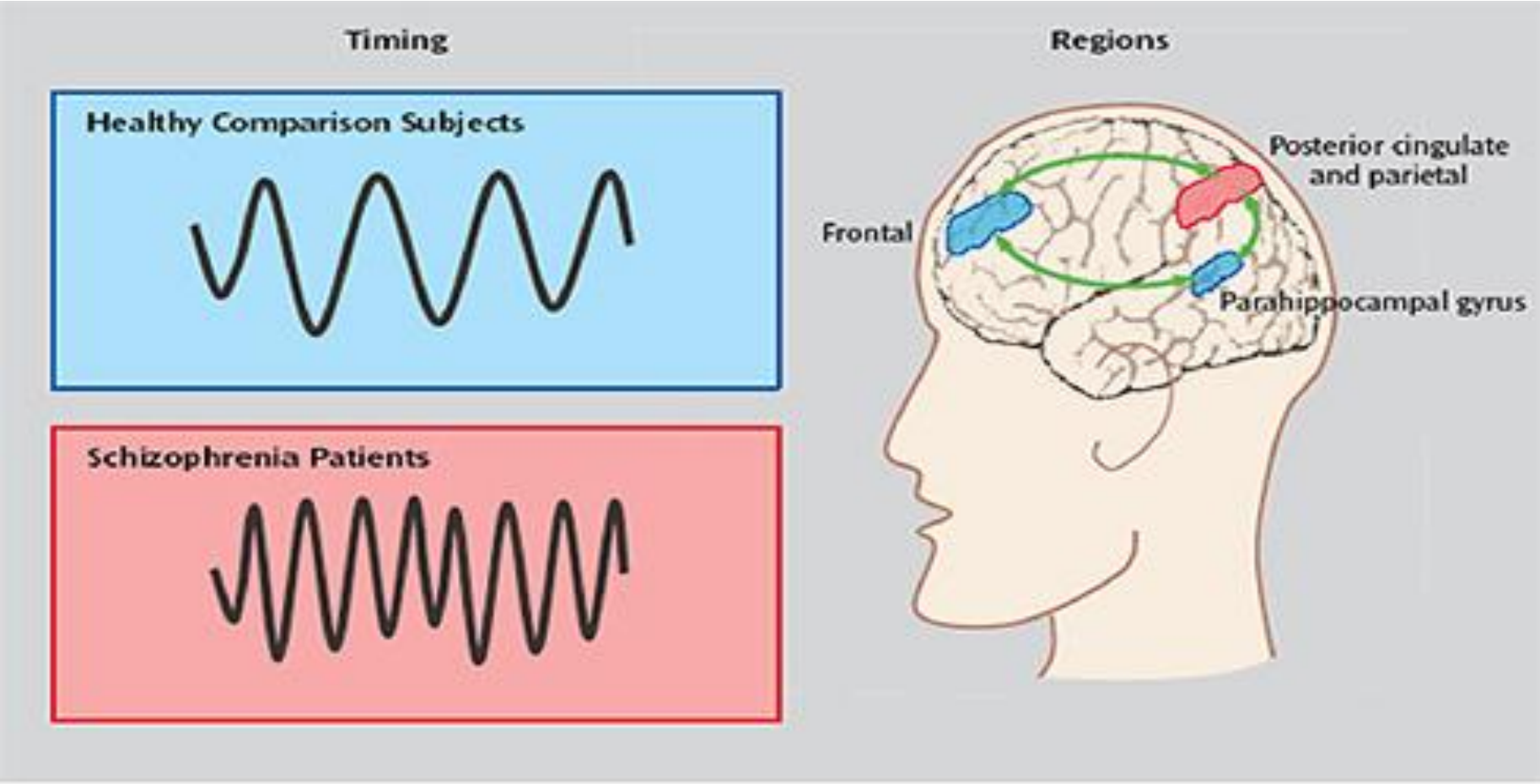

Figure 3. Comparison between healthy subjects and schizophrenia patients network as measured by its blood flow with fMRI. Taken from Garrity et al., 2007.

These pathological disturbances in intrinsic activity have also been defined as related to the severity of disease (Fox & Raichle, 2007). That suggests that this intrinsic network is sensitive to pathophysiologic alterations in brain function and structure (Broyd et al., 2009).

Furthermore, aberrant default mode connectivity has been reported also in persons at genetic risk for such illnesses. A recent study has also demonstrated that connectivity within the DMN is influenced by genetic factors (Glahn et al., 2010). The amount of synchrony, which reflects the strength of functional connections between the different areas, is increased in patients with schizophrenia. First-degree relatives of persons with the illness also show some increase, although less than patients (see figure 4).

The above described results suggested that the intrinsic activity may hold valuable diagnostic and prognostic informations and changes in fc can be used as an early marker of some diseases (Rogers et al., 2007).

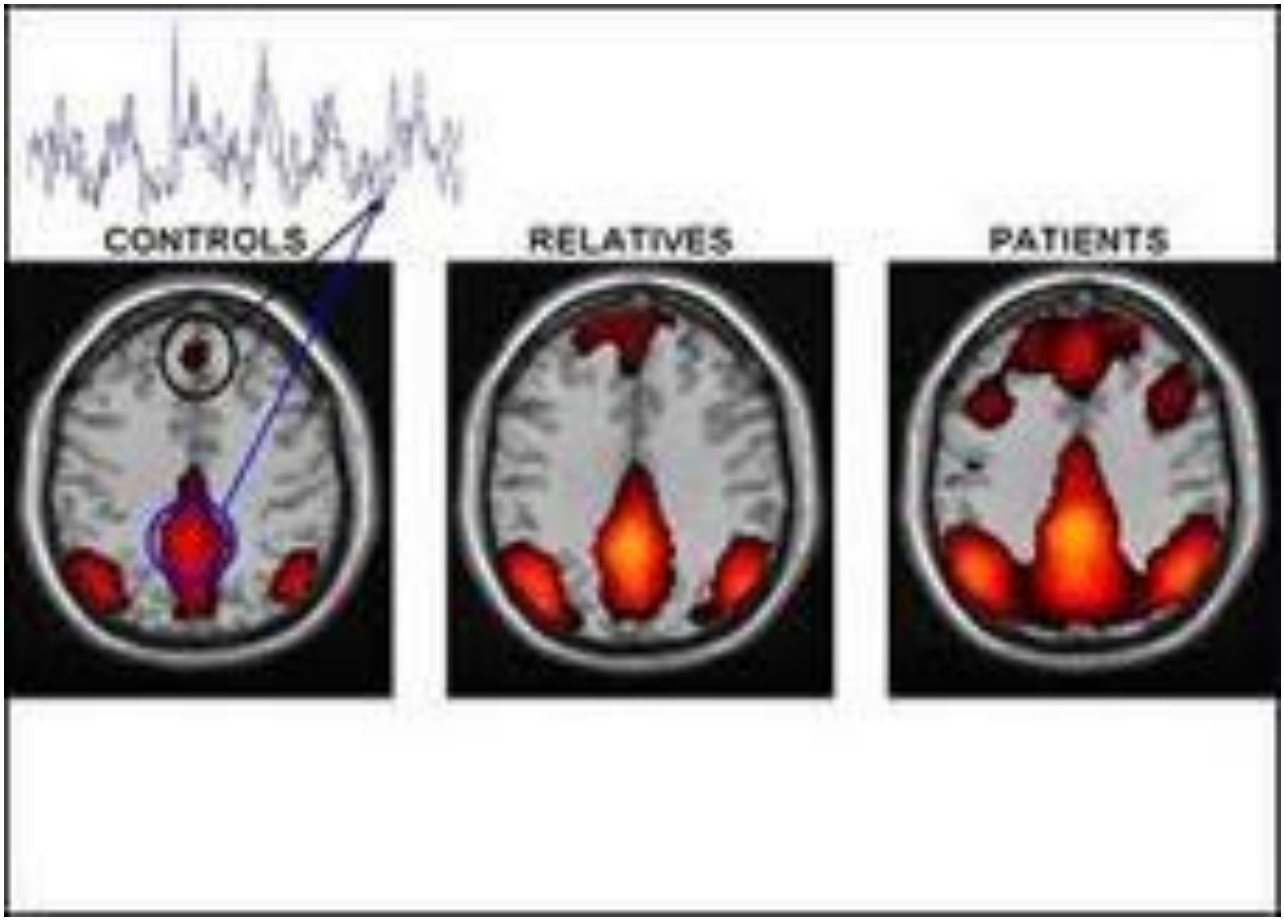

Figure 4. Altered brain connectivity of default brain network in persons with schizophrenia and first-degree relatives. Colored areas represent an interconnected network of brain regions that show synchronized activity when subjects rest and allow their minds to wander. Taken from http://www.schizophreniaforum.org/new/detail.asp?id=1486.

2.1.4 Drug-induced alterations of DMN

Changes in the correlation structure of spontaneous activity of the brain resting-state can be assessed across development, sleep, anaesthesia and pharmacological manipulation (Fox et al., 2007). Knowledge on drug-induced alteration of fc has been seen as an important field to be investigated in order to enhance our understanding on psychopharmacological action. The potential

to study pharmacological effects on low frequency fluctuations was first shown by Anderson et al. (2006) and Auer (2008). Further pharmacological resting-state studies were carried out in humans and in laboratory animals; during these studies drug-induced changes in the correlation structure of spontaneous activity of the brain resting-state were detected (Achard et al., 2007; Anand et al., 2005). Interestingly, significant effects have been observed with antipsychotic drugs (Schwarz et al., 2007) in temporal, parietal and cortical regions and in the dorsal cingulate gyrus. A study carried out with administration of a BZD, midazolam, highlighted that drug-induced sedation was associated with increased sensorimotor low frequency BOLD fluctuations (Auer, 2008; Kiviniemi et al., 2005). Anand et al. (2005) showed that chronic antidepressant therapy restored cortico-limbic connectivity in depressed patients at rest.

Such effects are predicted to occur with drugs that pass through the BBB and that potentiate glutammatergic activity, thus strengthening synaptic connections. We hypothesised that these effects are reflected in increased fc, particularly in hippocampal connections with the prefrontal cortex and that they correlate with levels of receptor occupancy of the drug in the brain.

2.2 *Motor task state*

The fc MRI analysis was applied to investigate spatio-temporal correlations of spontaneous signal fluctuations in the brain both in resting state and tasks state studies. By applying this technique it was observed that resting-state networks tend to group according to functionalities with prominent spatio-temporal synchronizations within the DMN, the motor, the visual and auditory system and the other resting state networks (Auer, 2008). There is accumulating evidence that low frequency BOLD fluctuations are modulated by specific task-related neuronal activity. The majority of studies reveal task-related increases in fc in the respective functional networks and decreases in unrelated networks (Auer, 2008; Newton et al.,2007; Hampson et al., 2004).

The motor network has been well investigated in a vast number of fMRI studies to evaluate the human brain function during the simple movements, like movement of one or more finger, left and/or right hands (Amann et al., 2009). Changes of spontaneous fluctuations of resting state during motor task condition allowed to identify a brain activity in motor areas, corresponding to the specific movements. According to this evidence, composite maps of the brain may be generated, demonstrating distinct motor-related activation in primary sensori-motor cortex, in premotor areas and in supplementary motor areas. That could increase knowledge of human brain topography and function.

