

"Advances in neuroimaging and pharmacology: an example using nicotine"

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Dissertation

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Abstract

The outline of the dissertation consists of four parts. First, the general framework about the recent impact that neuroimaging is having with respect to CNS pharmacology will be presented (Chapter 1). Subsequently, an EEG experiment based on the administration of nicotine during a resting-state task in healthy non-smokers will be introduced. The research question regarded whether nicotine impacts vigilance. The same data set and the same within-subject experimental design were used for conducting three different analyses. Each method of each analysis was considered appropriate for detecting vigilance modulations in the brain. In Chapter 2, the results of the first analysis based on source reconstruction (Current Source Density, CSD) in the whole-brain will be shown. In Chapter 3 two further analyses were based on the extraction of time-series. There it will be shown the results of these last two analyses: connectivity analysis by renormalized Partial Directed Coherence (rPDC); phase-amplitude coupling analysis by Mean Resultant Vector Length (VL). In the final chapter, the results of the three analyses will be compared. It will be emphasized that all three results converged in the finding that nicotine significantly increased vigilance. It will then be suggested that nicotine might unravel its cognitive-enhancing properties through its ability to counteract drowsiness. In conclusion, the present dissertation aims to show recent progress in the field of CNS pharmacology by using state-of-the-art non-invasive neuroimaging techniques and by using nicotine as a pharmacological probe in healthy subjects.

Zusammenfassung

Die vorliegende Dissertation ist in vier Teile gegliedert. Zunächst wird dargestellt, welche Rolle funktionelle Bildgebung zur Untersuchung von Pharmaka Effekten im zentralen Nervensystem (ZNS) hat (Kapitel 1). Anschließend wird ein EEG- Experiment vorgestellt, Einfluss die neuronale Bildgebung auf die Zentrales Nervensystem (ZNS)-Pharmakologie hat (Kapitel 1). Anschließend wird ein EEG- Experiment vorgestellt, welches die Applikation von Nikotin während einer Ruhezustandsaufgabe (sog.“resting-state“) bei gesunden Nichtrauchern untersucht. Dabei soll der Einfluss von Nikotin auf die Vigilanz erforscht werden. Der gleiche Datensatz wurde dann für drei unterschiedliche „within-subject“ Analysen verwendet. Jede der drei Analysemethoden eignet sich zur Detektion der Vigilanzmodulation im Gehirn. Im zweiten Kapitel werden die Ergebnisse der ersten Analyse gezeigt, welche auf „whole-brain source reconstruction“ basieren (Current Source Density, CSD). Im dritten Kapitel werden zwei weitere Methoden vorgestellt, welche auf der Extraktion von Zeitreihen beruhen. Die Ergebnisse dieser letzten zwei Analysen; einer Konnektivitätsanalyse via „renormalized Partial Directed Coherence“ (rPDC) sowie der Analyse der Phasen-Amplituden Kopplung mittels „mean resultant vector length“ (VL) werden hier vorgestellt. Im letzten Kapitel werden die Ergebnisse der drei Analysen verglichen. Es wird hervorgehoben, dass alle drei Ergebnisse übereinstimmend einen signifikanten Anstieg der Vigilanz als Folge von Nikotin zeigen. Darauf basierend wird vorgeschlagen, dass die gesteigerte Kognitionsleistung durch Nikotinauf eine müdigkeitsmindernde Wirkung zurückzuführen ist. Zusammenfassend wird mit der vorliegenden Dissertation beabsichtigt, unter der Verwendung neuester nicht-invasiver bildgebender Methode und Nikotin als Pharmazeutikum in gesunden Probanden die aktuelle Entwicklung der ZNS-Pharmakologie aufzuzeigen.

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Chapter 1: General Introduction

1.1 Introduction

The main goal of the present dissertation will be to convince the reader that non-invasive neuroimaging is slowly revolutionizing Central Nervous System (CNS) pharmacology. Indeed, it never happened before that we can track the effect of a psychoactive drug on-line (when the drug is actually in action) and in the end-user (human beings) (Wong *et al.* (2009)).

In this paragraph, I will discuss the rationale behind the claim that non-invasive neuroimaging offers an unprecedented opportunity to shape next generation of CNS pharmacology. At the beginning I will provide a working definition and a historical perspective of CNS pharmacology, highlighting the recent improvement driven by neuroimaging (Paragraph 1.2). Further, some warnings will be issued regarding the problem of reproducibility in pharmacology and in neuroscience (Paragraph 1.3). I will address the recent potential of neuroimaging for the pharmaceutical industry, namely in terms of early drug development (Paragraph 1.4).

1.2 Definition and historical perspective of Central Nervous System pharmacology

In terms of definitions, at the moment there is no agreed terminology to define the field which stands at the intersection of the two disciplines of pharmacology and non-invasive neuroimaging. With non-invasive neuroimaging I mean the use of various imaging techniques for detecting brain dynamics. Electroencephalography (EEG) belongs to neuroimaging and it is the main technique used in the analyses presented in Chapter 2 and Chapter 3.

One of the reasons for missing an agreed terminology could be the lack of novel methods which guarantees a stand-alone discipline. Indeed, such a field blends together methods and techniques which belonged originally to either pharmacology or neuroimaging. For example, a dose-response assessment or the recording of brain oscillatory activity are solid methods that belong to either pharmacology or neuroimaging, respectively. Hence, pharmacology and neuroimaging could be considered a sub-discipline, which originates from the two independent disciplines of either pharmacology or neuroimaging. I will not coin a new term (e.g. pharmaco-neuroimaging) for such sub-discipline. Therefore the terms *pharmacology and neuroimaging* will be used throughout the manuscript, referring to the bulk of work which has been created by combining these two disciplines.

In order to appreciate the revolution which is taking place within CNS pharmacology, I need to put it into perspective. Therefore I will briefly describe the historical landmarks which shaped CNS pharmacology up to modern ages. The choice of landmarks is personal and reflects a subjective way of organizing new discoveries and new techniques in CNS pharmacology. Each landmark triggered a sort of revolution in the bulk of knowledge about CNS pharmacology. In other words, each landmark provides a further layer of knowledge in characterizing a psychoactive molecule. For example, nicotine action on the CNS can be described by a behavioral, biochemical, genetic or neuroimaging point of view. Each point of view provides a new layer of knowledge which adds to the previous one. Having all layers of knowledge together helps with “drug profiling”: all the amount of knowledge which characterizes a molecule is gathered, and a coherent picture is attempted.

CNS pharmacology became a rigorous science around 1950. Back then, trained physicians observed the overt **behavior** (signs and symptoms) of people under the influence of CNS drugs. By observing behaviors, researchers could - for example - discover either an antipsychotic or an antidepressant effect of a specific molecule. Indeed, chlorpromazine as an antipsychotic and imipramine as an antidepressant were discovered serendipitously by behavioral observations only. Lastly, the taxonomy of CNS drugs (e.g. anxiolytics; antipsychotics etc.) was developed in those years. These examples demonstrate how observing behaviors can provide insight to the understanding of the “rough” effects that CNS drugs can have.

Around 1980, **biochemistry** started to revolutionize CNS pharmacology. For example, metabotropic receptors (called also G-protein-coupled receptors) were discovered. Indeed, the signaling pathway (namely, a complex protein phosphorylation) triggered by the binding between a molecule and its receptor is a good example of how pharmacology was advanced by biochemistry. Further, the development of radioligands helped to characterize the receptors to which a molecule binds. A modern version of the radioligand methodology applied to CNS pharmacology is the Positron Emission Tomography (PET), today widely used in drug development.

Around 1990, **pharmacogenomics** started to come into play in CNS pharmacology. Indeed, it was clear that the genetic milieu could play a role in characterizing the pharmacokinetics of a molecule (e.g. liver enzymes which distinguish a “poor metabolizer” from “good metabolizer” of a specific molecule). Another application

of genetic to CNS pharmacology was the use of knock-out mice. Lastly, optogenetics from 2000 began to inform CNS pharmacology as well.

At the beginning of the new millennium (around 2000), **non-invasive neuroimaging** started to push CNS pharmacology to the next level of understanding. This led into an astonishing time for CNS pharmacology, since we have never had such an accuracy: studying how CNS drugs work on the CNS of a living human being. Among the different neuroimaging techniques currently available, functional Magnetic Resonance Imaging (fMRI), was the most successful in convincing the pharmaceutical industries that neuroimaging does help the early drug development process. Indeed, one of the first papers showing a potential application of fMRI as a tool for early drug development was published by Borsook *et al.* (2002). Another worthwhile new technique which is starting to be used by CNS pharmacologists is Magnetic Resonance Spectroscopy (MRS). It can detect the modification of neurotransmitters by a psychoactive drug in the human brain non-invasively (Waschkies *et al.* (2014).

1.3 Non-invasive neuroimaging is revolutionizing CNS pharmacology

As mentioned in the previous paragraph, non-invasive neuroimaging is slowly reshaping modern CNS pharmacology. Regarding EEG and Magnetoencephalography (MEG), in recent years a big interest has been raised in detecting how pharmacological challenges could modify brain oscillatory activity. In current neuroscience, *neuronal circuits* seem to be the interface between brain biochemistry, oscillations and behaviors (Whittington *et al.* (2011), (Womelsdorf *et al.* , 2014)). What oscillations measured by EEG and MEG can realistically tell to CNS pharmacology is confined mainly to what we know about the correlation between oscillations and behavior. Interestingly, oscillations can correlate very well with sedation (e.g. slow waves which characterize either deep sleep or general anesthesia). Particularly, EEG and MEG are powerful in detecting on-line drowsiness (Sander *et al.* (2015)). Such a property could be used to the advantage of CNS pharmacology: EEG and MEG can precisely detect whether a drug has either increased vigilance (e.g. psychostimulants) or decreased vigilance (e.g. sedatives). The two studies presented (Chapter 2 and Chapter 3) will exploit a similar rationale: asking the subjects to close their eyes triggers a drowsy brain state. Such a brain state can be probed by the administration of a psychostimulant (i.e. nicotine). The combination of a drowsy brain

state and a psychostimulant is called an *orthogonal experimental design*: it guarantees the maximization of the effect by comparing two opposite experimental conditions.

1.4 Current problems in pharmacology and neuroimaging

In science as a whole there is a big problem of replicating results from other laboratories (Collins and Tabak (2014)). In the whole neuroscience, this problem - and countermeasures to alleviate it - have been addressed previously (Button *et al.* (2013) and Hartshorne and Schachner (2012)). Overall it seems that there are six main causes which converge in the abovementioned lack of reproducibility (namely, idiosyncratic results produced by the same scientific field). The causes seem to be *lack of standardization*, *inter-subject variability* and *intra-subject variability*. Such causes are also linked with the overall problem of *defining objective biomarkers* in CNS pharmacology, which translate to the current *inability to make predictions* about the individual's response to a CNS molecule. Lastly, there are still some problems for a smooth translation of results regarding the effect of the same CNS drug measured from different species (*translational neuroscience*). These six potential sources of the lack of replication will be discussed in details later.

The immediate consequences which emerge in an industrial setting because of lack of replication affect early drug development. The effects of lack of replicability emerge clearly in a multi-site research environment. Specifically, the consequences of the lack of replicability make it difficult to meet go/no-go decisions in early drug development (Leiser *et al.* (2011)). Indeed, lack of consistent results regarding a specific CNS molecule can make it complex to decide whether the molecule is worth more investment or the project should be halted. More about the go/no-go decision in early drug development will be discussed in paragraph 1.5.3.

As follows, each of the six potential sources of the lack of replication which affect CNS pharmacology will be addressed separately. A set of possible remedies for each source will be addressed as well. When possible, I will show how we attempted to implement some of the remedies in our two studies about the nicotine effect in the brain.

1.4.1 Lack of standardization

Regarding CNS pharmacology *lack of standardization* is defined as a problem which hinders the chances to obtain replicable results among different laboratories (Wilson *et al.*, 2014). Indeed, the lack of standardization can be a problem because pieces of research of a drug candidate are run in different laboratories around the world. Often these pieces of information can be hardly combined together, even if the same drug molecule is studied.

A possible remedy is to develop a standardized experimental setting in order to obtain replicable results among different laboratories. For example guidelines like Jobert *et al.* (2012) should help the neuroscientific community to comply with standards when studying CNS molecules. At large, there is a need in biological sciences to follow agreed-upon protocols and benchmarks (Collins and Tabak (2014)).

Another problem linked with the lack of standards in the design of an experiment is the cognitive task chosen to probe the pharmacological activity of a CNS drug. Indeed, huge variety exists in terms of cognitive tasks. Also the “versions” of a specific cognitive task could vary a lot.

One strategy suggested in Jobert *et al.* (2012) to overcome such a problem is the use of a "minimalistic" benchmark based on the resting-state: eyes-open (EO) and eyes-closed (EC). This should help running head-to-head comparisons of different psychoactive drugs since it avoids the variability intrinsic to the choice of cognitive tasks. Indeed, the variability intrinsic with the CNS drug could interact with the variability in terms of cognitive task chosen, thus jeopardizing an easy interpretation how the drug impacts the CNS. Further, implementing resting-state design is pretty straightforward in terms of programming burden.

The drawback in opting for resting-state only is less flexibility in the experimental design. Indeed, when using only eyes-open and eyes-closed designs we force the experiment to overlook the interaction between a CNS molecule and a cognitive task. Unless there are agreements on a standard cognitive task to be used, I think that is better to limit the explorative research to resting-state activity.