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3 AIMS OF THE STUDY

The main object of the project was to set-up a model which allows to test if a new drug can pass through the BBB or not. In the past several drugs were developed in order to have effect on CNS, but only when they were in an advanced development phase it was showed that they could not have the expected clinical effect, because they did not pass through the BBB. That caused a waste of time, money and resources for the researchers.

The model that we decided to set-up was the “Resting state test” or “resting test”.

The application of this model to humans would have to be feasible, reliable and valid, with a commonly used and non-invasive radiological technique. Furthermore, this model could be applied during the early development phase of a drug which is being studied to act on CNS. That could allow to save time, money and resources.

- The primary aim of the study was to set-up the resting state test and to test its reproducibility in healthy subjects.

- The secondary aims were:

    - to explore the effects of a single dose of alprazolam, a BZD drug, which affects the CNS, on brain activity in resting state,

    - to explore the effects of a single dose of alprazolam in motor task step.

4 MATERIALS AND METHODS

4.1 Imaging Method: BOLD fMRI

The radiological technique used for this study was the BOLD fMRI. It has been developed in early nineties and it become an important tool to explore the spatio-temporal correlations of spontaneous signal fluctuations in the brain. It provides the unique contribution of a combined spatial and temporal resolution in a non-invasive technique and it also provides whole-brain coverage at spatial resolutions of millimeters.

In details, the BOLD signal of fMRI arises from the magnetic properties of haemoglobin and the manner in which brain metabolism and blood flow are related to changes in neuronal activity. It is not a direct measure of neuronal activity, but reflects local fluctuations in de-oxyhaemoglobin (dHb) concentration that are determined by a combination of blood flow, blood volume and oxygen metabolism. During activation there is an increase in blood flow in the brain with a consequent oxygen delivery. Fully oxygenated haemoglobin in arteries has little effect on the magnetic field of an MRI scanner i.e. it has diamagnetic properties with a small magnetic susceptibility effect. However, when haemoglobin loses oxygen to the tissue as it passes through the capillaries of the brain the resulting de-oxygenated haemoglobin disrupts the MRI magnetic field in proportion to the amount of oxygen lost i.e. it has paramagnetic properties. When brain activity increases, blood flow and glucose consumption increase much more than oxygen consumption. As a result the amount of de-oxygenated haemoglobin decreases in the area of increased activity and the relationship between oxygen delivery and utilization decreases and the BOLD signal is enhanced (see figure 5 and 6).

The net effect is a surplus in the amount of oxygenated haemoglobin delivered to any activated voxel. As the delivered oxygen exceeds local demands, the capillary and venous beds fill with a

larger ratio of oxygenated to deoxygenated haemoglobin compared to when the cortex was at rest. This larger amount of diamagnetic oxyhaemoglobin will mean less effect from the field-altering deoxyhaemoglobin and to an increased signal on the images.

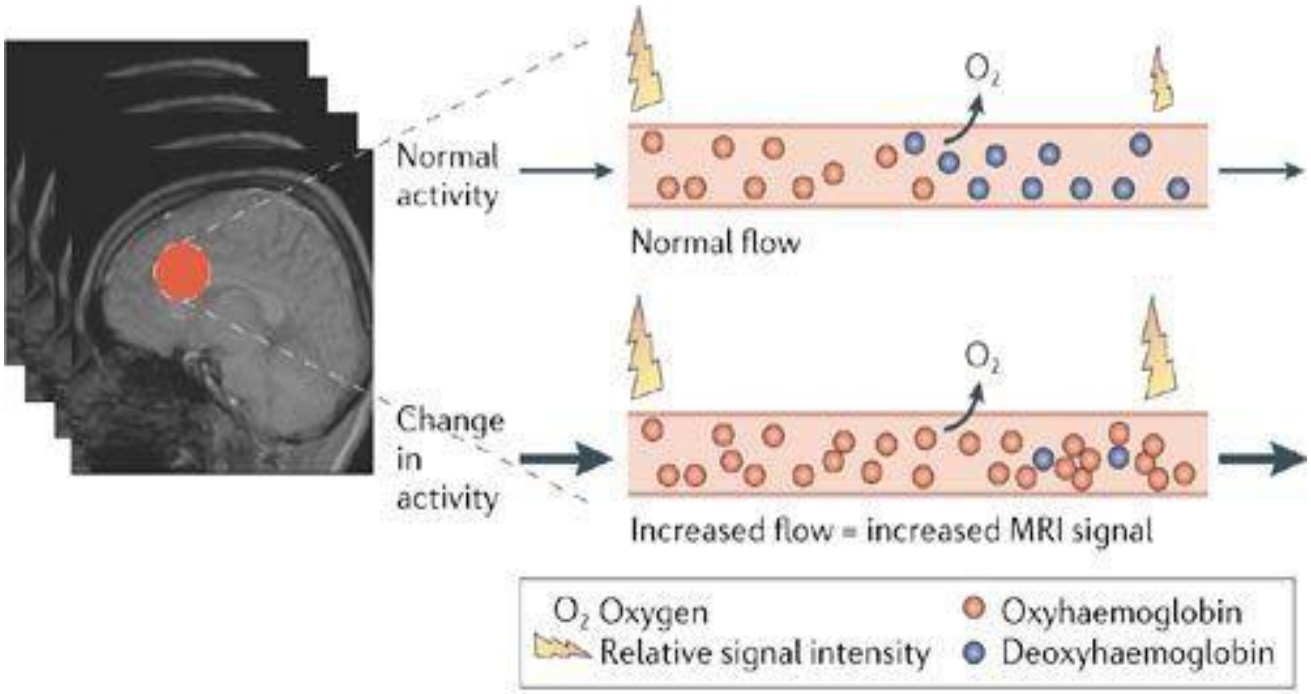


Figure 5. Changes in magnetic resonance intensity (MRI) signal as result of change in brain activity. Taken from Borsook et al, 2006.

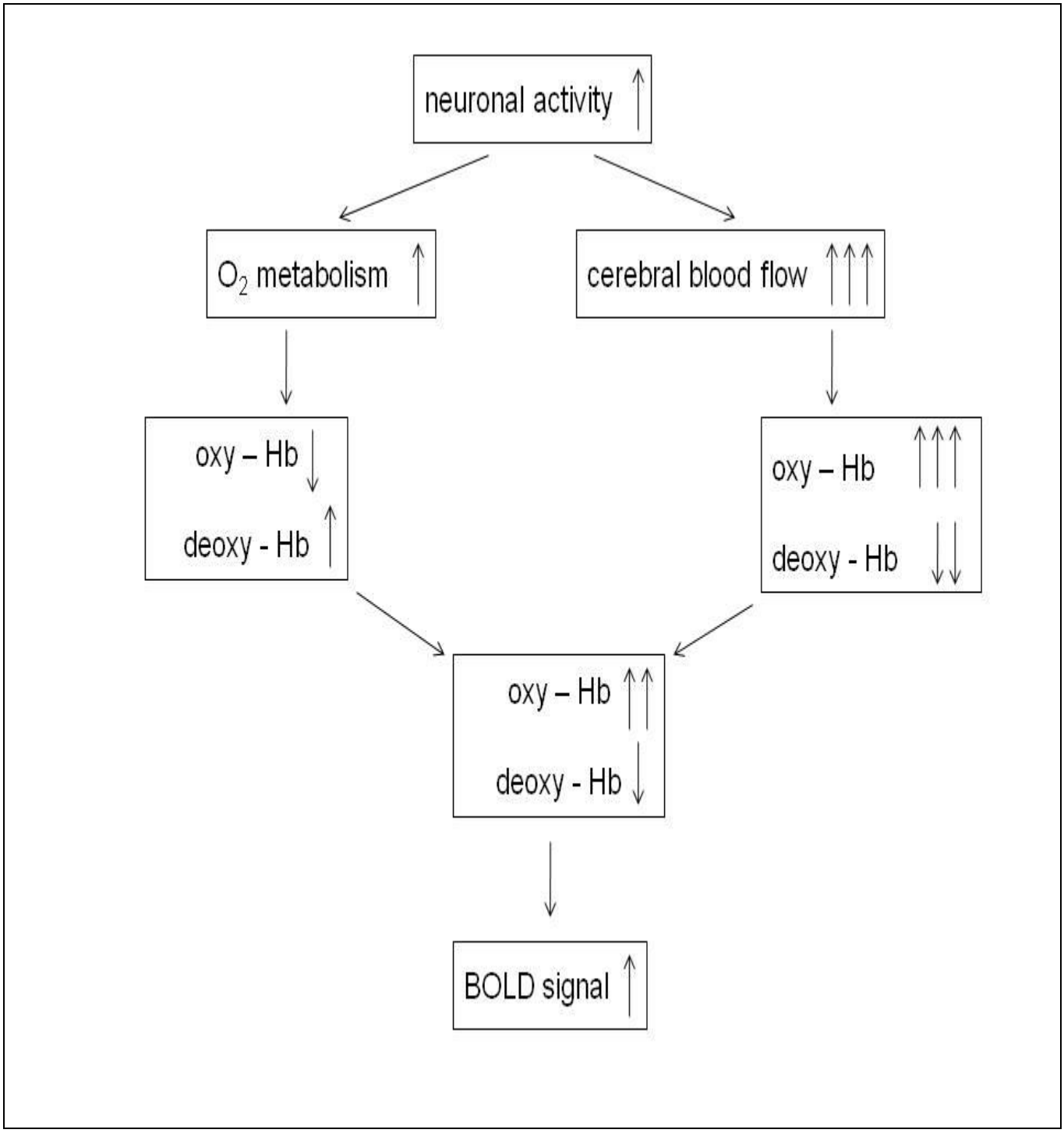


Figure 6. Physiology of the hemodynamic response during increased neuronal activity. Modified from Lindauer U. et al., 2010.

The BOLD peak activity can be observed within 3-5 seconds after starting of the event to be investigated. That is an important aspect for the data sampling.

To summarize, "BOLD fMRI" technique has several advantages compared to other radiological techniques:

- it is a noninvasive method based on an endogen contrast and it can be applied to young and old subjects,
- it studies the quantitative variation of de-oxyhaemoglobin and that allows the method to be less subjected to BIAS,
- the time for sampling is short: the BOLD effect appears only 5-10 seconds after the activation.

4.2 Study design

This investigation was undertaken as a randomized, double-blind, single dose, placebo – controlled, 2 way-crossover study. It was performed in a single site, at "Centro Ricerche Cliniche di Verona" (CRC). Before starting, it was approved by local research Ethics Committee. All participants individuals provided written, informed consent prior to their study participation and the study was conducted in accordance with Good Clinical Practice and the Declaration of Helsinky.

Subjects attended two study occasions and during each one they underwent a first scan session between 7.00 to 8.00 o'clock in the morning. The BOLD fMRI results consisted in the baseline data.

After the first scan session subjects were assigned to receive either alprazolam 0,75 mg or placebo in accordance with the randomization schedule generated by CRC. The time of second scan session was scheduled at approximately t-max of the alprazolam, after 2 hrs ±30 min post dosing. That was defined in order to capture the maximum effect of the drug.

Subjects were screened within 21 days of first dose and the two study occasions were separated by a wash-out period of at least 5 days. On both study occasions, subjects were admitted to the clinic on Day 1 and remained until at least 8 hours after dosing. A telephonic follow – up interview was performed 7-14 days after the administration of the last dose.

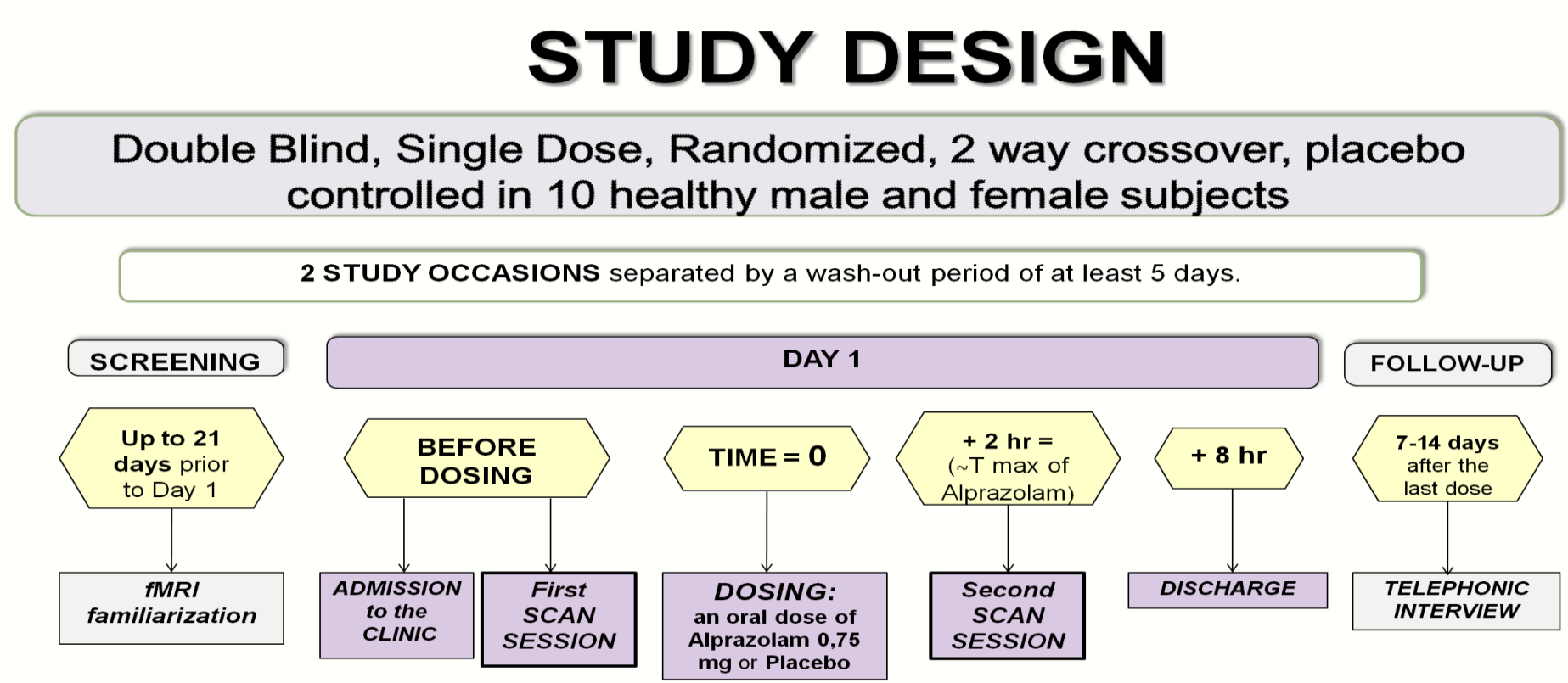


Figure 7. The figure shows a schematic description of study design as defined into the protocol. Under “screening”, “day 1” and “follow-up” are reported the main procedures to be performed during the visit and their time points according to the trial design.

4.2.1 Endpoints

In this study it was proposed to evaluate the reproducibility of the "resting test" in healthy volunteers and to explore the effects of a drug that acts on CNS on brain activity in resting state and in motor task step.

The end-point used was the "Brain resting-state functional-connectivity" measurements.

Primary endpoint was Brain resting-state functional-connectivity at baseline in two different occasions.

Secondary endpoint was Brain resting-state functional-connectivity and Brain Motor-Task connectivity at baseline and after administration of placebo and alprazolam.

4.2.2 Safety assessments

Adverse events (AEs) were assessed continuously. A physician was present at dosing thereafter, the medical cover was determined by CRC policy.

4.3 Subjects

A minimum of 10 subjects had to complete the clinical trial and to perform a total of four fMRI scan sessions each ones. The decision to enroll 10 subjects was taken by considering data reported in literature: significant results were collected during "resting state" clinical trials carried out in a sample of 10 subjects.

Male or female participants were recruited if aged 18-50 years, in general good health and in absence of any mental illness. They had to be with body weight ≥ 50 kg and BMI within the range of 19-29 kg/m2. All of them were capable to give written informed consent.

Key Exclusion criteria were family medical history positive for Alzheimer disease and Parkinson's disease; history or presence of neurological or psychiatric conditions (e.g., stroke, significant traumatic brain injury, epilepsy, space occupying lesions, multiple sclerosis, Parkinson's disease, vascular dementia, transient ischemic attack, any unexplained loss of consciousness, schizophrenia, major depression etc) that may influence the outcome or analysis of the scan results; presence of electronic device or ferromagnetic metal foreign bodies in vulnerable positions that contraindicates the MRI performance; history or presence of glaucoma, angle-closure; history of or suffers from claustrophobia or felt that they would be unable to lie in the MRI; presence of significant cardiac disease or cardiac conduction disorder; QTc interval >450 ms, and/or a PR interval outside the range 120 to 200 msec or an ECG not suitable for QT; resting pulse rate <40 or >100 bpm or a systolic blood pressure >140 or <90 or a diastolic blood pressure >90 or <50; history of pre-study Hepatitis B surface antigen or positive Hepatitis C antibody result within 3 months of screening; previously history of sensitivity to any of the study medications or components thereof, or a history of drug or other allergy that, in the opinion of the investigator contraindicates their participation; history of smoking > 10 cigarettes a day in the last three months; positive pre-study drug/alcohol screen; history of regular alcohol consumption within 6 months of the study; women of childbearing potential who were not using adequate contraception as defined by the Protocol; previous exposure to drugs-within 7 days or 5 half-lives prior to the first dose of study medication; previous participating in a clinical trial and having received an investigational product within 6 month period prior to the first dosing day in the current study.

During the study each subject had to observe the fasting state from 10 p.m on day -1 until "2 hrs post-dosing" fMRI scan and they had to abstain from drinking water from 1 hr before dosing to "post-dosing" fMRI scan, except 200 ml water requested for dosing; they had to abstain from consumption of poppy-seed-containing food, or any other food or drink containing quinine,

grapefruit or grapefruit juice red wine, Seville oranges, apple juice, vegetables from the mustard green family and charbroiled meats, from 7 days prior to the first dose of study medication until the last dose. Subjects had to abstain from strenuous exercise for 24 hours prior to each study occasion and they had to abstain from driving car or motorcycle for 24 hours after dosing.

During each dosing session, subjects were asked to abstain from ingesting caffeine- or xanthine - containing products for 24 hours prior to and after dosing during each session and to abstain from alcohol for 24 hours prior to dosing until discharge.

4.4 Drug treatment

Alprazolam was chosen as active drug to be administered during the study.
We hypothesized that modulation of activity by compound may change the pattern of default activity captured by the resting state and motor task acquisition protocols compared with baseline conditions. While some limited evidence of the effects of psychotropic drugs on the fMRI resting state fc has been reported in the literature, this pharmacodynamic approach has to be considered exploratory.

It is recognized that alprazolam acts on CNS after being passed through the BBB. It facilitates the inhibitory action of gamma-aminobutyric acid (GABA) neurotransmitter, which mediates both pre and post synaptic inhibition in the CNS with a dose dependent anxiolitic effect.

In details, the drug has a high affinity for the BZD binding sites on $GABA_A$. The $GABA_A$ receptor is a protein complex located in the synapses of neurons. All $GABA_A$ receptors contain an ion channel that conducts chloride ions across neuronal cell membranes and two binding sites for the neurotransmitter GABA, while a subset of $GABA_A$ receptor complexes also contain a single binding site for BZD. Binding of BZD to this receptor complex promotes binding of GABA (see

figures 8), which in turn increases the conduction of chloride ions across the neuronal cell membrane. This increased conductance raises the membrane potential of the neuron resulting in inhibition of neuronal firing (see figure 9).

Furthermore, alprazolam is readily absorbed: following oral administration, peak concentration in the plasma occurs after 1-2 hours and the mean half-life is 12-15 hours. The drug and its metabolites are excreted primarily in the urine.

The dose of alprazolam used in the study was 0,75 mg, given as single oral dose. It is the medium dose used to treat moderate anxiety disorders and it has been demonstrated to be safe and well tolerated.