For the above reasons we decided to run our nicotine experiment focusing only on resting-state activity (eyes-open and eyes-closed).

1.4.2 Inter-subject variability

Inter-subject variability is a long-standing problem in pharmacology. Particularly, the difference between responders and non-responders to a CNS drug is a major issue in the field. Indeed, different individual will often react differently to the same CNS drug.

The simplest way to deal with such problem is to stratify subjects in responders and non-responders. This could be done by using end-points like rising blood pressure in responders versus non-responders of a nicotine treatment.

There are also some methods to predict beforehand whether a specific subject is either responder or non-responder. Pharmacogenomics (e.g. detecting how liver enzymes are different in different individuals) has been suggested as a method to stratify subjects before running the experiment.

Beyond pharmacogenomics, also neuroimaging methods have tried to predict responders versus non-responders of a drug treatment. Some early attempts used EEG source reconstruction for predicting who will either respond or not to the antidepressant nortriptyline (Pizzagalli *et al.* (2001)). More recently Support Vector Machine (SVM) techniques applied to fMRI has being used for stratifying the subjects in a predictive fashion (Doyle *et al.* (2015)).

In our study we have not used any technique for stratifying the group of subjects who received nicotine. Such analysis has been done elsewhere using cardiovascular measurements after nicotine administration (Logemann *et al.* (2014)). We have also not used predictive ways of stratifying subjects, for example by using complex SVM methods.

1.4.3 Intra-subject variability

Intra-subject variability regards how one subject's brain activity changes from one session to another. Such a difference between the two session recorded in the same subject can be present even within the same day. That is puzzling since the same CNS drug has been administered in the two different sessions in the same subject. As for inter-subject variability, also intra-subject variability could generate discrepancy in a pharmacological study. As it will be explained later, a key issue of intra-subject variability concerns the estimation of single-subject variability. Single-subject variability is a combination of intra-subject variability (e.g. single-subject's homeostatic responses

and own biology) and within-session errors of measurement (Sinkkonen and Tervaniemi (2000)).

A *mixed-effects design* can be used for tackling intra-subject variability. Indeed, it effectively estimates at first single-subject variability (Friston *et al.* (2005)), then inter-subject variability (Stelzer *et al.* (2014)). In simple words, a mixed-effects design is an evolution of a longitudinal design (also called “repeated measures” design). For example, a key step at the foundation of pharmacological research is modeling at first single-subject variability and then computing variability at a population level. Such *hierarchical statistical modeling* (Friston *et al.* (2005)) stands at the core of a mixed-effects design. Within a pharmacological framework, single-subject variability is a combination of the individual reaction with respect to the administration of a drug and the within-session errors of measurement (Lavielle (2015)). Lastly, Bernal-Rusiel *et al.* (2013) demonstrated how a mixed-effects analysis outperforms the more traditional repeated measures analysis. In simple words, among the two factors of a basic pharmacological experiment (time x drug) the mixed-effects design allows to sort the drug effect out, by properly estimating the effect of time (time is measured in a longitudinal fashion).

Worth mentioning recent approaches in CNS pharmacology aimed to improve the detection of the pharmacodynamics by neuroimaging tools (Brain *et al.* (2014)). One of these approaches is generalized semi-linear canonical correlation analysis (GSLCCA). It is based on correlating the single-subject’s pharmacokinetics (i.e. level of the drug in the blood) with the single-subject’s pharmacodynamics (e.g. EEG changes in alpha-power). In simple words, it provides a better estimate of intra-subject variability (e.g. via normalization of response), thus improving the analysis at the population level. This happens because after normalization the two groups are now more homogenous. Intra-subject variability is called elsewhere “single-subject analysis”, whereas population level analysis is called also “group-level analysis”.

The first study (Chapter 2) shows the use of a mixed-effect design in a pharmacological experiment.

1.4.4 Defining objective biomarkers

One of the challenges of CNS pharmacology is to find *objective biomarkers* which should be considered as a “common brain signature” of how a specific molecule works in the brain. An even more complex problem is finding biomarkers of signs and symptoms of mental diseases. For the present discussion I avoid dealing with the topic of biomarkers for mental diseases. Instead, I will focus only on the topic of pharmacological biomarkers (e.g. the pattern that a CNS drug systematically triggers in the brain of healthy subjects).

In recent terminology there is an interest in *biomarkers candidates*: it is a less strict definition with respect to biomarkers. Indeed, a biomarkers candidate implies some correlation between the neuroimaging pattern and the effect of a CNS drug. In pharmacological terms is called “Proof of concept” validation, and it entails the concept of *surrogate end-points*: e.g. a pain-killer should show some decrease of activity exactly in the area of the brain stem where a painful stimulus has previously generated an increase of activity (Wanigasekera *et al.* (2016)). Therefore, with surrogate end-points a mechanistic explanation how the drug works is not necessary. Indeed, Wanigasekera *et al.* (2016) showed how it is possible to use neuroimaging in a head-to-head comparison of two pain-killers (ibuprofen and gabapentin) and see which one of the two is superior. Therefore with surrogate end-points the drug under investigation has to change some parameters in the direction expected. This is enough for claiming that the drug is efficacious. The concept of surrogate end-points, is identical to what in clinical neuroimaging is called the *key-lock principle* (Saletu *et al.* (2010)): the medication should resolve the brain pattern which is correlated with a neurological/mental disease. Then the terminology “key-lock”, since the CNS medication should act only in areas considered dysfunctional, bringing the brain activity - possibly - back to normal. At the moment, scientific evidence of the existence of the key-lock principle is present mainly in pain research.

Further, the effect recorded in the brain can be tracked by its *dose-response curve*. Such a curve shows how pharmacodynamics is linked with the dose of the drug molecule. This means that “common brain signature” (response) changes according to the amount of molecule administered (dose). A beautiful example in fMRI literature of a linear dose-response curve using anfenitanil is Fig. 1 in Oertel *et al.* (2008). Just as a reminder, anfenitanil is a synthetic opioid. Such linear dose-response curve shows clearly how the brain pattern changes in a linear fashion according to the dose of drug. Instead examples

of classical sigmoidal dose-response curves detected by EEG are Fig. 5 and 6 in Bewernitz and Derendorf (2012). In Fig. 5 they used sevoflurane - an anesthetic -, whereas in Fig. 6 they used midazolam and diazepam - benzodiazepines -. Neuroimaging can also unravel very complex pharmacodynamics processes. Indeed, Fig. 4 in Barbanoj *et al.* (2006) showed the pharmacological effect of *hyteresis* (a lagged dose-response curve). Further, Barbanoj *et al.* (2006) showed that EEG can detect subtle *drug-drug interactions*: a compound with its metabolites (e.g. midazolam with its main metabolite 1-hydroxymidazolam); different enantiomers of the same compounds (e.g. S-ketamine and R-ketamine); a compound with another compound (e.g. paroxetine and alpraxolam). More recently, advanced EEG pharmacodynamics approaches based on canonical correlation analysis were able to show consistency in the temporal structure (pharmacokinetics) of the effect of remifentanil (Brain *et al.* (2014)). For example, at 3 min after administration, the peak of remifentanil in blood was paralleled by the strongest brain modulation.

Such strong correlation between pharmacokinetics (level of drug in the blood) and pharmacodynamics (brain response) allows consistency among results recorded by using different neuroimaging techniques. Although anfentanil and remifentanil have a different chemical structure, they can be considered structural analogs; therefore for our purpose they have comparable properties. What was striking that both fMRI (anfentanil, Oertel *et al.* (2008)) and EEG (remifentanil, Brain *et al.* (2014)) showed the strongest correlation at 3 min after administration between their peaks in the blood (pharmacokinetics) and the strongest brain modifications (pharmacodynamics). Therefore the temporal structure of the effect (e.g. 3 min after administration) of the drug class is shared by all neuroimaging techniques. Lastly, it was discussed above that almost all neuroimaging techniques can detect both basic and complex pharmacodynamics properties. Detecting such pharmacodynamics is key for making neuroimaging appealing for CNS pharmacology.

Summarizing, not mechanistic but simple correlational analysis between pharmacokinetics and pharmacodynamics can provide a *common brain signature* (Paulus and Stein (2007)), which identifies the effect on the brain of a specific CNS drug. Also the common brain signature could be very useful in terms of early drug development (Paulus and Stein (2007)), because of its intrinsic predictability potential. Early drug development will be addressed in paragraph 1.5.

So far the field of neuroscience which showed the most reliable biomarkers candidates is pain research. Indeed, neuroimaging was there successful in identifying

“fingerprint of CNS drug efficacy signatures”, as called by Hargreaves *et al.* (2015). Such concept is equivalent to the above mentioned common brain signature. Further Hargreaves *et al.* (2015) proposed to develop surrogate end-points as early as possible in the early drug development. Possibly, already in Phase 1 clinical trials should be a *surrogate end-point* (namely, something quantitative to detect the efficacy of the molecule) created. This is particularly true for PET research where radioligands should be developed in parallel as the new molecule. Radioligands are radioactive molecules bound to the research molecule. Using radioligands should show *target engagement*, which means that the research molecule binds the receptors which are supposed to be targeted. Such phenomenon is called also “proof of concept”. Showing that the compound can enter the target area is sufficient for considering it efficacious and then move to the next phase of the clinical trials (Wong *et al.* (2009)). Note that target engagement is another example of surrogate end-points.

Target engagement is only possible in vivo by using PET in humans or fMRI and contrast agents in animals. Target engagement is a pharmacodynamics measure which should be investigated prior to other pharmacodynamics measures (e.g. deactivation of amygdala by fMRI or vigilance assessment by EEG and MEG). In a nutshell, we have different pharmacodynamics parameters which can be extracted by either the same or different neuroimaging techniques. These pharmacodynamics parameters are also called surrogate end-points since a mechanistic explanation is not required. These surrogate end-points reflect nevertheless different priorities: target engagement should be investigated prior to any other surrogate end-points in order to be at least sure that the molecule binds expected receptors.

In our experiment (Chapter 2, CSD study) we tried to replicate at first previous results. Indeed, Fisher *et al.* (2012) found that nicotine impacts left frontal cortex power during eyes-open condition. We found the same results during EO condition (see Chapter 2.). Therefore, such replication was considered like a biomarker candidate of the effect of nicotine: each time we introduce nicotine into the system, it should show its peculiar “signature” in terms of brain activity.

In Chapter 4, I will address further replications of our CSD results. In a nutshell, our results matched a recent fMRI meta-analysis (Sutherland *et al.* (2015)) which showed that left frontal cortex and anterior cingulate cortex are involved in the effect of nicotine. In simple words, I think that the consistency of our results with previous results are a good example of a biomarker candidate: every time we administered nicotine we should

expect changes in power (by EEG) or in BOLD (by fMRI) at the very same locations (i.e. left frontal cortex and anterior cingulate cortex).

1.4.5 Inability to make predictions

Overall, the *inability to make predictions* about the response of an individual to a CNS molecule is still affecting neuroimaging. In simple words, the inability to make predictions means that a “trial and error” approach for administering a specific CNS drug at specific dosages is still widely used. This problem is hindering the field of personalized medicine applied to CNS pharmacology. Ideally, personalized medicine should help the clinician during the administration process of a specific CNS drug. This should translate to the adagio of giving “the right pill, at the right dosage to the right person”. Up to now such level of precision in CNS pharmacology is at the moment not possible.

Nevertheless, new approaches are emerging in order to fill the gap in terms of predictability potential of neuroimaging techniques. For example, a simple correlational approach (see paragraph 1.4.4) offers the ability to use biomarker candidates in a predictive way. Further, SVM algorithms can predict the effect of a new CNS drug by using old neuroimaging datasets of similar compounds (Doyle *et al.* (2015) and Khodayari-Rostamabad *et al.* (2013)). The last approach will be discussed in paragraph 1.5.5.

Neither predictability issues or SVM approaches have been addressed in the two studies with nicotine.

1.4.6 Translational neuroscience

Another hurdle of CNS pharmacology and neuroimaging is the *translational* component. With this I mean the difficulty to translate the results from one species to the other, even though the same CNS molecule is studied. For example, eyes-closed resting-state with humans is different from eyes-closed resting-state in rats. To such extent it seems that more potential for translation resides in cognitive tasks other than resting-state - specifically eyes-closed activity - : engaging all primates in the same task (e.g. fixating a cross on the screen) could force all of them to be in the same brain state. In summary, the resting-state paradigm could be difficult to implement in animals. For such a reason a

standardized cognitive task done both in humans and animals could help in translating results from one species to the other.

Although some old neuroimaging techniques (e.g. EEG and PET) were not able to succeed yet in making a smooth transition between results from one species to the other, recent efforts hold promises. Indeed, almost all neuroimaging techniques are making effort to close the gap between the species difference: PET (Finnema *et al.* (2015)); fMRI (Smucny *et al.* (2014)) and EEG (Drinkenburg *et al.* (2015)). Therefore a bigger potential for translational neuroscience in CNS pharmacology seems to be likely to occur in the near future.

In both studies (Chapter 2 and Chapter 3) we made some connections of our results with the animal literature about the effect of nicotine in the brain. I strongly believe that CNS pharmacology should be multi-disciplinary and researchers should be trained both in human models as well as in animal models. Certainly, there is a strong common thread between animal models and human models: the very same CNS drug (namely, the very same chemical structure) has been used in both types of models. This should ease the comparison between the effects of a CNS drug on different species.