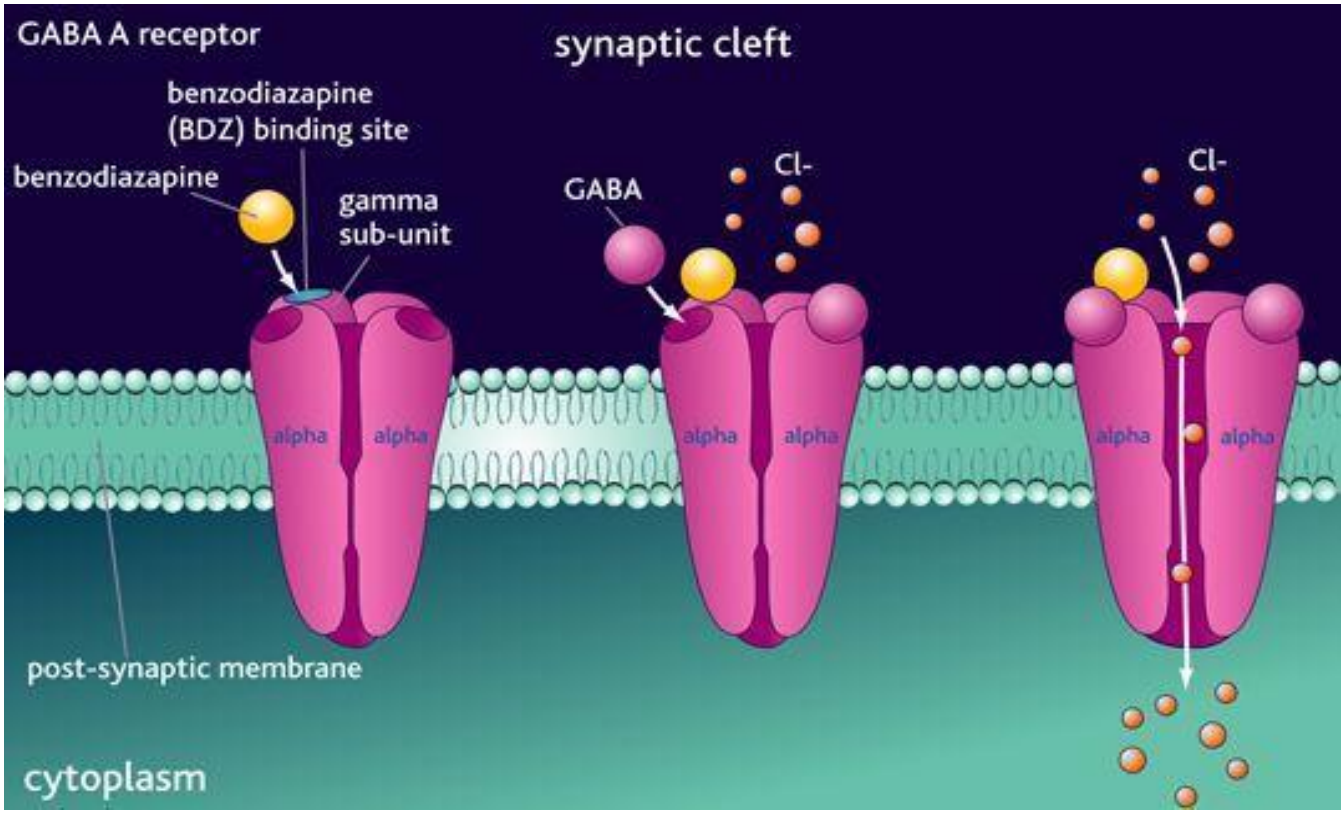


Figure 8. Description of BZD binding to $GABA_A$ receptor sited in neurons in the brain. $GABA_A$ receptor contains an ion channel that conducts chloride ions across neuronal cell membranes and two binding sites for the neurotransmitter GABA, while a subset of $GABA_A$ receptor complexes also contain a single binding site for BZD. Binding of BZD to this receptor complex promotes binding of GABA which in turn increases the conduction of chloride ions across the neuronal cell membrane. Taken from http://medicastore.com/apotik_online/image/obat_benzodiazepine.jpg

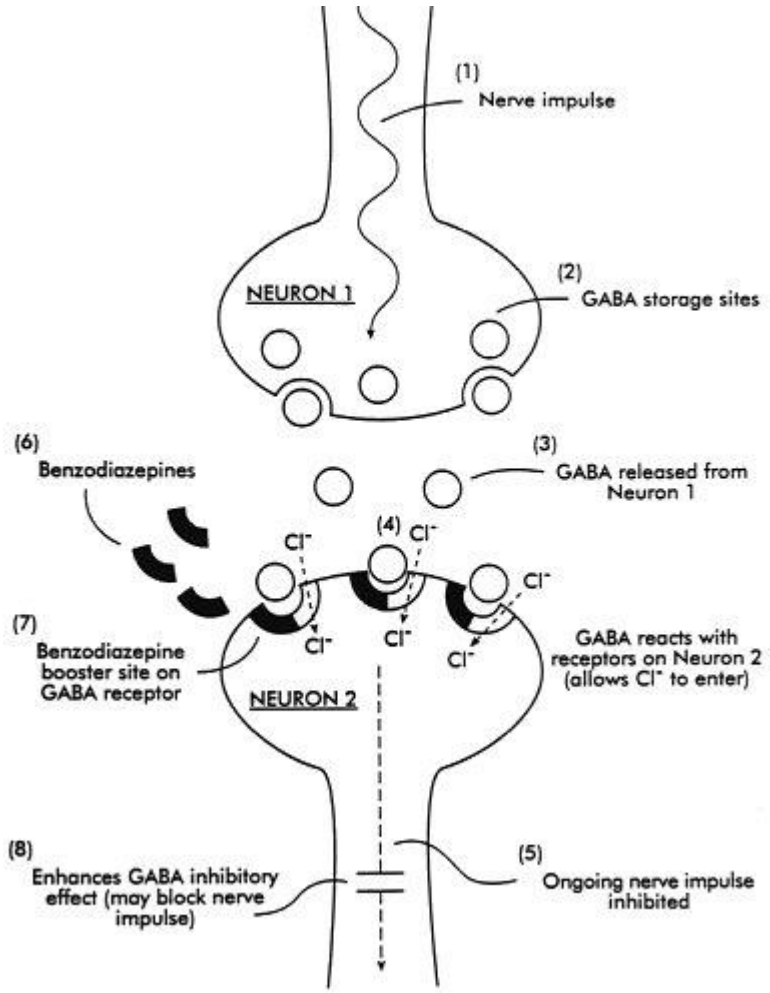


Figure 9. Diagram of mechanism of action of neurotransmitter GABA and BZD on nerve cells (neurons) in the brain. Binding of BZD to GABA receptor promotes binding of GABA which increases the conduction of chloride ions across the neuronal cell membrane and this increased conductance raises the membrane potential of the neuron resulting in inhibition of neuronal firing. Taken from The Ashton Manual, 2008.

4.5 Imaging protocol

Scans were acquired at the “Radiology Department”, Policlinico ”GB Rossi” - Verona, on a 1.5 T Magnetom Symphony Maestro class syngo MR 2002B (Siemens, Erlangen, Germany).

The collection of details necessary to allow an anatomical three dimensional reconstruction it has been obtained through performing the following sequence:

“T1_(3D)MPR_NS: TR 9 ms; TE 3,33 ms; Flip angle 12°; slices: 144 (trasversal);

thick 1 mm; FOV 235 mm; 1 acq.; matrice 256 x 256.”

fMRI scan sessions were performed while the subject was conscious. Each session consisted in two parts and the entire procedure lasted 15-25 minutes approximately.

4.5.1 First part: “resting state” test

During the first part the aim was to assess the “resting state” test.

The subject was asked to stay in wakeful, motionless and with eyes closed.

The sequence performed was EPI_2D_BOLD: TR 2000 ms; TE 50 ms; slices: 22 (trasversal);

thick 4,5 mm; FOV 225 mm; 1 acq.; 180 misurations; matrix 64 x 64; Fat Sat: yes;

4.5.2 Second part: "motor task" step

In the *second part* the subject performed the "motor task" step.

The motor task consists of two parts: during the first one, the subject was asked to stay wakeful, with eyes closed and to open and close the hands alternatively for 10 times; during the second part, the subject was asked to stay wakeful, motionless and with eyes closed for the same period of the first part. The motor task was repeated for 5 times.

The sequence performed was EPI_2D_BOLD (for motor task): TR 3500 ms; TE 50 ms; slices: 36 (trasversal); thick 3 mm; FOV 225 mm; 1 acq.; 100 misurations; matrix 64 x 64; Fat Sat: yes; Task paradigm size: 20.

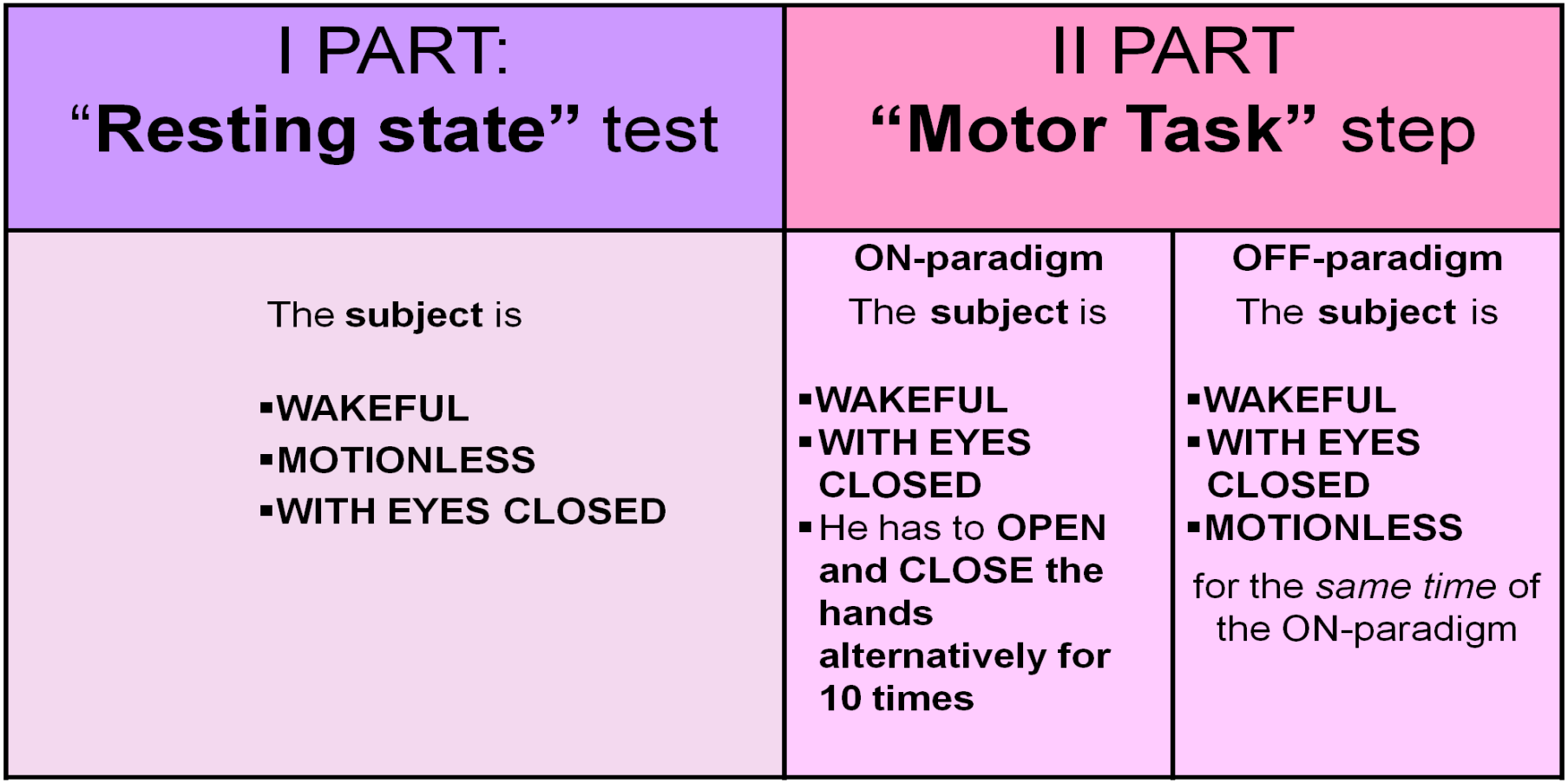


Figure 10. Schematic description of fMRI scan session: "Resting state" test and "Motor Task" step.

4.6 Data analysis

Brain resting-state functional-connectivity was measured in terms of pair wise correlations between low-frequency BOLD fluctuations in different brain areas.

Reproducibility of resting state test was evaluated by comparing the MRI results performed at pre (baseline) and post placebo conditions and at baseline in two different occasions.

The Δ between brain resting-state functional-connectivity at baseline and after administration of alprazolam was measured in terms of pair wise correlations between low-frequency BOLD fluctuations in different brain regions pre and post drug administration. This result was compared versus result collected after placebo administration.

The Δ between brain Motor-Task connectivity at baseline and after administration of alprazolam was measured in terms of pair wise correlations between low-frequency BOLD fluctuations in different brain regions pre and post drug administration. This result was compared versus result collected after placebo administration.

The functional data collected were analyzed using software FSL (FMRIB's Software Library, www.fmrib.ox.ac.uk/fsl). The technical details of analysis are reported below.