1.5 Current applications of neuroimaging to early drug development

The second aim of such chapter is to try to justify how the implementation of the neuroimaging framework to CNS pharmacology fits the necessities of the pharmaceutical industries.

Connecting with what said in paragraph 1.4.4, the use of biomarkers candidates in CNS pharmacology - and their predictive potential - could be useful for two purposes: one is personalized medicine and the other is early drug development. For personalized medicine I mean again the right amount of knowledge which should be used for administering the right CNS molecule at the right dose to a specific subject. I will not talk about the use of neuroimaging for personalized medicine here, rather about the use of neuroimaging for early drug development.

Specifically, it will be discussed the role of neuroimaging in light of modern early drug development for CNS drugs.

1.5.1 Early drug Development

As described in paragraph 1.4.4 the potential of neuroimaging with respect to early drug development is its superior ability of tracking pharmacodynamics. Indeed, questionnaires (e.g. depression checklists) usually don't track pharmacodynamics (namely, an improvement of symptoms) as well as neuroimaging (Oertel *et al.* (2008)). Therefore, the superiority of neuroimaging in terms of detecting important pharmacodynamics processes makes it appealing for CNS drug evaluation within an industrial setting.

A key problem in drug development for new CNS drugs is the huge attrition ratio: very few new compounds can survive the research and development process, thus being brought to the market. This translates to huge research costs undertaken by the pharmaceutical industries (Hargreaves *et al.* (2015)). Therefore the pharmaceutical industries are asking to the neuroimaging community to figure out methods in order to decrease costs.

As follows four main strategies for decreasing costs/increasing revenues could be potentially addressed by neuroimaging: *escalation studies*; *go/no-go decisions*; *repurposing*; *predictability of drug action*.

1.5.2 Escalation studies

The escalation studies are typically Phase I clinical trials: the dose is gradually increased in order to test tolerability. At Phase I, the right and safest dose is looked for. Some researchers postulated the use of neuroimaging already at the level of Phase I clinical trials. Indeed, neuroimaging can be helpful in finding the right dosages (Doyle *et al.* (2015)). Therefore neuroimaging could have an impact in measuring pharmacokinetics as well as pharmacodynamics.

Further, I already discussed in paragraph 1.5.1 that many neuroimaging techniques have superior sensitivity in delineating dose-response curves as questionnaires (Oertel *et al.* (2008)). Therefore neuroimaging has a remarkable potential in measuring pharmacodynamics. This could have an impact in Phase II and Phase III clinical trials, as it will be explained in the next paragraph.

1.5.3 Go/no-go decisions

The efficacy studies are called also “pharmacodynamics studies” and they traditionally belonged to the Phase II and Phase III clinical trials. Further, traditionally during Phase III clinical trials go/no-go decisions (called also “cost-benefit analysis”) are achieved (see below).

I already discussed in paragraph 1.5.1 that many neuroimaging techniques have superior sensitivity in delineating dose-response curves as questionnaires (Oertel *et al.* (2008). Therefore neuroimaging has a remarkable potential in measuring pharmacodynamics.

There is a recent trend in running efficacy studies as soon as possible. There is a proposal to reach go/no-go decisions already at the Phase II clinical trial, instead of Phase III clinical trial (Wilson and Danjou (2015)). This maneuver should help saving costs. Go/no-go decision should be made at each intermediate step of a clinical trial, as well (e.g. from Phase IIa to Phase IIb clinical trial). The continuous monitoring guarantees online tracking of the progresses in terms of efficacy of the drug (i.e. marketable potential of the compound).

Early *go decisions* have the function of increasing the confidence that the molecule under investigation is promising and deserves further investments. Early *no-go decisions* have the advantage in decreasing the costs, focusing the investment on the most promising molecule only. For EEG analysis Gilles and Luthringer (2007) showed how EEG can effectively ascertain “go decision” regarding novel compounds. More recently, Wilson and Danjou (2015) described how it is also possible to use EEG for effective no-go decisions. The parameters which are chosen as surrogate end-points are selected on a case-by-case basis. It could be that ERPs are better for a specific molecule (e.g. cognitive enhancers), whereas power spectrum of source reconstructed data could be optimal for another molecule (e.g. antidepressants). Previous literature coming from both animal and human models can guide the selection of the parameters to be measured with respect to the molecule under investigation. This is the case when the same (e.g. in animal models) or a similar (e.g. in human models) molecule have been previously studied.

1.5.4 Repurposing

Another strategy which leverages the pharmacodynamic superiority (namely, detecting drug efficacy) of neuroimaging with respect to questionnaires, regards repurposing an old compound for a new clinical entity. The goal of such repurposing study is to find a new market for the old drug. A recent example is the use of quetiapine for treating sleep disorders, even if it was originally marketed as an antipsychotic.

This could be now done in Phase III or earlier, when looking for drug efficacy. Traditionally, repurposing happened in Phase IV clinical trials (post-marketing). As for the go/no-go decisions, repurposing should be done as soon as possible in terms of clinical trials. The reason is the long amount of time before getting FDA approval for the second usage of the drug (e.g. sleep-inducing properties of quetiapine), in case the first usage (e.g. antipsychotic properties of quetiapine) would not be commercially profitable. This holds also for further expanding commercial domains and having parallel income from two distinct marketplaces.

The use of biomarker candidates could hamper the repurposing process since it forces the neuroimaging researcher to look to the specific effect expected. An example of using biomarker candidates by fMRI is subgenueal area 25 in case of a study with a molecule with potential antidepressant properties. Therefore some aside effects of the drug under investigation could be overlooked. It is therefore important to use multidisciplinary approach. Indeed, using EEG simultaneously with fMRI could provide extra knowledge of the secondary effect of the compound. Taking the example of a molecule with alleged antidepressant properties we formulate a case: from an fMRI looks like an antidepressant, but the EEG showed it has also a sleep-inducing effect. Practically, if the revenues of the molecule are low when it is sold as antidepressant, it can be re-sold and re-branded as a sleeping pill.

In summary, neuroimaging can enhance repurposing and thus offers a “plan B” to the pharmaceutical industry. Indeed, it can help finding an alternative “niche” in the market, making it profitable whenever previous attempts showed to be unsuccessful.

1.5.5 Predictability of drug action

There are SVM approaches used for predicting whether a new investigational molecule will be efficacious with respect to a particular disease/problem. In a nutshell, an old dataset of an already marketed drug can provide the ability to make predictions how a new drug is going to work in the brain. Such methods have been mainly produced within the SVM community (Doyle *et al.* (2015), Duff *et al.* (2015) and Khodayari-Rostamabad *et al.* (2013)). Such an approach does hold promises for early drug development.

A more simple approach based on correlation is impinged on the concept of biomarkers candidates (see paragraph 1.4.4). Indeed, biomarkers candidates should have potential for the predictability of the efficacy of the new CNS drug themselves (Hargreaves *et al.* (2015)).

Another flavor of the SVM approach regarded the stratification of subjects. Doyle *et al.* (2015) claimed that stratification via SVM can produce a more homogenous dataset which could be advantageous for early drug development. Specifically, it could be used for selecting responders and non-responders, and finding the active dose (done in Phase I clinical trial) only in the responders. The drawback of stratification is the lack generalization of the results to the whole population.

1.6 Outline of the dissertation

In Chapter 2 and Chapter 3, practical applications of neuroimaging to pharmacology will be provided. How nicotine impacts brain activity will be presented. The modulation of brain activity was detected by EEG. Specifically, a source reconstruction analysis was used in order to estimate the brain activity from scalp electrodes. The first study (Chapter 2) has been already published as Ranzi *et al.* (2016), whereas the second study (Chapter 3) is currently (May 2016) under review.

In Chapter 2, the first study regarding how nicotine affects the current source density (CSD) using a source reconstruction method will be shown. To avoid confusion, CSD is in essence a power spectrum analysis. In terms of source reconstruction the Minimum Norm algorithm (Hamalainen and Ilmoniemi (1994)) was used. Then whole-brain CSD was extracted.

In Chapter 3 we conducted two further analyses. In terms of source reconstruction the eLORETA algorithm (Pascual-Marqui *et al.* (2011)) was used and thirteen time-series

representing thirteen sources belonging to the resting-state network were extracted (Chen *et al.* (2013)). A connectivity analysis using the renormalized Partial Directed Coherence (rPDC) algorithm (Schelter *et al.* (2009)) was first adopted with the thirteen time-series. Then a phase-amplitude coupling analysis utilizing the Mean Resultant Vector Length (VL) algorithm (Miyakoshi *et al.* , 2013) was computed on these thirteen time-series. In conclusion, two studies will be presented. Overall, three analyses have been carried out on the same dataset: CSD (first study, Chapter 2); rPDC and VL (second study, Chapter 3).

In Chapter 4 I will present the concluding remarks about the dissertation. First, I will try to converge the results of the three analyses. I will explain how the three analyses showed that nicotine does increase vigilance. I will then try to sum up the material presented in the introduction, by reinforcing the concept that non-invasive neuroimaging can boost the development of modern CNS pharmacology.

1.7 Experimental design of the nicotine experiment

We run three analyses of the same experiment. First analysis (CSD) is in Chapter 2, whereas second (rPDC) and third (VL) analyses are located in Chapter 3. All three analyses share the common experimental design. For this reason I will introduce now the common experimental design adopted.

The *research question* here was whether the well-known nicotine-induced increase of vigilance (Gilbert *et al.* (2000)) could be captured by neuroimaging techniques. In order to answer such question the focus on brain oscillations was considered appropriate, since their well known correlation with drowsiness (Sander *et al.* (2015)). Therefore using EEG was considered paramount since its ability to detect oscillatory patterns which correlate with drowsiness. The choice of nicotine was due the decision to study whether a well-known cognitive enhancer as nicotine (Thiel *et al.* (2005)) exploits its properties by increasing vigilance. Such question was so far unexplored by previous neuroscientific research.

The use of three EEG techniques (CSD, rPDC and VL) was justified by the fact that previous literature used such techniques for detecting online modulations of vigilance. In simple words, they correspond to three different biomarkers of drowsiness. Then we tested whether nicotine could possibly change such biomarkers, thus unraveling how the molecule modulates vigilance. As follows, I will explain in detail the rationale.

CSD analysis was used because its ability to detect the phenomenon of anteriorization of alpha (Olbrich *et al.* (2009)). *Anteriorization of alpha* is a natural phenomenon that happens during eyes-closed, when alpha migrates from occipital region to frontal region within minutes. When such a process occurs, it means that the subject gets drowsy. Further, we found out that a connectivity measure (rPDC) is also sensitive to an online decrease of vigilance (Maksimow *et al.* (2014)). Specifically, the authors were able to observe a specific direction of the effect: when we are pharmacologically drowsy the forward connectivity increases, whereas the backward connectivity decreases. Indeed, they discovered that the two types of connectivity are anti-correlated. Lastly, we found in the literature that phase-amplitude coupling is influenced by drowsiness (Blain-Moraes *et al.* (2015)). Specifically, they found that a decrease of phase-amplitude coupling correlates with a decrease of vigilance. Therefore, we used VL technique in order to detect the effect that nicotine can have on vigilance. In conclusion, as a first step we use previous literature to guide us in the selection of three methods/biomarkers which are more sensitive in detecting modulations of vigilance. As a second step, we analyzed the impact of nicotine on these three methods selected.

Regarding the tasks, resting-state tasks were adopted: eyes-open fixation on a cross (EO) and eyes-closed (EC) conditions were employed. Indeed, research on model organisms with small neuronal circuits suggests that the physiological state of a network impacts the effects of neuromodulators (Marder *et al.* (2014)). Therefore effects of centrally acting drugs on oscillatory activity may thus depend on the resting-state condition employed. The EO condition was considered ideal for testing the effect of nicotine, because nicotine appears to target attentional networks (Lawrence *et al.* (2002), Thiel *et al.* (2005)). The EC condition was considered also important since nicotine impacts this condition as well (Bowers *et al.* (2015)). The rationale behind the EC condition was to induce a state of drowsiness by asking the subjects to close their eyes (Olbrich *et al.* (2009)). Regarding the terminology, drowsiness, sleepiness, tiredness and fatigue are all synonyms for reduced wakefulness (Sander *et al.* (2015)). For consistency reason, the term drowsiness will be used throughout the dissertation.

According to the previous literature (Bowers *et al.* (2015), Fisher *et al.* (2012) and Foulds *et al.* (1994)) it seems that nicotine preferentially operates within three frequency ranges during eyes-open and eyes-closed activity in non-smokers: α_1 , 8.5-10.4 Hz; α_2 , 10.5-12.4Hz; β_1 , 12.5-18.4Hz. Such previous knowledge helped us in selecting *a priori* the frequency ranges where a nicotine effect was expected. In conclusion, the present study

represents a standardized benchmark for conducting pharmacological research (nicotine) non-invasively in humans.

1.8 Conclusion

Despite of the problems that are cursing the field (e.g. lack of standardization), I think that a revolution is taking place within CNS pharmacology. Such revolution is mainly driven by non-invasive neuroimaging. A direct consequence of the marriage between CNS pharmacology and neuroimaging is a commercial implementation of neuroimaging in early drug development. I discussed thoroughly the process of early drug development in CNS drugs and the role of neuroimaging is having in it.