4.6.1 Pre – processing

fMRI data processing was carried out using FEAT (fMRI Expert Analysis Tool) Version 5.98, part of FSL. The following pre-statistics processing was applied: motion correction using MCFLIRT (Jenkinson 2002); non-brain removal using BET (Smith 2002); spatial smoothing using a Gaussian kernel of FWHM 5mm; grand-mean intensity normalization of the entire 4D dataset by a single

multiplicative factor; high pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with sigma=50.0s).

4.6.2 Post – processing and statistical analysis: "resting state" - DMN

The "resting state-DMN" analysis was carried out using Probabilistic Independent Component Analysis (ICA) (Beckmann & Smith, 2004) as implemented in MELODIC (Multivariate Exploratory Linear Decomposition into Independent Components) Version 3.10, part of FSL. ICA is a statistical technique that separates a set of signals into independent—uncorrelated and non-Gaussian—spatiotemporal components (Beckmann & Smith, 2004). When applied to the T2* signal of fMRI, ICA allows not only for the removal of artifact (Grecius et al., 2009; McKeown et al. 1998; Quigley et al. 2002) but also for the isolation of task-activated neural networks (McKeown et al. 1998; Gu et al. 2001; Calhoun et al. 2002). Most recently, ICA has been used to identify neural networks, including the DMN, during task-free or cognitively undemanding fMRI scans (Greicius et al. 2004; van de Ven et al. 2004; Beckmann et al. 2005).

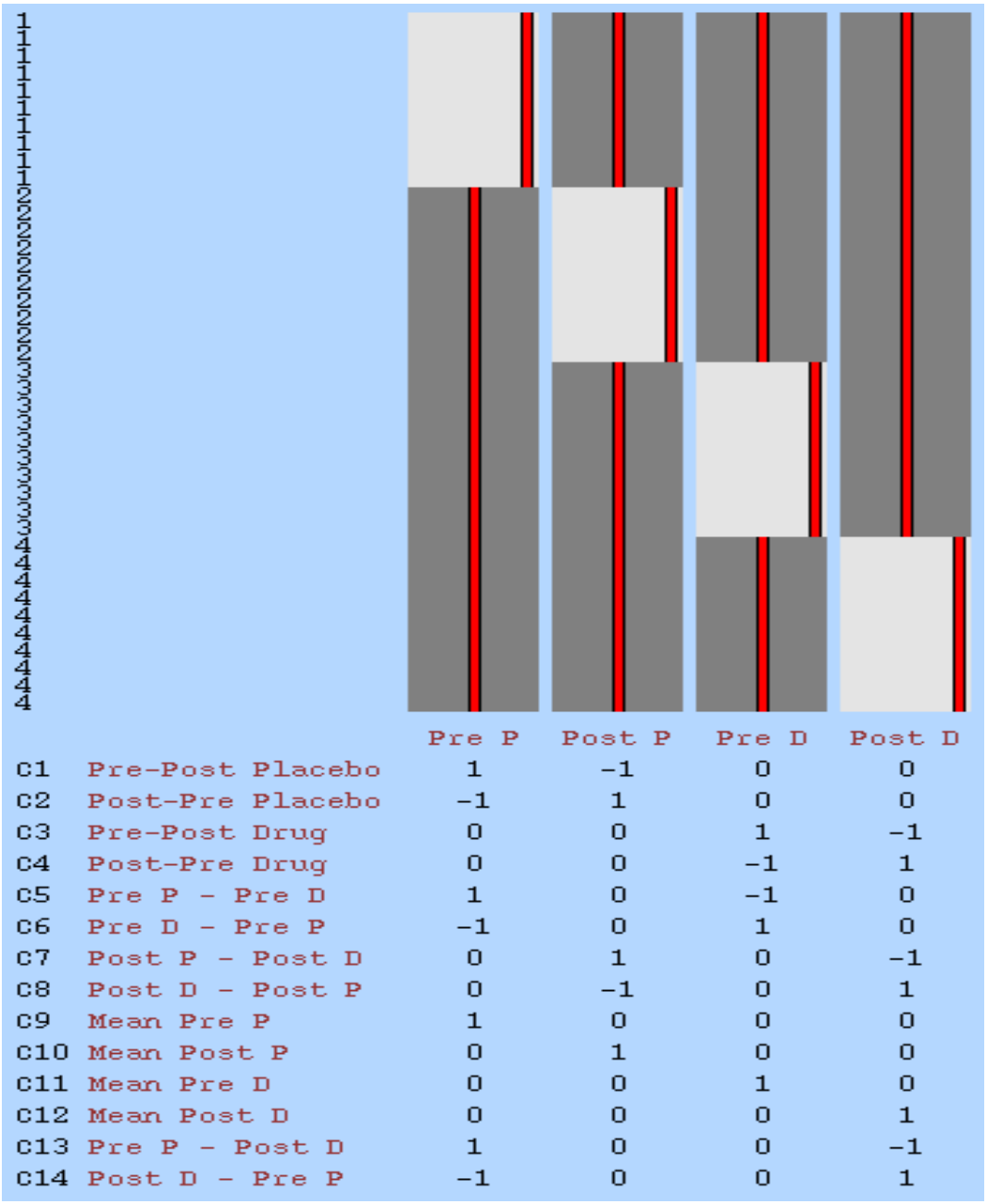
The following data pre-processing was applied to the input data: masking of non-brain voxels; voxel-wise de-meaning of the data; normalization of the voxel-wise variance; Pre-processed data were whitened and projected into a 35-dimensional subspace using Principal Component Analysis. The whitened observations were decomposed into sets of vectors which describe signal variation across the temporal domain (time-courses), the session/subject domain and across the spatial domain (maps) by optimizing for non-Gaussian spatial source distributions using a fixed-point iteration technique (Hyvärinen, 1999). Estimated Component maps were divided by the standard deviation of the residual noise and thresholded by fitting a mixture model to the histogram of intensity values (Beckmann & Smith, 2004).

The between-subject analysis of the resting data was carried out using a regression technique (dual regression) that allows for voxel-wise comparisons of resting fc (Beckmann, 2009; Filippini et al.,2009).

The first step was the use of Multi Session Temporal Concatenation in MELODIC FSL component in which multiple fMRI data sets are concatenated temporally and ICA is applied in order to identify large-scale patterns of fc in the subjects. At this level, it was also applied an F-test to verify if there are global differences in the DMN between the groups.

The next step was the dual regression technique, that allows to find out which spatial maps and associated time courses corresponding to the multi-subject ICA components, using a contrast matrix designed previously.

The last step was to collect the different sets of spatial maps across subjects and to analyze them by using a non-parametric 5000 permutation testing. The results were spatial maps defining the between-subject group-consistency and/or between-subject group-differences. The maps were thresholded with p value < 0,05 and visualized on T1 anatomic standard brain template (2 mm isovoxels) contained in FSL.



		Pre P	Post P	Pre D	Post D
C1	Pre-Post Placebo	1	-1	0	0
C2	Post-Pre Placebo	-1	1	0	0
C3	Pre-Post Drug	0	0	1	-1
C4	Post-Pre Drug	0	0	-1	1
C5	Pre P - Pre D	1	0	-1	0
C6	Pre D - Pre P	-1	0	1	0
C7	Post P - Post D	0	1	0	-1
C8	Post D - Post P	0	-1	0	1
C9	Mean Pre P	1	0	0	0
C10	Mean Post P	0	1	0	0
C11	Mean Pre D	0	0	1	0
C12	Mean Post D	0	0	0	1
C13	Pre P - Post D	1	0	0	-1
C14	Post D - Pre P	-1	0	0	1

Fig. 11. Dual-Regression contrast Matrix. The first part (upper) represents the subdivision of fMRI scan sessions undergone by subjects during the study: group n.1 corresponds to “pre placebo” scan sessions, group n.2 to “post placebo” ones, group n.3 to “pre drug” ones and group n.4 to “post drug” ones. On the left it’s reported the list of groups numbers and on the right the graphical representation of groups: each group is represented by a light gray section with a single red line. The second part (lower) reports the various contrasts set by the operator and the means. In the contrasts each number with minus sign corresponds to contrast subtracted.

4.6.3 Post - processing and statistical analysis: "motor task"

fMRI data processing was carried out using FEAT (FMRI Expert Analysis Tool) Version 5.98, part of FSL. Z (Gaussianised T/F) statistic images were thresholded using clusters determined by Z>2.3 and a (corrected) cluster significance threshold of P=0.05 (Worsley, 2003).

The first level of analysis consisted of designing the stimulation protocol of the motor task in FSL (35 seconds off, 35 seconds on, period 70 seconds, square wave time, gamma convolution) which was then applied to all the 40 fMRI sessions in order to obtain the cluster thresholded z-map per each subject.

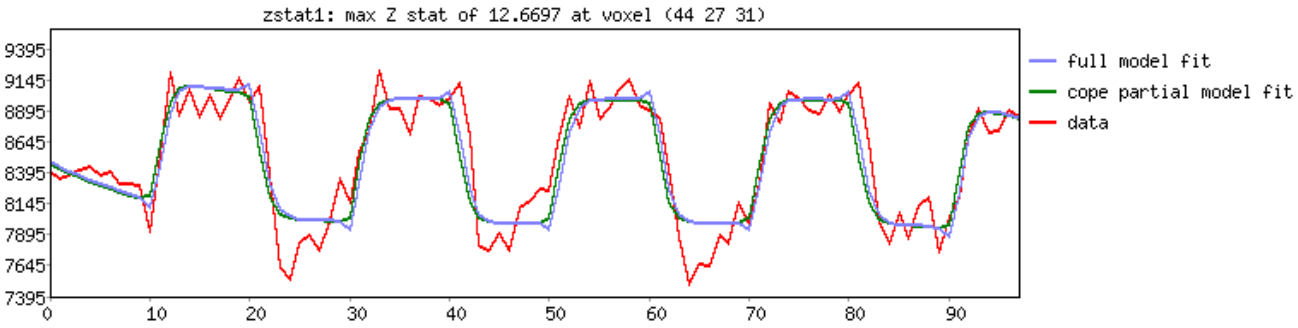


Fig. 12. Time series plot example (X axis, time in seconds; Y axis, intensity of the signal). The purple and green lines represent the full and partial mode fit, whereas the red line represents the data acquired.

After that step, one fMRI pre-placebo data was excluded by the analysis, because of the corruption of the data itself. Consequently, the higher level analysis was carried out on 39 fMRI data session applying a GLM design in order to find out the between-subject group-consistency thresholded z-map.