In the following, I will present one experiment using nicotine administered in healthy subjects. Such experiment represents the optimal use of a standardized experimental design for conducting pharmacological research non-invasively in humans. Indeed, we tried to develop an experimental design suited for mitigating the problems listed in paragraph 1.4. The experiment consisted in three analyses (CSD, rPDC and VL) which provide different hence converging perspectives in characterizing the effect of nicotine in the brain. Chapter 2 will present the CSD analysis, whereas Chapter 3 is dedicated to rPDC and VL analyses. The common thread to the three analyses was the research question whether nicotine modulates vigilance. Further in common, the three analyses have all used EO and EC resting-state activity for testing the pharmacological activity of nicotine in male healthy non-smokers. In Chapter 4 I will sum up and I will show how the results of the three analyses converged. Anticipating the results, I will demonstrate that nicotine does increase vigilance.

1.9 Acknowledgments

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Chapter 2: Study 1, Current Source Density (CSD)

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2.1 Abstract

Modern psychopharmacological research in humans focuses on how specific psychoactive molecules modulate oscillatory brain activity. Here we present state-of-the-art EEG methods applied in a resting-state drug study.

Thirty healthy male non-smokers were randomly allocated either to a nicotine group (14 subjects, 7 mg transdermal nicotine) or to a placebo group (16 subjects). EEG activity was recorded in an eyes-open and eyes-closed condition before and after drug administration. A source reconstruction (Minimum Norm algorithm) analysis was conducted within a frequency range of 8.5 to 18.4 Hz subdivided into three different frequency bands.

During eyes-open, nicotine reduced the power of oscillatory activity in the 12.5-18.4 Hz frequency band in left middle frontal gyrus. In contrast, in the eyes-closed condition, nicotine reduced the power in the 8.5-10.4 Hz frequency band in superior frontal gyri and in the 10.5-12.4 Hz and 12.5-18.4 Hz frequency bands in supplementary motor areas. In summary, nicotine reduced power of the 12.5-18.4 Hz band in the left middle frontal gyrus during eyes-open, and it reduced power from 8.5 to 18.4 Hz in a brain area spanning from superior frontal gyri to supplementary motor areas during eyes-closed.

In conclusion, the results suggest that nicotine counteracts the phenomenon of anteriorization of alpha activity, hence potentially increasing the level of vigilance.

2.2 Introduction

In recent years, much interest has been raised in detecting how pharmacological challenges could modify oscillatory brain activity. Nonetheless, a problem in current neuroscience is low replicability (Button *et al.* (2013), Hartshorne and Schachner (2012)). Three key factors in designing an experiment are essential: replicability (i.e. finding the same results among different labs); reproducibility (i.e. using a simplified experimental design, describing protocols transparently and adhering to agreed benchmarks); reliability (i.e. the correlation between a measurement and the true value). By maximizing reproducibility and reliability, an increase of replicability is expected. In terms of experimental design, two recommendations are here suggested to alleviate the problem of low replicability which complement the guidelines presented in Jobert *et al.* (2012).

The first recommendation aims to increase reproducibility. There's a need to follow agreed-upon protocols and benchmarks (Collins and Tabak (2014)), which is key to increasing reproducibility. Likewise, pharmaco-electroencephalographical research is currently affected by lack of standardization (Wilson *et al.* (2014)). Further, a simplified design (i.e. resting-state) has the advantage of high translational potential for drug research (Marder *et al.* (2014), Smucny *et al.* (2014)). Functional magnetic resonance imaging studies suggest that the patterns of brain activation during resting-state differ between eyes-closed, eyes-open or fixation conditions (Patriat *et al.* (2013)). Between the two alternatives of either eyes-open fixating on a cross or eyes-open without fixation, the first was considered the ideal condition for testing the effect of nicotine, since nicotine appears to target attentional networks (Lawrence *et al.* (2002), Thiel *et al.* (2005)).

The second recommendation aims to increase reliability. Among many factors which determines reliability, a key issue concerns the correct estimation of intra-subject variability. Shortly, intra-subject variability corresponds to a combination of intra-subject variability (i.e. single-subject's homeostatic responses and own biology) and within-session errors measurement (Sinkkonen and Tervaniemi (2000)). A reliable way of estimating such intra-subject variability is to run a repeated measures design (Sinkkonen and Tervaniemi (2000)). A recent departure from the repeated measures design is the mixed-effects design, which seems to outperform the more traditional repeated measures analysis (Bernal-Rusiel *et al.* (2013)). Further, a hierarchical mixed-effects design can effectively estimate at first intra-subject variability (Friston *et al.* (2005), Mumford and Nichols (2009)) and then inter-subject variability (Stelzer *et al.* (2014)). In conclusion, a

hierarchical mixed-effects design seems particularly useful in increasing reliability in a pharmacological study, since the appropriate estimation of individual reaction with respect to the administration of a drug is of fundamental importance (Lavielle (2015)).

Regarding the neuroimaging technique used, source reconstruction on EEG time-series was employed, since it was considered a privileged window for looking at the drug-induced modulations of brain oscillations (Saletu *et al.* (2010)). In short, the technique is based on the estimation of dipolar sources which reflect the power of brain oscillations. Each dipolar source corresponds to the activity generated from 10.000 to 50.000 synchronized pyramidal cells (Murakami and Okada (2006)).

A previous study with non-smokers (Foulds *et al.* (1994)) investigated whether nicotine would impact EEG brain activity during eyes-open resting-state. The authors found a significant increase in the single-subject's dominant alpha (namely, the highest spectral power within the 8-13 Hz range), induced by nicotine. A more recent study with non-smokers (Fisher *et al.* (2012)) investigated the effect of nicotine when the subjects fixated on a cross. They found a significant nicotine-induced increase in power within the frequency range of 10.5-13.0 Hz at a left frontal electrode (electrode F₃).

Particularly intriguing is the question what generates the already established nicotine-induced cognitive enhancing properties (Giessing and Thiel (2012)). Our hypothesis was that nicotine induces a cognitive improvement via an increase of vigilance. To test such hypothesis we used the eyes-closed activity as a benchmark. Indeed, it was already demonstrated that eyes-closed activity induces a decrease of vigilance itself and this can be monitored by EEG (Olbrich *et al.* (2009)). Therefore our hypothesis was that nicotine could counteract a naturally induced decrease of vigilance. For detecting vigilance modulations we use EEG as technique of choice.

In summary, the aim of the present paper is twofold. On the one hand, two recommendations (e.g. increasing standardization of experimental design; employing mixed-effects designs) will be implemented with the assumption that they can alleviate the problem of low replicability in current psychopharmacological EEG studies. On the other hand, we present a state-of-the-art source reconstruction analysis approach to localize drug-induced changes of oscillatory activity. Given prior evidence of the effects of nicotine on brain activity in the alpha frequency range, we focused our analysis on the alpha and lower beta band and assessed the drug effect in eyes-closed (EC) and eyes-open (EO) resting-state conditions. The hypothesis to be tested is whether nicotine would induce an increment of vigilance, particularly within the alpha range during EC. To the

best of the authors' knowledge, this is the first paper using a source reconstruction analysis to investigate the effects of nicotine in an eyes-closed resting-state in healthy non-smoker male subjects.

2.3 Methods

2.3.1 Subjects

Thirty right-handed, nonsmoking male subjects (age: 27 years \pm 3, weight: 81.1 kg \pm 9.5, height: 1.82 meter \pm 0.05) participated in this study. Subjects were recruited by advertisement from the local university. Subjects were not on any kind of medication nor reported any history of major medical illness or neurological or psychiatric disorders. Non-smokers (no more than 10 cigarettes consumed during their whole life) were studied to avoid confounding effects of withdrawal symptoms (Fisher *et al.* (2012)). We recruited only male subjects to minimize gender-related confounds (Jausovec and Jausovec (2010)) and possible hormonal interaction with nicotine (Duncan and Northoff (2013)). All subjects gave written informed consent. The study was approved by the Ethics Committee of the German Psychological Association.

2.3.2 Drugs

All subjects refrained from taking any legal psychoactive drugs such as alcohol, caffeine 24 hours before the experiment. Following a double-blind procedure, each subject was randomly allocated to receive either nicotine (NIC, n=14) or placebo (PLA, n=16). A 7 mg nicotine patch (Niquitin® Clear 7 mg, GlaxoSmithKline Consumer Healthcare GmbH) and a matched placebo (plaster of same shape and thickness). The patches were administered by a third person, not otherwise involved in the study, onto the subject's lower back, covered with a standard plaster and removed after 50 min. In order to minimize side effects in non smokers, the patch was administered for 50 min only and then removed prior to the second EEG recording session. A similar procedure was previously used by Breckel *et al.* (2015) and Potter and Newhouse (2008) and yielded significant behavioural effects of nicotine. Since very little time elapsed between the

removal of the patch and EEG recording (~10 min refilling the electrodes + 14 min EO + 7 min EC), nicotine level in the blood is expected to be stable during the whole EEG recording session (Benowitz *et al.* (2009)).

2.3.3 Experimental design

We employed a mixed-effects design, which investigates the effects of nicotine both, within and across subjects. Subjects were randomly allocated to the placebo or nicotine group (between-subject factor drug) and measured in two sessions, before (PRE) and after (POST) the respective intervention (within-subject factor time), namely immediately after removal of the placebo or nicotine patch. Each EEG recording session consisted of 14-min eyes-open fixation on a cross condition (EO) as well as a 7-min eyes-closed condition (EC). The EC session always followed the EO session. A 40-min cognitive task was also performed thereafter, but was not analyzed due to a systematic software error. Testing took place at the same time of day (3:00 pm) for all subjects in order to minimize the influence of circadian rhythms. Subjects remained with the EEG cap mounted during the whole experiment. Before the second recording, the gel in the electrodes was refilled in order to return their impedance to 10 k Ω for each electrode (see section 2.5).

2.3.4 Subjective and physiological measures

To investigate subjective and cardiovascular effects of nicotine, mood rating scales as well as heart rate and blood pressure were assessed two times: upon arrival (PRE) and immediately after removal of the placebo or nicotine patch (POST). Subjective mood was assessed with visual analogue scales (Bond and Lader (1974)). Rating scores were grouped into the three factors 'alertness', 'contentedness', and 'calmness', according to Bond and Lader (1974). Immediately after removal of the placebo or nicotine patch, subjects were also asked to assess whether they received the nicotine or placebo patch.

2.3.5 EEG recording

The EEG data were recorded with 63 Ag/AgCl-electrodes attached to an elastic cap (Easycap GmbH, Herrsching-Breitbrunn, Germany) with standard 10-20 montage. The reference was placed at the tip of the nose and electrode AFz set as ground (see standard 10-20 montage). One EOG electrode was positioned on the external canthus of the right eye, again referenced to the tip of the nose. Impedances were kept below 10 k Ω for each electrode. The EEG signal was sampled with 500 Hz and amplified using a BrainAmp system (Brain Products, Munich, Germany). Data were recorded and digitalized with the BrainVision Recorder (Brain Products, Munich, Germany). Data were stored in a computer and analyzed off-line.

All recordings were performed in an electrically shielded, sound insulated and dimly-lit chamber. Subjects were seated in a comfortable chair with firm armrests up to the wrists. During the EO condition the subjects were asked to keep their eyes-open and fixate their gaze on a cross located on the screen. The cross was light grey (size 0.86°) and superimposed on a dark-grey background. During EC the cross was kept on the screen, but the subject was asked to close his eyes and to keep them closed until the experimenter asked to open them again.

2.3.6 EEG data preprocessing

Preprocessing was performed using EEGLAB (Delorme and Makeig (2004)), version 12.0.2.5b and included the following steps: first, raw time-series from BrainVision Analyzer (Brain Products, Munich, Germany) were converted to an EEGLAB compatible format. Afterwards, PRE and POST time-series belonging to the same subject were concatenated (i.e. 14 min + 14 min = 28 min length time-series). The assumption was that a concatenated time-series belonging to the same subject provides a better estimate of ICA-rejected artifacts than a single time-series (i.e. only 14 min), according to Tsai *et al.* (2014). The two conditions (EO and EC) were concatenated separately. Data were then high-pass symmetric FIR filtered at 2 Hz with Blackman windows (transition band 0.9 Hz, filter order 1000), downsampled to 250 Hz and low-pass symmetric FIR filtered at 40 Hz with Blackman windows (transition band 5 Hz, filter order 276). The channel location according to standard 10-20 montage was then added (necessary step for generating ICA topographies). ICA estimation was computed. Then ICA-based artifacts rejection (i.e.

blinks; muscular movements; heart-beats;) was performed in a semi-automated way on a subject-by-subject basis by considering the concatenated data according to published guidelines (Jung *et al.* (2000)). It should be noted that using ICA-based artifact rejection instead of manually rejecting corrupted epochs, guarantees no data loss. This means that the clean EO and EC time-series always have the same length as the raw time-series. The ICA-based artifact rejection was considered particularly important for source reconstruction analysis: such a procedure does significantly improve source estimation (Fatima *et al.* (2013)). The concatenated file was then split back into the two original time-series (i.e. from 28 min length time-series back again to two separated 14 min time-series), now cleaned of artifacts. Further, the EOG channel was deleted from each time-series. Lastly, each time-series was split into 5-sec epochs (i.e. 14 min = 168 epochs of 5 sec each).

2.3.7 Source reconstruction analysis and statistical inference on sources

Source reconstruction was carried out using the Minimum Norm algorithm implemented in SPM12 version 6225 (Litvak *et al.* (2011)). The 5-sec epochs from EEGLAB were first converted to an SPM-compatible format. In order to create the head model, an ICBM152 template was used according to Litvak *et al.* (2011). For all subjects a cortical mesh was extracted by using a three-layer Boundary Element Model (BEM) head model. Therefore all subjects had the very same cortical mesh. The computation of the sources was band-limited according to standard frequency ranges 8.5-10.4 Hz, α_1 ; 10.5-12.4 Hz, α_2 ; 12.5-18.4 Hz, β_1 according to Jobert *et al.* (2012). Standard frequency ranges - instead of custom frequency ranges - were chosen in order to maximize reproducibility while minimizing circular inference (Kriegeskorte *et al.* (2009)). The estimation of sources was computed within each frequency range. The Minimum Norm algorithm (Hamalainen and Ilmoniemi (1994)), as implemented in SPM, was used. No mask was used for restricting sources to a particular location. A smoothing kernel of 32 mm was set. This resulted in a 32-bit image representing the power of estimated sources at a specific frequency range within a 5-sec epoch. All the images for each subject and for each condition were later averaged using the 'spm_reslice.m' Matlab function with 7th Degree B-Spline interpolation available in SPM. This resulted in a single averaged image per subject per session (either PRE or POST) and per condition (either EO or EC).

Statistical inference on the sources was performed with Statistical NonParametric Mapping version 13 (SnPM13, Nichols and Holmes (2002)). The averaged single subject images of the PRE and POST condition in the PLA and NIC group entered into a mixed-effects analysis based on non-parametric statistics and permutation. The mixed-effects design relies on a hierarchical generalized linear model (GLM) according to Friston *et al.* (2005).

To illustrate the effect of EO and EC we first validated the pipeline by performing a two-sample paired T-test by SnPM13 using the data of 29 subjects of the PRE condition only and comparing between EO and EC. Only 29 subjects instead of 30 were computed due to software limitation. Note that only the first 7 min of EO were considered, to have a similar length as the EC condition. This procedure of pipeline-validation follows the same rationale of standard pipeline-validation used in fMRI research (Liu *et al.* (2013)): if the pipeline can detect the often reported EO versus EC differences (i.e. typical increase of occipital alpha in EEG during EC), then it should also be possible to gauge drug effects. Our validation yielded the typical increase of occipital alpha (see Chen *et al.* (2008)).

Then, in order to isolate drug effects, the single subject data in the EO and EC condition were entered separately into the '2 Groups: Test diff of response; 2 conditions, 1 scan per condition' option in SnPM13. This analysis tests for an interaction and represents a special case of a mixed-effect design which holds when exactly two measurements per subject are available (Mumford and Nichols (2009)). Note that the procedure is equivalent to a two-sample unpaired T-test on the PRE-POST differences (Mumford and Nichols, 2009). For each condition (e.g. EC) a total of 60 images (2 images per subject, 1 PRE and 1 POST) were used as input for SnPM13. Here the whole time points were used for either EO (14 min) or EC (7 min), without data loss. Significant differences between PLA and NIC were then estimated using the following two planned contrasts for each frequency range in the EO and EC condition: (NIC PRE-NIC POST) - (PLA PRE-PLA POST) to isolate stronger decreases of power under nicotine and (PLA PRE-PLA POST) - (NIC PRE-NIC POST) to isolate stronger increases of power under nicotine. The number of permutations for all analyses was set to 5000, according to Douaud *et al.* (2013). The statistical inference chosen was a cluster-inference analysis on pseudo t-statistics values (Nichols and Holmes (2002)). Although, cluster-inference analysis suffers from low spatial specificity when significant clusters are large (Woo *et al.* (2014)), this is not considered an issue for the already low spatial resolution of EEG source reconstruction methods (Akalin Acar and Makeig (2013)). The smoothing at

statistical level was again set at 32 mm in order to comply with the smoothing already used for computing the sources. Note that even though high smoothing was applied, it should have not biased the results. Indeed, permutation-based methods - like SnPM13 - are resistant to changes of smoothing values (Pantazis *et al.* (2005)). All *P*-values reported and discussed were significant at *P*-value < 0.05 FWE-corrected. MNI coordinates of the significant clusters were translated to anatomical labels according to the Automated Anatomical Labeling (AAL) (Tzourio-Mazoyer *et al.* (2002)). Further, the AAL labels were plugged into the Online Brain Atlas Reconciliation Tool (OBART) (Bohland *et al.* (2009)) in order to obtain standardized names of anatomical labels.

The pipeline used here (by using SPM and SnPM) is very similar to the pipeline implemented in the famous LORETA software (Pascual-Marqui *et al.* (1994)), both from a source reconstruction algorithm point of view as from a nonparametric permutation-based statistic standpoint. We opted for SPM and SnPM since they are implemented in a MATLAB environment, thus providing more flexibility with the analysis. In conclusion, we believe that the pipeline used and the pipeline already implemented in LORETA software should convey approximately the same results, hence they are comparable.

The direction of the nicotine effect was computed according to Douaud *et al.* (2013). In short, the summary statistics image was used as mask for all raw images and voxel intensity at those locations were extracted per subject and condition and averaged. A value of 100 % was attributed at the PRE image, whereas the POST image provided a difference in percentage with respect to the PRE image.

2.4 Results

2.4.1 Subjective and physiological measures

A Two-way ANOVA (Weighted Least Square algorithm) was performed with the between-subject factor drug (either placebo or nicotine) and the within-subject factor time (PRE and POST) both for cardiovascular measures and mood ratings. Cardiovascular measures are depicted in Table 1.

Table 1: *Cardiovascular measures pre and post nicotine/placebo.*

Measure	Nicotine		Placebo	
	PRE	POST	PRE	POST
Systolic pressure	129 (12)	125 (12)	132 (11)	122 (10)
Diastolic pressure	81 (8)	82 (8)	81 (7)	82 (8)
Heart rate	78 (15)	65 (16)	79 (14)	60 (9)

Note. Systolic and diastolic pressure in mmHg. Heart rate in bpm. Means and standard deviation (in parentheses).

For systolic blood pressure we found a significant interaction between the two factors ($F_{1,28} = 4.33, p < 0.047$), which was related to a stronger decrease of systolic blood pressure under placebo as compared to nicotine. There was neither a significant main effect of drug nor a significant main effect of time. The heart rate showed a tendency for a significant interaction between the two factors showing a stronger decrease under placebo as compared to nicotine. The heart rate showed neither a significant main effect of drug nor a significant main effect of time. No significant effects (neither main effects nor interaction) were found for diastolic blood pressure.

We found no significant interaction between the two factors on mood ratings with respect to alertness ($F_{1,28} = 0.844, p < 0.367$), contentedness ($F_{1,28} = 0.140, p < 0.720$) or calmness ($F_{1,28} = 0.170, p < 0.685$). There was neither a significant main effect of drug nor a significant main effect of time. A Two-way ANOVA (Ordinary Least Square algorithm) was performed with the between-subject factor drug (either placebo or nicotine) both for vertigo and nausea after the patch was removed. Neither for vertigo nor nausea a significant was found. Lastly, we found a significant effect with respect to the subjects' guesses whether they received either placebo or nicotine. Indeed, Chi-square showed a significant effect ($\chi^2 = 8.134, p < 0.004$).

2.4.2 Source reconstruction analysis

To validate our data analysis we compared oscillatory activity in the EC and EO condition with the data from prior to the intervention (PRE). A drug by time interaction was computed on the data acquired prior to drug/placebo administration comparing EC and EO.

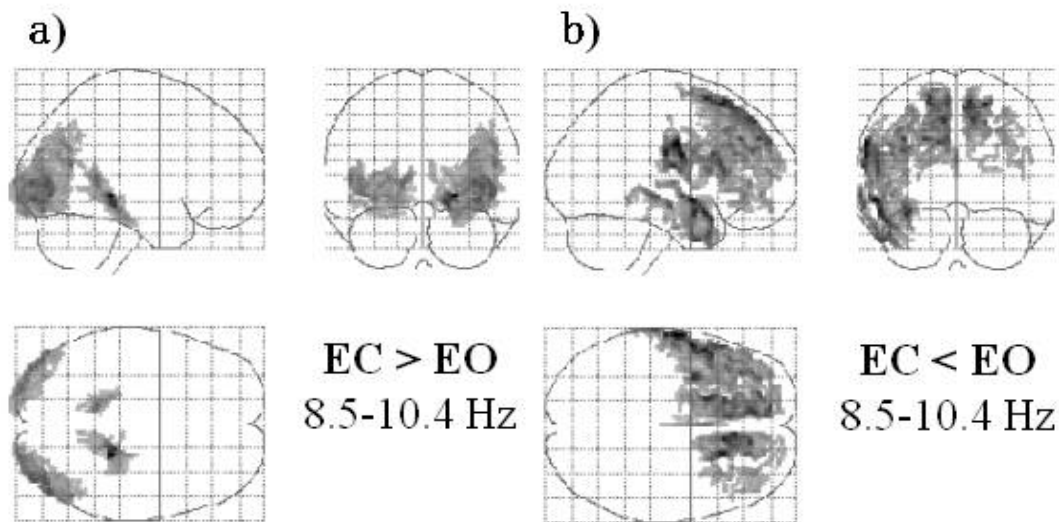


Figure 1: Eyes-open (EO) vs eyes-closed (EC) condition. Data were taken from the session prior (PRE) to drug/placebo administration. a, Occipital increase of power within 8.5-10.4 Hz during EC as compared to EO. b, Frontal decrease of power within 8.5-10.4 Hz during EC as compared to EO. The figures show FWE-corrected cluster-inference results at $p < 0.05$ (two-sample paired T-test).

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First, we found an increase of power within 8.5-10.4 Hz in the occipital lobe during EC with respect to EO (Fig. 1a). Further, we found a decrease of power within 8.5-10.4 Hz in the frontal lobe during EC with respect to EO (Fig. 1b).

Table 2: *FWE-corrected P-values of the nicotine-induced power changes in eyes-open (EO) and eyes-closed (EC).*

Eyes-open (EO), decrease of power under nicotine;

Frequency range	<i>P</i> -value
12.5-18.4	0.0148
10.5-12.4	ns
8.5-10.4	ns

Eyes-open (EO), increase of power under nicotine;

Frequency range	<i>P</i> -value
12.5-18.4	ns
10.5-12.4	ns
8.5-10.4	ns

Eyes-closed (EC), decrease of power under nicotine;

Frequency range	<i>P</i> -value
12.5-18.4	0.0024
10.5-12.4	0.0008
8.5-10.4	0.0022

Eyes-closed (EC), increase of power under nicotine;

Frequency range	<i>P</i> -value
12.5-18.4	ns
10.5-12.4	ns
8.5-10.4	ns

Note. Frequency range in Hz. All *P*-values are FWE-corrected for whole brain comparisons and Bonferroni-corrected ($p < 0.016$) for each direction (either increase or decrease). 'ns' means not significant ($p > 0.016$).

To investigate the effects of nicotine on resting-state oscillations, we analyzed the factor drug (nicotine/placebo) by factor time (PRE/POST) interaction using a mixed-effects design. In the EO condition, we found a significant decrease of power from the PRE to the POST session under nicotine as compared to placebo within the frequency range of 12.5-18.4 Hz in the orbital part of the left middle frontal gyrus ($p = 0.0148$, FWE-corrected; MNI coordinates - x,y,z - -44, 48, -6; Fig. 2a). Further, a tendency for a significant decrease of power was found in the left angular gyrus ($p = 0.093$, FWE-corrected; MNI coordinates - x,y,z - -40, -70, 44; figure not shown). No further decreases or increases in power were found for other frequency ranges (see Table 2).

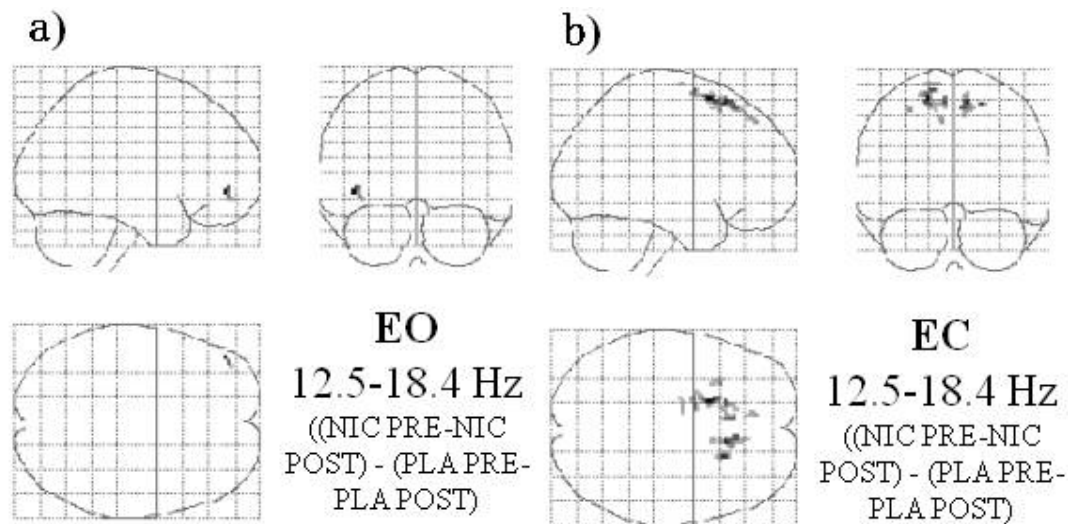


Figure 2: Effects of nicotine in the 12.5-18.4 Hz frequency range. a, EO condition. Decrease of power within the frequency range of 12.5-18.4 Hz in left middle frontal gyrus comparing the PRE and POST condition under NIC with PLA; b, EC condition. Decrease of power within the frequency range of 12.5-18.4 Hz in right supplementary motor area comparing the PRE and POST condition under NIC with PLA. Both figures show FWE-corrected cluster-inference results at $p < 0.05$.