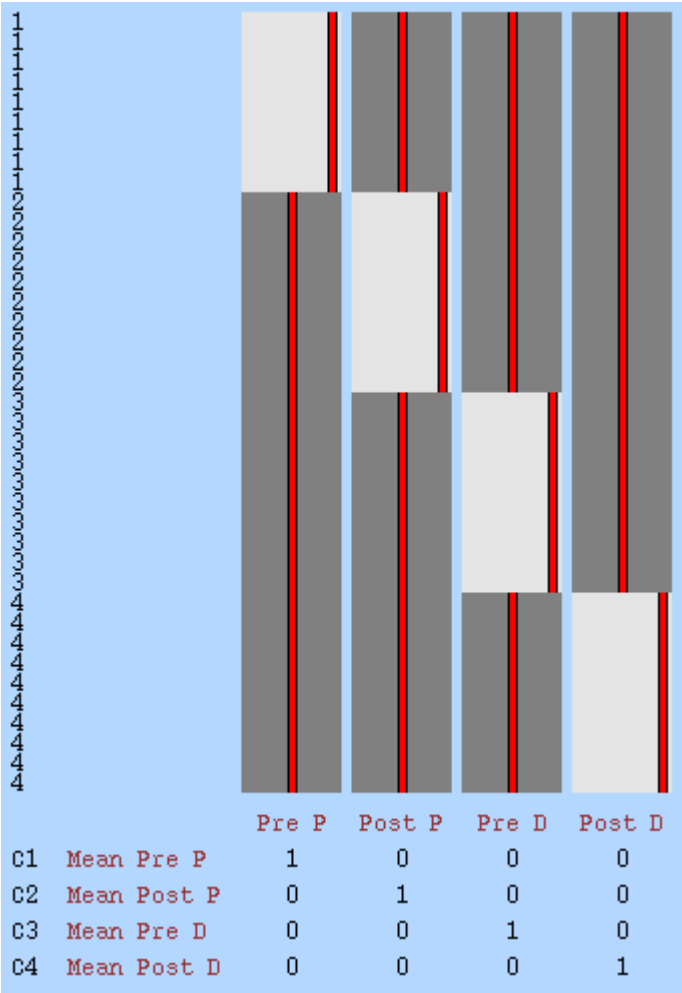


Fig. 13. GLM FEAT higher level analysis. The first part(upper) represents the subdivision of fMRI scan sessions undergone by subjects during the study: group n.1 corresponds to “pre placebo” scan sessions, group n.2 to “post placebo” ones, group n.3 to “pre drug” ones and group n.4 to “post drug” ones. On the left it’s reported the list of groups numbers and on the right the graphical representation of groups: each group is represented by a light gray section with a single red line. The second part consists in the setting of mean calculation per each group.

5 RESULTS

5.1 Clinical trial

A total of 14 healthy volunteers were screened, 11 subjects were enrolled into the study and one of them withdrawn consent before starting second study part. Therefore a further subject was enrolled and a total of 10 subjects completed the study. Only data collected from subjects who completed both sessions were considered for data analysis.

The baseline demographic characteristics (age and BMI) were similar among participants and there was a proportion between female and male subjects. The mean age ranged from 27 to 37 years, the mean weight ranged from 50.4 to 86.5 Kg and the mean BMI ranged from 19,30 to 27,60 Kg/m2.

In both part 1 and 2 subjects underwent “pre-dose fMRI scan” session between 7:00 and 7:30 a.m. and they received a single dose of alprazolam 0,75 mg or placebo between 8:00 and 8:30 a.m. All “post-dose fMRI scan” sessions were performed between 9:55 and 10:50 a.m. at approximately t-max of alprazolam (2hrs after dosing). During each fMRI scan session each subject attended the “resting state” test and the “motor task” step and the entire procedure lasted no more than 30 minutes as planned.

No serious adverse events (AEs) occurred. A total of 12 AEs were reported by 6 subjects. The majority of them were considered to be mild in intensity and they were mainly reported after alprazolam administration.

5.2 “Resting state” test – DMN

The maps of DMN obtained by applying the Multi Session Temporal Concatenation in MELODIC FSL component showed a common pattern across all the 40 sessions of fMRI, composed by the anterior, posterior and lateral components as represented in figure 14.

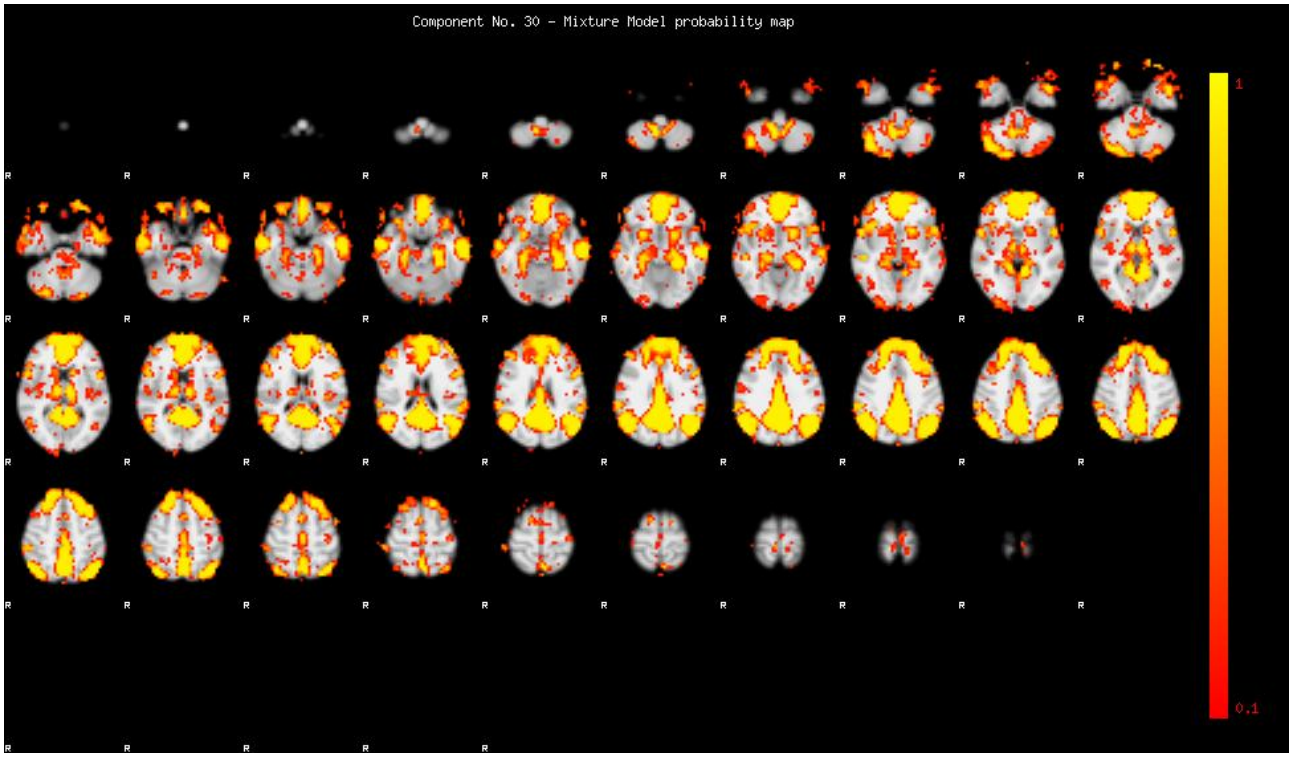


Fig. 14. The DMN representation across all fMRI sessions; maps obtained with Multi Session Temporal Concatenation in MELODIC FSL component and corresponding to different axial sections of brain. Colored brain areas correspond to DMN components. The gradation of color from orange to light yellow is proportional to the increase of probability of connectivity.

The distribution of BOLD fMRI low frequency fluctuations of the DMN resulted with a mean of about 1,4 Hz, defining the typical DMN powerspectrum timecourse.

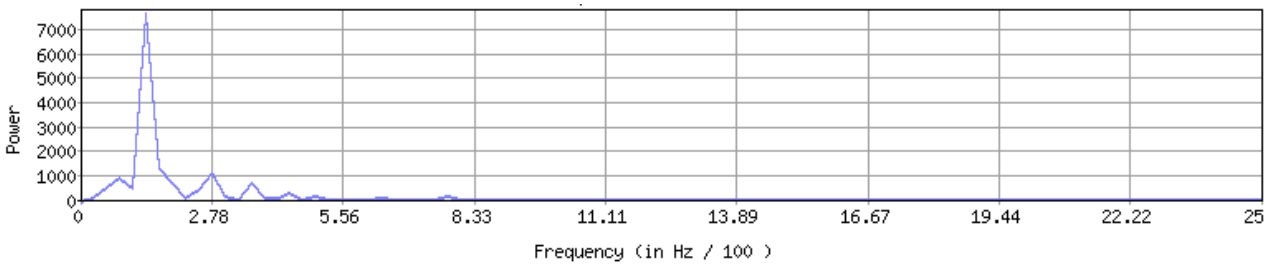


Fig. 15. Powerspectrum timecourse of all 40 fMRI sessions. In abscissa it's reported the frequency of BOLD fluctuations and in ordinate the intensity of signal. It's visible the peak at 1,4Hz.

The reproducibility of DMN across all subjects was first demonstrated by the contrast of two baseline conditions, pre placebo vs. pre alprazolam. The F-Test ($p < 0,05$) applied showed a non-statistically significant difference between these two conditions, with a $p < 0,11242$, as reported in Figure 16.

F-test on full model fit ($p < 0,05$) **40 sessions**	**Contrasts ($p < 0,05$)** **Groups 10 sessions each**	
F = 1.00 p < 0.42197	Pre-post placebo: Post alprazolam: Pre-pre: Post-post:	p < 0.11226 p < 0.48742 p < 0.11242 p < 0.48776

Figure 16. Table with summarized the p values obtained by application of statistical F-test to all contrasts evaluated.

The dual-regression approach permitted to explore local differences of connectivity. In this context various contrasts were examined in order to clarify the final role of alprazolam in modifying, or not,

the DMN. However the results obtained provided also evidences regarding reproducibility of DMN across subjects.

Dual regression: mean value across the groups

The dual regression method applied to the mean value per each group showed a very similar condition between pre-post placebo and pre alprazolam maps, whereas in post alprazolam map several differences were observed.

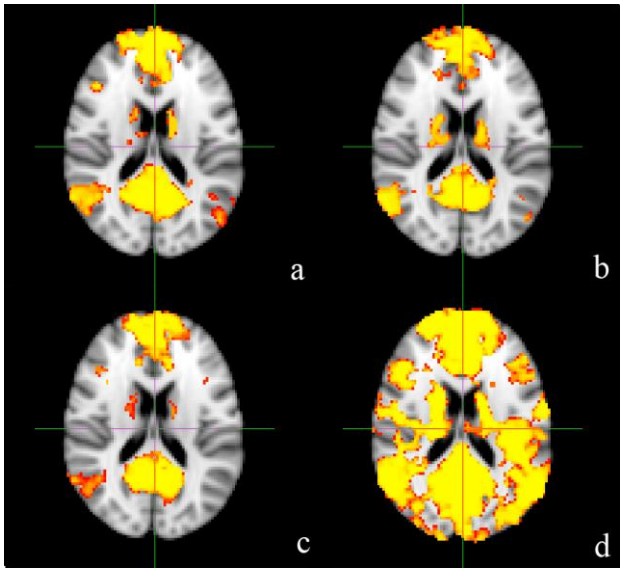


Figure 17. Dual regression z-maps on MRI T1 anatomic axial brain template. Maps show mean values of connectivity in the four groups. Pre-placebo (a), post-placebo (b), pre-alprazolam (c), post-alprazolam (d). The maps were thresholded with p value < 0,05. The gradation of color from orange to light yellow is proportional to the increase of connectivity. In the post alprazolam map (d) the light yellow is more intense and diffuse due to a widespread and increased connectivity.

In order to understand how the connectivity was modified by the drug, the dual regression approach was applied in contrast between groups. The most representative images were reported below even if the analysis was performed by considering all axial coronal and sagittal images.