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In the EC condition, we found a significant decrease of power from the PRE to the POST condition under nicotine as compared to placebo: within frequency range of 8.5-10.4 Hz in right and left superior frontal gyrus (both areas $p = 0.002$, FWE-corrected; MNI

coordinates - x,y,z - either 20, 20, 58 or -18, 10, 62; Fig. 3a). A significant decrease of power was also present within the 10.5-12.4 frequency range in the right supplementary motor area (most significant cluster $p = 0.0008$, FWE-corrected; MNI coordinates - x,y,z - 10, 22, 60; Fig. 3b) and in the 12.5-18.4 Hz frequency range in right supplementary motor area (most significant cluster $p = 0.0024$, FWE-corrected, MNI coordinates - x,y,z - 10, 22, 60; Fig. 2b). Hence, in the EC condition widespread frontal decreases in power were observed in alpha and beta frequency bands. Further, a tendency for a significant increase of power was found within 12.5-18.4 Hz in left and right middle occipital gyri (both areas $p = 0.076$, FWE-corrected; MNI coordinates - x,y,z - either -50, -78, 6 or 38, -84, 26; figure not shown). No further decrease or increase in power was found in the investigated frequency ranges (see Table 2).

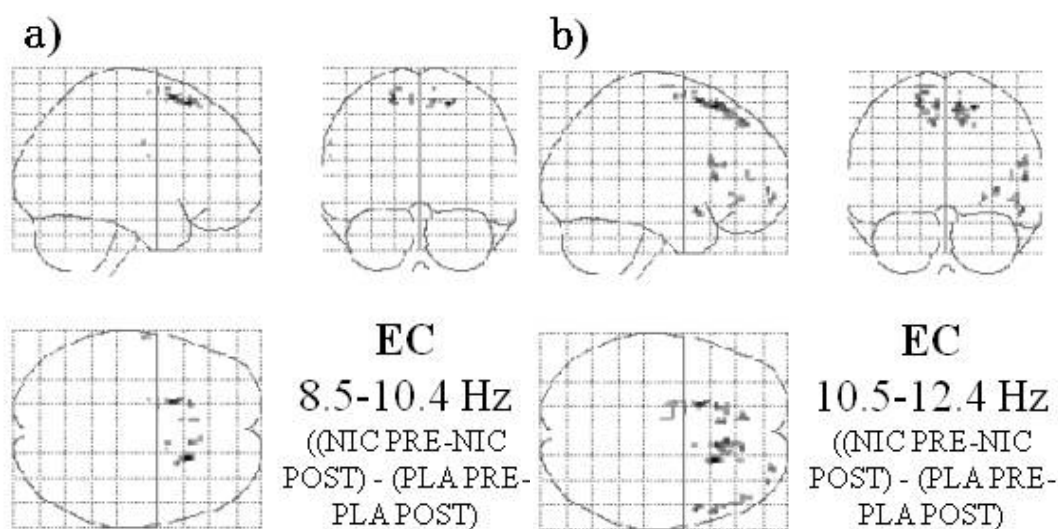


Figure 3: Effects of nicotine on 8.5-10.4 Hz and 10.5-12.4 Hz frequency ranges in the EC condition. a, Decrease of power within the frequency range of 8.5-10.4 Hz in right and left superior frontal gyrus comparing the PRE and POST condition under NIC with PLA; b, Decrease of power within the frequency range of 10.5-12.4 Hz in right supplementary motor area comparing the PRE and POST condition under NIC with PLA. Note that no effect of nicotine was found in these frequency ranges in the EO condition. Both figures show FWE-corrected cluster-inference results with $p < 0.05$.

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The direction of the nicotine effect was computed to further illustrate the nicotine-induced decrease of brain oscillatory power with respect to PLA. The box plot representing the frequency range from 10.5 to 12.4 Hz during EC condition is shown in Fig. 4. All other frequency bands which were significant in Table 2, showed the same pattern as Fig. 4 after computing their respective box plots (not shown).

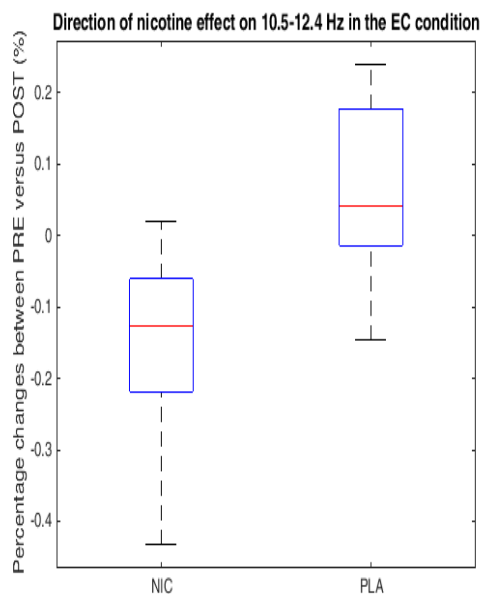


Figure 4: Direction of the nicotine effect on 10.5-12.4 Hz in the EC condition. The x-axis refers to the condition of either NIC or PLA. The y-axis refers to percentage change between PRE and POST for each condition. The figure shows a significant decrease (negative percentage values) from 10.5 to 12.4 Hz of NIC with respect to PLA. On each box plot, the central mark is the median, whereas the edges of the box are the 25th and 75th percentiles and the whiskers extend to the most extremes data points not considered outliers.

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2.5 Discussion

The aim of the article was to establish and test a standardized benchmark procedure for studying the drug-induced modulation of oscillatory brain activity by EEG. As an aside, it has been briefly discussed about practical steps in order to incentivize replicability in pharmacological research.

Regarding the cardiovascular effects of nicotine are in line with many prior findings showing a relative increase of heart rate and/or blood pressure under nicotine (Fisher *et al.* (2012), Thiel *et al.* (2005), Ahrens *et al.* (2015), Logemann *et al.* (2014)).

Regarding the pipeline-validation, the increase of power within 8.5-10.4 Hz in the occipital lobe during EC with respect to EO replicates previous results quite accurately (Chen *et al.* (2008)). We also found a decrease of power within 8.5-10.4 Hz in the frontal lobe during EC with respect to EO. Overall, the increase of power occipitally during EC and the decrease of it frontally during EO, resembles the different correlation of EEG alpha activity and BOLD activity pattern found in Sadaghiani *et al.* (2010). These authors used simultaneous EEG-fMRI recordings and focused specifically on the alpha frequency. In their data, occipital alpha amplitude during an eyes-closed condition correlated negatively with BOLD activity fluctuations while frontal alpha amplitude correlated positively with BOLD activity fluctuations. These findings seem to be in line with our data.

Regarding the EEG analyses of the pharmacological manipulation, a nicotine-induced modulation of power was found during EO (decrease within the frequency range of 12.5-18.4 Hz in the left middle frontal gyrus) and during EC (decrease within a frequency range of 8.5-10.4 Hz in the frontal gyri and decrease within 10.5-12.4 Hz and 12.5-18.4 Hz in the supplementary motor areas).

Although one previous study examined the effect of nicotine during EC and EO by EEG (Gilbert *et al.* (2000)), their analysis was limited to smokers. Therefore, a direct comparison with either the present study is not possible. Instead, the present study is very similar to Fisher *et al.* (2012) and a more direct comparison is therefore possible.

As a comment, the significant decrease within the frequency range of 12.5-18.4 Hz in the left middle frontal gyrus during EO condition is somewhat in line with the nicotine-induced changes in the higher alpha-band over left frontal electrodes reported by Fisher *et al.* (2012). The direction of the effect was however different with increases reported by Fisher *et al.* (2012). Five reasons may have contributed to this discrepancy.

First the different definition of frequency ranges, either 12.5-18.4 Hz (present paper) or 10.5-13 Hz (Fisher *et al.* (2012)). Second the lower plasma nicotine concentration that is obtained by a 7 mg patch (present paper) as compared to a 6 mg nicotine gum used by Fisher *et al.* (2012) (see Table 1 in Benowitz *et al.* (2009)). Indeed, a non-linear dose-response pattern could lead to completely opposite effects (i.e. in terms of vigilance) (Husain and Mehta (2011)). A third source of discrepancy could have been a difference in sampling the groups of subjects. Indeed, only males were used in the present paper, instead of a mixed sample of males and females (Fisher *et al.* (2012)). Fourth, the different statistical models used: a mixed-effects design with fourteen NIC repeated measures and sixteen PLA repeated measures was used in the present paper. Instead, in Fisher *et al.* (2012) twenty subjects were assessed in a within-subject design both under the influence of nicotine and under placebo. Fifth, the discrepancy could be generated by using different measurements of resting-state activity of 14 min (present paper) as compared to 2 min in Fisher *et al.* (2012).

In contrast to the EO condition, the EC condition showed novel results. Namely, the decrease in power in a range from 8.5 to 18.5 Hz during EC spread through the frontal lobe and was stronger than the effect of nicotine during EO. Indeed, the significant *P*-values are smaller in EC compared to EO, notwithstanding halved signal-to-noise ratio (SNR) during EC (7 min) with respect to EO (14 min).

As follows, we provide an interpretation of the results which will be limited to the EC condition since existing scientific evidence about a clear link between frontal alpha and sedation. The phenomenon we are referring to is called anteriorization of alpha (see below) and it appears simultaneously as a decrease of vigilance experienced by the subject. First, Olbrich *et al.* (2009) demonstrated that during 30 minutes eyes-closed (no drug) the brain activity tends to naturally move from higher vigilance stages to lower vigilance stages. One of the key issues of such process is the phenomenon of anteriorization of alpha: using a predefined alpha range between 8 to 12 Hz, the authors showed that the power within such frequency range gradually decreases in the occipital lobe but increases frontally during eyes-closed. This pattern can be observed already a few minutes after closing the eyes. Second, Vijayan *et al.* (2013) showed that the anesthetic propofol during eyes-closed could artificially reproduce the abovementioned anteriorization of alpha in healthy subjects. This pattern was correlated - together with an increase of < 1Hz oscillation -, with the gradual loss of responsiveness to stimuli as the anesthetic dose was increased.

Note that a prior study found a phenomenon similar to anteriorization of alpha during eyes-closed after smoking (Knott, 1989). There were however several differences in the design of that study and our study that may account for these conflicting results. For example, EEG recordings with eyes-closed were limited to 3 min which may not be enough to detect the anteriorization of alpha. Further, smokers were studied and opposite effects of nicotine on brain activity in smokers and non-smokers have been described previously (Ernst et al. (2001)).

Summarizing the nicotine effect during the EC condition, it seems that nicotine counteracts a well preserved pattern which leads to a decrease of vigilance level. In conclusion, it seems that the decrease in power shown during EC could reflect a nicotine-induced increase of vigilance.

The frontal location of our nicotine effect is in line with Picard *et al.* (2013). These authors used PET and radioligands for localizing the $\alpha 4\beta 2$ nicotinic receptors in humans. Particularly interesting, they combined their result with the brain areas which were involved during eyes-closed activity within alpha activity in a previous EEG-fMRI study (Sadaghiani *et al.* (2010)). The authors (Picard *et al.*, 2013) were able to find that the brain areas with highest concentration of $\alpha 4\beta 2$ receptors were located within the insular and anterior cingulate cortex. Note that our EC results showed the most significant clusters (the darkest spots in the figures) located in the region that could correspond perfectly to the CI: they are located close to the midline. Therefore we speculate that nicotine binds to the $\alpha 4\beta 2$ receptors directly located in CI. The consequence of such binding could be a decrease of oscillatory power recorded by EEG. Such interpretation is further corroborated by an *in vitro* study showing that a low concentration of nicotine can activate $\alpha 4\beta 2$ receptors and decrease the overall oscillatory power (Sigalas *et al.* , 2015). In conclusion, it is plausible that the decrease of power found fronto-centrally during EC in our experiment, reflects -at least partially- the direct action of low concentration of nicotine on $\alpha 4\beta 2$ receptors located in the cingulate cortex.

Worth mentioning are recent pharmacogenomic approaches which try to link the nicotine individual response with the COMT genotype in non-smokers (de la Salle *et al.* (2013)). A more recent study (Bowers *et al.* (2015)) investigated the modulatory role of COMT genotype on nicotine effects resting-state condition in non-smokers. They found an increase of upper alpha power frontocentrally in Met/Met carriers. Although the direction was opposite with respect to ours, the location and frequency range still converged.

Several limitations have occurred in the present experiment. The first limitation regards nicotine administration. Indeed, in nonsmokers, it relies for ethical reasons mainly on pharmacokinetic techniques based on a slow release of nicotine into the body. Instead, it would have been optimal to use an intravenous route of administration, since the slow absorption of nicotine may have favored nAChR desensitization, rather than activation (Grady *et al.* (2012)).

The second limitation was the lack of blood samples. Therefore individual pharmacokinetic variability is still an issue. Bewernitz and Derendorf (2012) showed how pharmacokinetics could inform pharmacodynamics (i.e. drug-induced brain activity). Therefore future pharmacological experiments may also include the collection of a blood sample for optimally investigating how the drug impacts brain activity. Note however, that plasma blood levels of acute nicotine in nonsmokers do not show strong correlations with behavioural and neural measures (Vossel *et al.* (2008)).

The third limitation relates to the organization of EO and EC sessions. At first we explain why the two conditions were not randomized. Having first EO and then EC precludes straightforward comparison between them. Nonetheless this was a necessary choice in order to avoid doubling the number of subjects, since a meaningful comparison would have needed a permutation of the two conditions. Second, we justify the different length of the two conditions: 14-min EO and 7-min for EC. The 14-min EO was chosen due to the high reliability in terms of frontal asymmetry during resting EEG when the recording is longer than 12 min (Hagemann (2004)). Indeed, the initial hypothesis designing the experiment, included the possibility that nicotine could have an effect on frontal asymmetry (analysis later not carried out). The author claimed that the same length should be used also during EC. Instead, it was agreed upon in the present study to limit the EC to 7 min, in order to prevent the subject from falling asleep, thus potentially increasing the muscular artifacts (Olbrich *et al.* (2009)).

The fourth limitation regards the fact that previous EEG literature (Akalin Acar and Makeig (2013)) suggested that a four-layer BEM head model wrapped on a single subject's MRI image should provide the minimal localization error (mean 5.4 mm error). Instead in the present study a three-layer BEM head model was wrapped on a MNI template image for all subjects. Therefore the configuration used in the present study should be affected by a mean localization error of 7.6 mm. Thus a difference of a 2.2 mm of mean localization error between the optimal configuration and the actual configuration was considered tolerable and the source estimates were considered valid.

The fifth limitation is the use of standardized frequency ranges, which could have smeared the effect along the whole frequency range. Nonetheless, it was a mandatory step due to the authors' decision to adhere as much as possible to published guidelines (Jobert *et al.* (2012)). The sixth limitation regards the lack of use of genetic test in order to stratify the subjects. Particularly, the COMT genotype seems to be important in determining the individual response to nicotine (Bowers *et al.* (2015)). The seventh limitation regards the moderate number of subjects used.

Additional comments regard the lack of stratification in terms of responders versus non-responders (present paper), by using nicotine-induced cardiovascular changes as done elsewhere (Logemann *et al.* (2014)). Nonetheless, both papers share the leveraging on intra-subject variability either by a mixed-effects design (present paper) or by stratifying the sample in responders versus non-responders (Logemann *et al.* (2014)). Lastly, the present study converged with Logemann *et al.* (2014) about the finding that subjects can properly guess above chance whether they have been administered either nicotine or placebo.

Independent of the limitations and comments, we would suggest that future studies conducting pharmacological research non-invasively in humans, would measure oscillatory activity in a eyes-open and eyes-closed conditions and use a mixed-effects design. We would further recommend to localize changes in oscillatory activity with a source reconstruction analysis.

In conclusion, we have found that during EO condition, the intake of nicotine reduced the power of oscillatory activity between 12.5-18.4 Hz in the orbital part of the left middle frontal gyrus, while during EC we found a nicotine-induced reduction of power from 8.5 to 18.4 Hz involving an area spanning from the supplementary motor areas to the superior frontal gyri. Altogether the results suggest that nicotine inhibits the phenomenon of anteriorization of alpha, thus potentially increasing the level of vigilance.

2.6 Acknowledgments

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Chapter 3: Study 2, renormalized Partial Direct Coherence (rPDC) and Vector Length (VL)

Published as:

Ranzi P, Freund JA, Thiel CM, Herrmann CS. Encephalography connectivity on sources in male non-smokers after nicotine administration during resting-state. *Neuropsychobiology*. 2016;74:48-59.

3.1 Abstract

We present an EEG connectivity study where thirty healthy male non-smokers were randomly allocated either to a nicotine group (14 subjects, 7 mg transdermal nicotine) or to a placebo group.

EEG activity was recorded in an eyes-open and eyes-closed condition before and after drug administration. This is a re-analysis of a previous dataset. Through a source reconstruction procedure we extracted thirteen time-series representing thirteen sources belonging to resting-state network. Here we conducted connectivity analysis (renormalized Partial Directed Coherence, rPDC) on sources, focusing on the frequency range of 8.5 to 18.4 Hz, subdivided into three frequency bands (α_1 , α_2 and β_1) with the hypothesis that an increase in vigilance would modulate connectivity. Further, a phase-amplitude coupling (Mean Resultant Vector Length, VL) analysis was performed investigating whether an increase of vigilance would modulate phase-amplitude coupling. In VL analysis we estimated the coupling of the phases of three low frequencies (α_1 , α_2 and β_1) respectively, with amplitude of high frequency oscillations (30 to 40 Hz, low γ).

With rPDC we found that during eyes-closed, nicotine decreased feedback connectivity (from precentral gyrus to precuneus, angular gyrus, cuneus and superior occipital gyrus) at 10.5-12.4 Hz. The VL analysis showed nicotine-induced increases in coupling at 10.5-18.4 Hz in precuneus, cuneus and superior occipital gyrus during eyes-closed. During eyes-open, no significant results were found neither in connectivity nor phase-amplitude coupling measures at any frequency range.

In conclusion, the results suggest that nicotine potentially increases the level of vigilance in the eyes-closed condition.

3.2 Introduction

New developments in pharmacology are based on non-invasive neuroimaging, particularly by leveraging and optimizing techniques and methodologies already validated in basic neuroscience. An example is the use of connectivity measures which provide a unique perspective on the interaction of oscillatory brain dynamics (Kometer *et al.* (2015)). Particularly interesting is the use of connectivity measures of electromagnetic oscillatory activity regarding the modulation of vigilance (Piantoni *et al.* (2013)). Indeed, there has been recent interest in directed connectivity detected by renormalized Partial Directed Coherence (rPDC), with a particular emphasis to the pharmacological modifications of vigilance (Maksimow *et al.* (2014)). Thus, the experimental question here was whether the well-known nicotine-induced increase of vigilance (Gilbert *et al.* (2000)) could be captured by connectivity analysis as well.

Another example of methods which can be transferred to pharmacology is the study on nested oscillations by phase-amplitude coupling (Monto (2012)). Indeed, there were already previous attempts to use phase-amplitude coupling in combination with anesthetics. For example, Kullback-Leibler divergence (Tort *et al.* (2010)) has been used for assessing the action of propofol in the brain (Mukamel *et al.* (2014)), as well as Canolty's Modulation Index (Canolty *et al.* (2006)) for assessing the effect of sevoflurane on brain dynamics (Blain-Moraes *et al.* (2015)). Although the correlation between rPDC estimates and vigilance is well-established (Maksimow *et al.* (2014)), less so is the correlation of phase-amplitude cross-frequency coupling (CFC) and vigilance. Therefore the main focus of the paper will be the connectivity analysis, whereas phase-amplitude CFC analysis is considered a catalyst for stimulating further research in the field.

For the purpose of the present paper, rPDC (Schelter *et al.* (2009)) was used for computing directed connectivity, due to its previously known sensitivity in detecting pharmacological manipulations (Maksimow *et al.* (2014)). In addition, for the phase-amplitude CFC analysis we employed Mean Resultant Vector Length algorithm (VL) (Miyakoshi *et al.* (2013)). Unfortunately, terminology is not consistent throughout literature: for instance, the "mean vector length" in Tort *et al.* (2010) is "Canolty's modulation index (MI)", while Tort's "modulation index (MI)" (Tort *et al.* (2010), Roux *et al.* (2013) and Aru *et al.* (2015)) is the Kullback-Leibler divergence. VL as defined in

Miyakoshi et al. (2013) is constructed in parallel to an index termed "phase-locking-value (PLV)" elsewhere. Although Kullback-Leibler divergence is considered the gold standard (Tort *et al.*, 2010)), a recent paper challenged its sensitivity to spectral changes (Aru *et al.* (2015)). We think that VL is a more robust measure of phase-amplitude CFC since restriction to the largest (top 2 %) of observed amplitudes probably causes a good tolerance to noise. In other words, VL should be more tolerant to changes in signal-to-noise ratio and to the effect of outliers. In a nutshell, rPDC and VL algorithms were employed in the present study on source-reconstructed EEG time-series.

Regarding the experimental design, eyes-open fixation on a cross (EO) and eyes-closed (EC) conditions were employed. The EO condition was considered ideal for testing the effect of nicotine, because nicotine appears to target attentional networks (Lawrence *et al.* (2002), Thiel *et al.* (2005)). The EC condition was considered also important since nicotine impacts this condition as well (Bowers *et al.* (2015)). Lastly, a mixed-effects design where subjects were randomly allocated to a drug or placebo condition and each subject was tested before and after drug or placebo administration. Such designs are reliable in decreasing intra-subject variability by estimating the individual reaction with respect to the administration of a drug (Lavielle (2015)).

According to the previous literature (Bowers *et al.* (2015), Fisher *et al.* (2012) and Foulds *et al.* (1994)) nicotine preferentially exploits its effect within three frequency ranges during eyes-open and eyes-closed activity in non-smokers: α_1 , 8.5-10.4 Hz; α_2 , 10.5-12.4Hz; β_1 , 12.5-18.4Hz. Such previous knowledge helped us in selecting the frequency ranges where a nicotine effect was expected. We have previously shown in the same dataset as used here that nicotine increased vigilance during eyes-closed activity (Ranzi *et al.* (2016)). The analysis presented here offers a new complementary perspective in the way the molecule modifies oscillatory brain activity. Further, not attempt of a mechanistic explanation of a causal relationship between nicotine and vigilance will be provided. Thus the study has to be considered descriptive in its nature, intending it as a guide for future research in the field.

In summary, we aimed to investigate whether nicotine-induced modulations of vigilance impact both connectivity (rPDC) and phase-amplitude CFC (VL) computed on EEG time-series during resting-state. Particularly for connectivity analysis, a plausible link between nicotine-induced modification of connectivity and vigilance is provided. To the best of the authors' knowledge, this is the first paper showing results of nicotine-

induced modification of connectivity during the resting-state condition in healthy non-smoker male subjects.

3.3 Subjects and methods

3.3.1 Subjects

Thirty right-handed, nonsmoking male subjects (age: 27 years \pm 3, weight: 81.1 kg \pm 9.5, height: 1.82 meter \pm 0.05) participated in this study. Subjects were not on any kind of medication nor reported any history of major medical illness or neurological or psychiatric disorders. Only non-smokers (no more than 10 cigarettes consumed during their whole life) were recruited to avoid confounding effects of withdrawal symptoms (Fisher *et al.* (2012)). We decided to use only male subjects in order to minimize gender-related confounds (Jausovec and Jausovec (2010)) and possible hormonal interaction with nicotine (Duncan and Northoff (2013)). All subjects gave written informed consent. The study was approved by the Ethics Committee of the German Psychological Association.

3.3.2 Drugs

All subjects were told to refrain from legal psychoactive drugs such as alcohol and caffeine 24 hours before the experiment. Following the double-blind procedure, each subject was randomly allocated to receive either nicotine (NIC, n=14) or placebo (PLA, n=16). A 7 mg nicotine patch (Niquitin® Clear 7 mg, GlaxoSmithKline Consumer Healthcare GmbH) and a matched placebo (plaster of same shape and thickness) were used. The patches were administered by a third person, not otherwise involved in the study, onto the subject's lower back, covered with a traditional plaster and removed after 50 min. In order to minimize side effects in non smokers, the patch was administered for 50 min only and then removed prior to the second EEG recording session. A comparable procedure was used in previous studies (Breckel *et al.* (2015), Potter and Newhouse (2008)) and provided significant behavioural effects of nicotine. Because very little time elapsed between the removal of the patch and EEG recording (~10 min refilling the

electrodes), nicotine level in the blood is expected to be stable during the whole EEG recording session (Benowitz *et al.* (2009)).

3.3.3 Experimental design

A mixed-effects design was employed, which investigates the effects of nicotine both within and across subjects. Subjects were randomly allocated to the placebo or nicotine group (between-subject factor drug) and measured in two sessions, before (PRE) and after (POST) the respective intervention (within-subject factor time), namely immediately after removal of the placebo or nicotine patch. Each EEG recording session consisted of 14-min eyes-open fixation on a cross condition (EO) and a 7-min eyes-closed condition (EC). The EC session always followed the EO session. A 40-min cognitive task was also performed thereafter, but was not analyzed due to a systematic software error. The same time of day (3:00 pm) was used for all subjects in order to minimize the influence of circadian rhythms. Subjects kept the EEG cap mounted during the whole experiment. Before the second recording, the gel in the electrodes was refilled in order to regain an impedance below 10 k Ω for each electrode (see section 2.5).

3.3.4 Subjective and physiological measures

To address subjective and cardiovascular effects of nicotine, mood rating scales as well as heart rate and blood pressure were assessed twice: upon arrival (PRE) and immediately after removal of the placebo or nicotine patch (POST). Subjective mood was measured by visual analogue scales (Bond and Lader (1974)). Rating scores were grouped into the three factors ‘alertness’, ‘contentedness’, and ‘calmness’, following Bond and Lader (1974).