Pre vs. post placebo

Figure 18 shows that the dual regression between pre and post placebo conditions did not point out a statistically significant difference: there is no evidence of areas where the fc is modified.

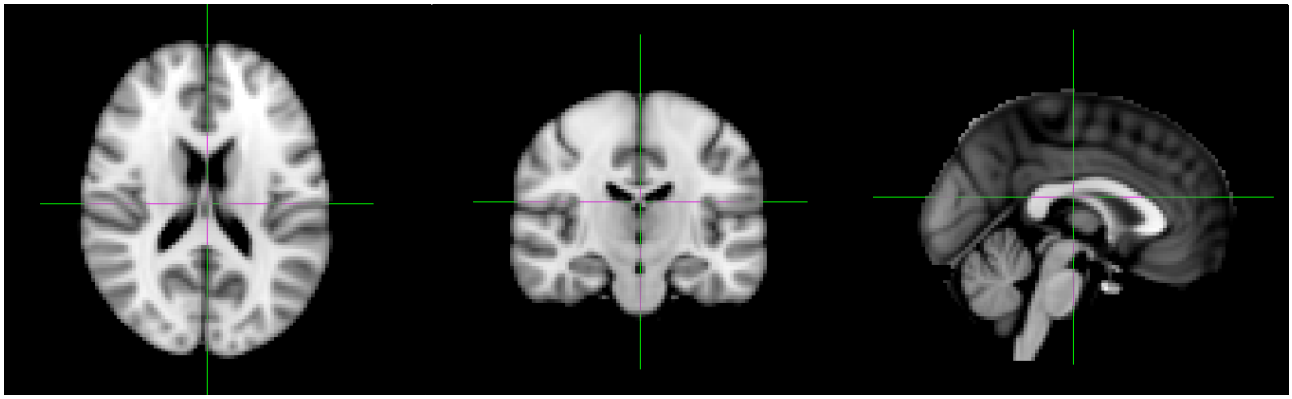


Fig. 18. Dual regression z-maps on MRI T1 brain template. Pre vs. post placebo (map thresholded with p value < 0,05). In this contrast there is not an increase of connectivity statistically significant: results are under threshold and no area is colored.

Pre vs. post alprazolam

The result obtained with dual regression between pre and post alprazolam showed a diffuse increased connectivity in post alprazolam condition (blue color), in particular in the posterior component of DMN (PCC), in the thalamus and bilateral parietal cortex. Pre alprazolam areas (red color) cannot be seen, because they are under threshold.

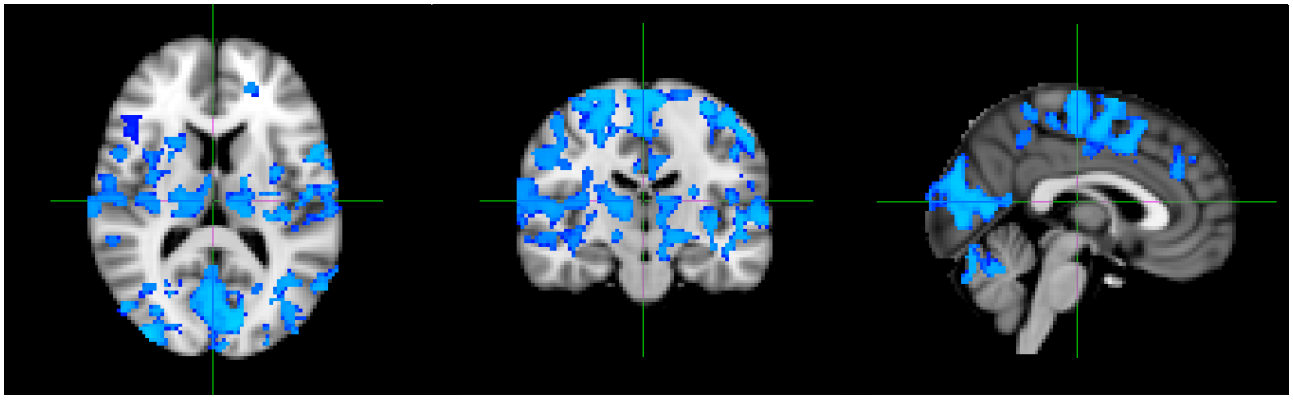


Fig. 19. Dual regression z-maps on MRI T1 brain template (map thresholded with p value < 0,05). The contrast post alprazolam-pre alprazolam (blue color) can be seen due to an increase of connectivity. The contrast pre alprazolam-post alprazolam (red color) can not be seen, because results are under threshold.

Pre placebo vs. pre alprazolam

The result obtained with between pre placebo vs pre alprazolam contrast did not show any statistically significant differences.

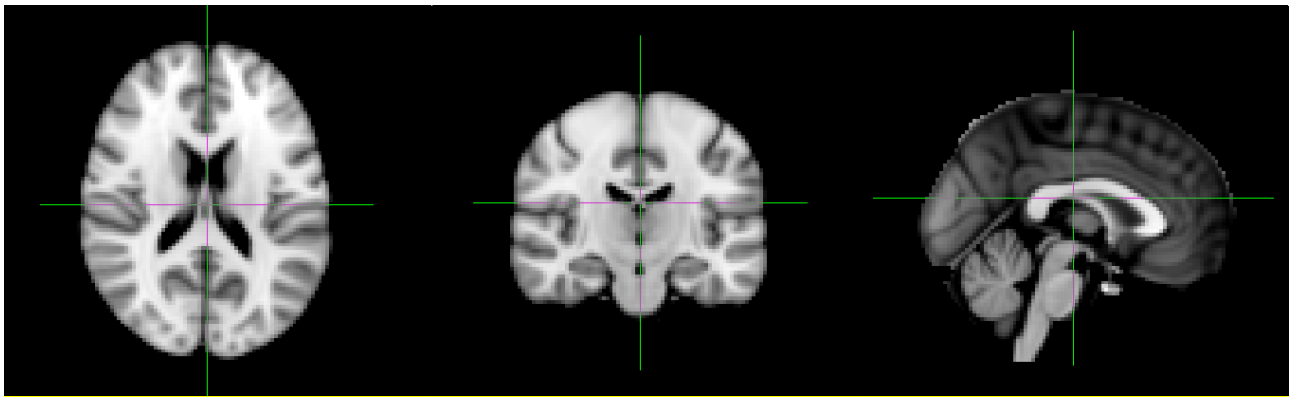


Fig. 20. Dual regression z-maps on MRI T1 brain template (map thresholded with p value < 0,05) pre placebo-pre alprazolam contrast. In this contrast there is not an increase of connectivity statistically significant: results are under threshold and no area is colored.

Post placebo vs. post alprazolam

The contrast between post placebo and post alprazolam revealed an increased connectivity as the contrast between pre and post alprazolam. Post placebo areas (red color) are under threshold.

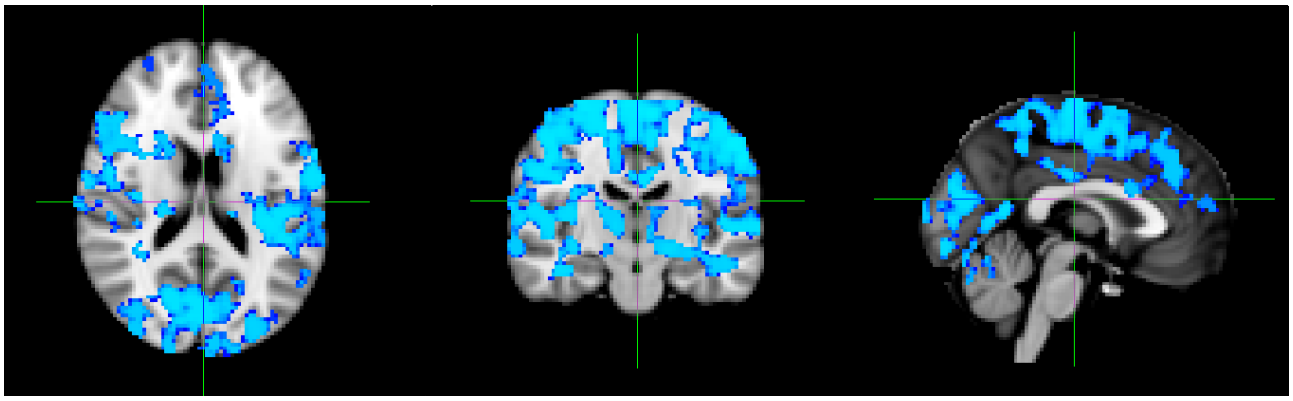


Fig. 21. Dual regression z-maps on MRI T1 brain template (map thresholded with p value < 0,05). The contrast post alprazolam-post placebo (blue color) can be seen due to an increase of connectivity. The contrast post placebo-post alprazolam (red color) can not be seen, because results are under threshold.

Pre placebo vs. post alprazolam

To complete the analysis, dual regression method was applied also between pre placebo and post – alprazolam conditions. The result confirmed an increase of connectivity in post drug condition.

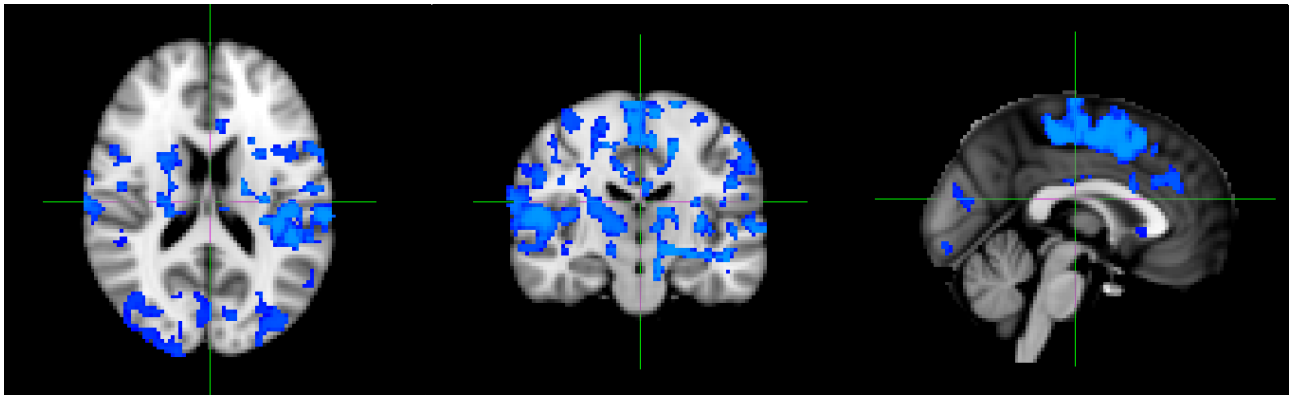


Fig. 22. Dual regression z-maps on MRI T1 brain template (map thresholded with p value < 0,05). The contrast post alprazolam-pre placebo (blue color) can be seen due to an increase of connectivity. The contrast pre placebo-post alprazolam (red color) can not be seen, because results are under threshold.

5.3 Motor task" step

The cluster thresholding (Z > 2.3, p = 0.05) showed a reduced activity in both post placebo and post alprazolam maps (see figures 23 and 24). However the reduction between pre and post alprazolam was far more greater than the placebo one. The number of voxel thresholded was 13779 in pre placebo and 11047 in post placebo, whereas was 17109 in pre alprazolam and 8157 in post alprazolam. The change of activity can be observed in primary motor area, bilateral primary somato-sensitive areas and bilateral cerebellar areas.

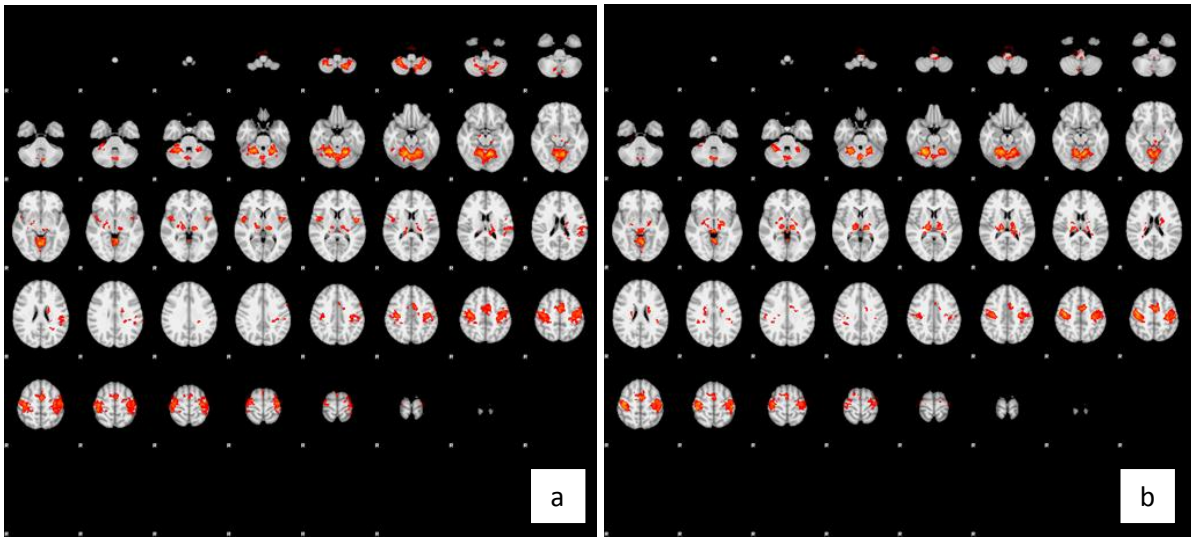


Fig. 23. Pre placebo (a) vs. post placebo (b). The orange red marks on a MRI T1 anatomic multiple slices axial template, correspond to areas activated by motor task.

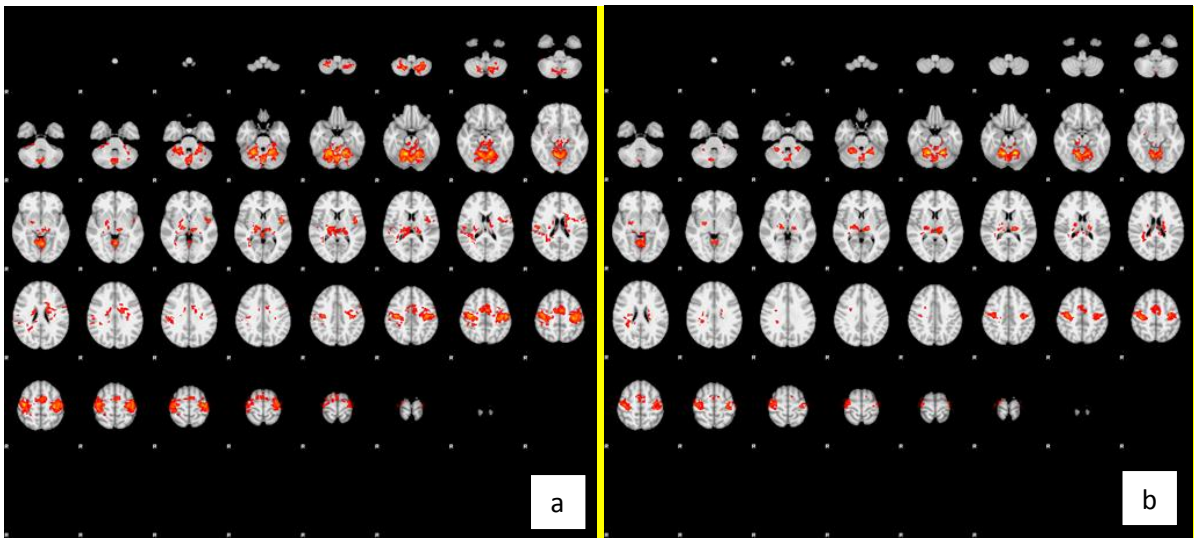


Fig 24. Pre alprazolam (a) vs. post alprazolam (b). The orange red marks on a MRI T1 anatomic multiple slices axial template, correspond to areas activated by motor task.

6 DISCUSSION

This project was designed and planned with a translational main object i.e. the development of a model, the "resting state" test, with an immediate application to clinical research. For this reason, resting state test was designed to be feasible applied to humans during early development phase of a drug with putative anxiolitic-antidepressant activity.

During last ten years several studies were carried out to investigate the presence of a spontaneous BOLD activity in animal and human brain during resting state. The observation that this spontaneous active state is not a random noise, but it is specifically organized in the resting human brain has generated a new avenue of neuroimaging research. The functional network organization of the brain is stable across species, thus paving the way for translational studies with particular potential for pharmacological efficacy assessment. There is also a strong interest in exploring the clinical potential of assessments supported by promising early results, feasibility of application and not needing for demanding patient co-operation. (Auer, 2008).

According to these considerations it was decided to set-up the resting state model by performing a clinical trial in healthy volunteers. To explore resting state in humans it was chosen BOLD fMRI technique. Compared to other radiological techniques (i.e. PET) this method is safe and not invasive: it can be applied to young and old subjects and it is based on an endogen contrast. According to that it was possible to ask subjects to perform a total of four scan sessions during the study without any impact on their health. As drug it was chosen a BZD, alprazolam: it is known that it can pass through the BBB and acts on CNS. Furthermore, given that the drug would have been taken by healthy subjects, it was decided to administer a single dose of it at a dosage considered as safe and well tolerated, 0,75 mg. Among resting state networks, it was decided to mainly investigate the DMN, due to his unique characteristic to show an high activity during rest

and deactivation during task. It is commonly accepted that this network can show aberrant patterns in fc in patients with psychiatric and neurological conditions or in patients with family medical history for Alzheimer's disease or Parkinson's disease; for this reason it was decided to exclude subjects which met those criteria.

The resting state paradigm was designed according to the common definition of resting state condition where human brain is wakeful and conscious, but relaxed and in absence of specific stimulus. The time for the execution of scan sessions was thought with the aim to detect a baseline condition and a condition where the DMN could have been potentially modified by the pharmacodynamic effect of the drug, i.e. time corresponding to t-max of alprazolam. To date, very little data is available on the reproducibility of fcMRI studies (Amman et al., 2009), for this reason it was thought to plan two identical study sessions, one with alprazolam and one with placebo according to a double blind, crossover design.

The decision to test motor task and effect of alprazolam in motor task step was taken in order to collect data regarding changes in brain motor areas. The set-up of motor task paradigm was more complicate than the resting state one. It was designed in order to have a symmetric and bilateral activation of brain areas through simple movements which could be performed in a MRI machine. Plus, the activation had not to be influenced by right or left hand. It was also discussed the total number of sequences when the subject had to open and close the hands in order to have a significant answer. The final decision was to ask subjects to stay wakeful, with eyes closed and to open and close both hands alternatively for ten times, then to ask them to stay wakeful, motionless and with eyes closed for the same period of the previous part; this task had to be repeated for 5 times.

The clinical phase of the study was carried out as scheduled. A higher number of healthy volunteers had to be screened in order to recruit subjects meeting study criteria. However, specialist knowledge and experience of CRC allowed to complete this step without any important delays. All procedures were carried out in agreement with the study protocol and the time schedule was respected. The "resting state" parts and the "motor task" steps were performed by applying the model defined and detailed into the protocol. No one protocol waiver was recorded. Alprazolam and study procedures were well tolerated. The majority of AEs occurred were considered to be mild in intensity and mainly reported after alprazolam administration.

A total number of 11 subjects had to be enrolled into the study, because one subject not completed both study sessions. Only data sets obtained by subjects who had completed both sessions were analyzed.

The choice of the more appropriate methodology to be applied for data analysis was taken by following most recent scientific articles. To perform the DMN post-processing and statistical analysis it was decided to require opinions of FSL forum experts and last steps of analysis were driven by their instructions.

The reproducibility of DMN across all subjects was first demonstrated by the contrast of two baseline conditions, pre placebo vs. pre alprazolam. The F-Test (thresholded per $p < 0,05$) showed a a statistically non-significant difference between these two conditions, with a $p < 0,11242$. The further analysis performed through dual-regression method did not point out a statistically significant difference as well. A very similar condition was showed between two baseline and pre-post placebo conditions. In details there were not areas where the fc had been modified.

The main result obtained from the analysis was a diffuse and increased fc highlighted in post alprazolam maps. Dual regression method allowed to detect these changes due to his peculiarity of focus on local differences rather than on global statistical maps. The maps obtained show an

increase of connectivity mainly in the posterior component rather than in the other components of DMN; plus these changes were detected also in brain areas out of DMN i.e. thalamus and bilateral parietal cortex. This important increase of connectivity can be explained through DMN main characteristic i.e. it is characterized through high activity during rest and deactivation during tasks and due to this reason it is also called "task-negative network". This increase of connectivity can be interpreted as a consequence of the administration of a drug with a sedative effect. This effect can further inhibit tasks and increase activity at rest. According to this interpretation, the DMN or "task-negative network" can tent to increase and it can be more evident. The presence of changes also in brain areas out of DMN is due to the binding of alprazolam to BZD receptors in CNS.

The results obtained from motor task steps showed an evident reduction of activity in brain motor areas. It can be observed changes in primary motor area, bilateral primary somato-sensitive areas and bilateral cerebellar areas. The changes on first two areas are related to physical movement functionality and the one on cerebellar areas is related to the function of coordination. These results are in agreement with characteristics of motor task paradigm as defined into the study protocol. A reduction of motor activity can be justified by the pharmacological effect of drug.

In summary both primary and secondary aims of the study have been achieved. Resting state test was set up and its reproducibility in healthy volunteers was demonstrated statistically (F test) and also by applying dual regression method. It was demonstrated that the effect of a single dose of alprazolam on brain activity in resting state consists in an increased and diffuse connectivity in DMN and in other cerebral areas. Alprazolam effects in motor task step consist in a reduction of activity in motor areas previously activated by motor task.

In conclusion, the modification of DMN after administration of a BZD was demonstrated through the study by the application of a common and not invasive technique. The model can be considered

set-up and applicable in humans during future clinical trials. The maps generated during motor task step increase knowledge of human brain topography and that is in line with previous studies.

In the future we can plan to apply the resting state test to investigate effect of drugs with putative anxiolitic-antidepressant activity during its early development phase. Plus we can also schedule to carry out a similar clinical trial in patients affected by psychiatric and neurological diseases and to compare results obtained in patients against the ones in healthy subjects.

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