3.3.5 EEG recording

The EEG data were recorded with 63 Ag/AgCl-electrodes attached to an elastic cap (Easycap GmbH, Herrsching-Breitbrunn, Germany). A standard 10-10 montage was used, where the reference was positioned at the tip of the nose and electrode AFz served as ground (see standard 10-10 montage). One EOG electrode was set on the external canthus of the right eye, again referenced to the tip of the nose. Impedances were kept below 10

k Ω for each electrode. The EEG signal was sampled with 500 Hz and amplified using a BrainAmp system (Brain Products, Munich, Germany). Data were recorded and digitalized with the BrainVision Recorder (Brain Products, Munich, Germany). Data were stored in a computer and analyzed off-line.

All recordings were conducted in an electrically-shielded, sound-insulated and dimly-lit chamber. Subjects were seated in a comfortable chair with firm armrests up to the wrists. During the EO condition the subjects were asked to keep their eyes-open and fixate their gaze on a cross located on the screen. The cross was light grey (size 0.86°). It was superimposed on a dark-grey background. During EC the cross was kept on the screen, but the subject was asked to close his eyes and to keep them closed until the experimenter asked to open them again.

3.3.6 EEG data preprocessing

Preprocessing was performed using EEGLAB (Delorme and Makeig (2004)), version 12.0.2.5b and included the following steps: first, raw time-series from BrainVision Analyzer (Brain Products, Munich, Germany) were converted to an EEGLAB format. Afterwards, PRE and POST time-series belonging to the same subject were concatenated (i.e. 14 min + 14 min = 28 min length time-series). According to Tsai *et al.* (2014) a concatenated time-series belonging to the same subject provides a better estimate of ICA-rejected artefacts than a single time-series (i.e. only 14 min). The two conditions (EO and EC) were concatenated separately. Data were then high-pass symmetric FIR filtered at 2 Hz with Blackman windows (transition band 0.9 Hz, filter order 1000), downsampled to 250 Hz and low-pass symmetric FIR filtered at 40 Hz with Blackman windows (transition band 5 Hz, filter order 276). The channel location according to standard 10-20 montage was then included (necessary step for generating ICA topographies). ICA estimation was computed. Then ICA-based artefacts rejection (i.e. blinks; muscular movements; heart-beats;) was performed in a semi-automated way on a subject-by-subject basis by considering the concatenated data according to Jung *et al.* (2000). It should be noted that using ICA-based artefact rejection instead of manually rejecting corrupted epochs, guarantees no data loss. This means that the clean EO and EC time-series always have the same length as the raw time-series. The ICA-based artefact rejection was considered important for source reconstruction analysis, since it does significantly improve source

estimation (Fatima *et al.* (2013)). The concatenated file was then split back into the two original time-series (i.e. from 28 min length time-series back again to two separated 14 min time-series), now cleaned from artefacts. Further, the EOG channel was deleted from each time-series. Lastly, each time-series was split into 5-sec epochs (i.e. 14 min = 168 epochs of 5 sec each).

3.3.7 Extraction of reconstructed sources

The 5-sec epochs from EEGLAB were first converted to an SPM-compatible format by SPM12 version 6225 (Litvak *et al.* (2011)). Source reconstruction was carried out using the eLORETA algorithm (Pascual-Marqui *et al.* (2011)) implemented using SPM12 beamforming toolbox (<https://code.google.com/p/spm-beamformingtoolbox/>). The regularization parameter for eLORETA was set to 0.05 (default in SPM12). In order to create the head model, an ICBM152 template was used according to Litvak *et al.* (2011). For all subjects a cortical mesh was extracted by using a three-layer Boundary Element Model (BEM) head model. Therefore all subjects had the very same cortical mesh which was MNI-aligned. The computation of the sources was band-limited to 4-40 Hz in order to avoid line noise (50 Hz). For all three analyses we further subdivided the time-series into standard frequency ranges of 8.5-10.4 Hz, α_1 ; 10.5-12.4 Hz, α_2 ; and 12.5-18.4 Hz, β_1 according to Jobert *et al.* (2012). Lastly, the 5-sec epochs were merged using EEGLAB and the whole time-series for each brain area was rebuilt.

<i>OBART terminology</i>	<i>Anatomical label (AAL)</i>	<i>MNI coordinates</i>		
		<i>x</i>	<i>y</i>	<i>z</i>
right superior frontal gyrus	Frontal_Mid_Orb_R	8	60	-9
left middle frontal gyrus	Frontal_Mid_L	-29	43	25
right middle frontal gyrus	Frontal_Mid_R	44	52	18
left superior frontal gyrus	Frontal_Sup_Medial_L	-3	53	26
left precentral gyrus	Precentral_L	-43	2	39
right precentral gyrus	Precentral_R	56	-3	36
left inferior parietal gyrus	Parietal_Inf_L	-41	-45	44
left precuneus	Precuneus_L	-4	-58	44
right inferior parietal gyrus	Parietal_Inf_R	45	-48	54
right angular gyrus	Angular_R	39	-60	44
left angular gyrus	Angular_L	-57	-62	27
right cuneus	Cuneus_R	7	-90	20
left superior occipital gyrus	Occipital_Sup_L	-20	-99	21

Table 3: Anatomical labels and corresponding MNI coordinates of 13 ROIs belonging to the resting-state network.

Note. Showing anatomical labels from either the Online Brain Atlas Reconciliation Tool (OBART) in the -leftmost column - or Automated Anatomical Labeling (AAL) - central column - corresponding to a specific MNI coordinates - rightmost columns-.

Regions of Interest (ROIs) were chosen according to Chen *et al.* (2013) who identified thirteen brain regions that show increased connectivity in the alpha band in an eyes-open state with respect to an eyes-closed resting-state. We centred a sphere (5 mm radius) at each specific MNI coordinate defining the respective ROI and we then extracted the time-series. The 13 time-series were then used as input for either rPDC or VL analyses. Since the areas in Chen *et al.* (2013) were in Talairach coordinates, a conversion to MNI

coordinates was conducted (Lancaster *et al.* (2007)), by using 'tal2icbm_spm.m' (see <http://www.brainmap.org/>). Further, SPM12 recognized some MNI coordinates as outside the brain, therefore a slight modification of the MNI coordinates outputted by 'tal2icbm_spm.m' was necessary. The MNI coordinates used in the present paper are shown in Table 3. These MNI coordinates were also used for the spatial dimensionality reduction needed for optimizing the source reconstruction procedure, as suggested in Oswal *et al.* (2014). Further, MNI coordinates for each ROI were translated to anatomical labels according to the Automated Anatomical Labeling (AAL) atlas (Tzourio-Mazoyer *et al.* (2002)), by using xjView toolbox (<http://www.alivelearn.net/xjview>). Lastly, the AAL labels were plugged in the Online Brain Atlas Reconciliation Tool (OBART) (Bohland *et al.* (2009)) in order to obtain standardized names of anatomical labels.

In summary, 13 time-series were extracted from 13 ROIs representing 13 brain areas which belong to the resting-state network. Table 3 displays 13 anatomical labels of the corresponding ROI centred at specific MNI coordinates.

3.3.8 Connectivity measure

The inference of *directed* connections between pairs of reconstructed sources from related time series must necessarily go beyond classical correlation measures which are symmetric under an exchange of pair constituents. A *vector autoregressive process of order p* (VAR[p]) serves as starting point for the inference. The order p must be sufficiently large to cover the time range of the suspected causal interaction. We deliberately chose the order $p=30$ which covers the time span $T = p/f_s = 30/250 \text{ Hz} = 0.12 \text{ secs}$ and roughly corresponds to one period of an 8 Hz signal. On the other hand, a VAR[30] for 13 sources requires the estimation of $30 \times 13 \times 13 = 5070$ parameters. To estimate these parameters we extracted epochs of 21 seconds which comprise $21 \text{ secs} \times 250 \text{ Hz} = 5250$ data samples per source. The time series of sources are thus segmented into 40 and 20 epochs for EO and EC, respectively (e.g. PRE_NIC_EC corresponds to 20 epochs per subject).

The so-called partial directed coherence PDC (Baccala and Sameshima (2001)) is a spectral equivalent of Granger-causality and based on the Fourier transform of the estimated VAR[30] coefficients. Since it includes the concept of partialization it can exclude *indirect* connections between a pair of sources; therefore it accounts for *directed*

direct connections. The final step is a renormalization of the PDC yielding the rPDC which allows for a statistical assessment of estimated values and makes them robust against spectral changes (details in Schelter *et al.* (2009)).

We thus obtained for each epoch an asymmetrical 13-by-13 array of rPDC curves over a spectral axis (Nyquist interval) for each epoch. For each of the $13 \times 12 = 156$ ordered unequal source pairs, i.e. the off-diagonal entries of the 13-by-13 array, rPDC averages over the subbands of interest ($\alpha_1=8.5-10.4$ Hz, $\alpha_2=10.5-12.4$ Hz, and $\beta_1=12.5-18.4$ Hz) were computed. In summary, for each subband ($\alpha_1, \alpha_2, \beta_1$), for each ordered source pair (13 x 12) and for each epoch (EO: 40 / EC: 20)) we collected related rPDC values and (without assessing statistical significance) passed these on to the statistical inference (see paragraph 2.11).

3.3.9 Phase-amplitude CFC

We considered only nested phase-amplitude CFC, i.e. the coupling between the amplitude of high frequency oscillations (hfo band low γ) and the phase of low-frequency oscillations (lfo bands $\alpha_1, \alpha_2, \beta_1$) within the same source. For a CFC a wider hfo band would surely be beneficial, however, also Monto (2012) found the most interesting phase-amplitude CFC phenomena during EC below 35 Hz.

All 13 source signals were segmented into 168 (EO) / 84 (EC) epochs of 5-sec each. Each segment was bandpass filtered with respect to lfo and hfo frequency bands using the Phase-Amplitude Coupling Toolbox (PACT <http://scn.ucsd.edu/wiki/PACT>). This was followed by a computation of the analytic signal and an extraction of the lfo phase and hfo amplitude by using built-in MATLAB's commands. To avoid edge effects resulting from the Hilbert transform we snipped off 0.5-sec long head and tail segments of each 5-sec epoch. The computation of the VL from remaining 4-sec was left to the PACT; in this toolbox hfo amplitudes are ranked and a certain percentage (selected by a threshold rate) of the largest are selected to compute the vector sum with related lfo phases. From a global pre-assessment of our data in the context of Rayleigh statistics we fixed the threshold rate at 2%.

In summary, for each of the 13 sources, for each subband ($\alpha_1, \alpha_2, \beta_1$) - $13 \times 3 = 39$ entries per matrix – and for each epoch (EO: 168 / EC: 84) we collected related VL

values and (without assessing statistical significance) passed these on to the statistical inference (see paragraph 2.11).

3.3.10 Statistical inference

LIMO EEG toolbox version 1.5 (Pernet *et al.* (2011)) provided the statistical inference for a hierarchical mixed-effects design (Friston *et al.* (2005)) based on robust regression (Wilcox (2012)). A Weighted Least Square algorithm was used for computing linear regression. A t-test (either paired or unpaired or one sample t-test, depending on the analysis) was employed.

Regarding the validation of the two pipelines, two separate methods were used in order to replicate previous results as much as possible. Regarding the validation of rPDC-pipeline, a paired t-test with bootstrapping was performed on the EO and EC conditions in all subjects prior to placebo or drug application. Note that only the first 7 min of EO were considered, in order to have the same length as the EC condition. This procedure of *methodological control* allowed the same rationale of standard pipeline-validation used in fMRI research (Liu *et al.* (2013)) and EEG research (Blain-Moraes *et al.* (2015)): if the pipeline can detect a previously reported effect (e.g. EO versus EC differences), then it should also be possible to gauge drug effects. The validation of the rPDC-pipeline aimed to replicate the findings of Piantoni *et al.* (2013). Our results were almost all significant. In order to generate an interpretable graphical output, only the strongest effect was considered. Threshold-free Cluster Enhancement (TFCE) (Smith and Nichols (2009)) was used for clustering the brain areas with the strongest effect. Specifically, the maximum (max) of TFCE scores (using t-values as input) was computed, which in practice is similar to the maximum-likelihood estimation approach (having a matrix of statistical values, the max within such matrix is taken). For the validation of VL-pipeline, a different procedure was undertaken in order to provide a replication as close as possible to the methods of Roux *et al.* (2013). Particularly, a one sample t-test with bootstrapping at a frequency range from 8 to 13 Hz was performed on the median (it is the most robust estimator with respect to outliers, Wilcox (2012)) of the PRE_EC values over all trials belonging to each subject. The results were all significant. To further select the strongest effect, the medians were used as input for computing TFCE scores. Lastly, the max of TFCE scores was computed, which practically resembles the maximum-likelihood estimation approach. Note that TFCE scores are sufficiently flexible to any statistical

input (Smith and Nichols (2009)). Further, TFCE scores are dimension-less, therefore they can have different ranges depending on the statistical values used as input.

Contrary to methodological control, two unpaired t-test were used on the PRE and POST data to gauge the effect of nicotine. Beta values for each subject were obtained with first-order statistics (within-subject factor time; input: raw values PRE and POST; output: two Betas, one for PRE, one for POST). These Beta values were entered into two second-order statistics using two unpaired t-tests (between-subject factor drug). Regarding the multiple comparison problem the cluster-based approach TFCE (Smith and Nichols (2009)) was implemented by percentile bootstrap (Pernet *et al.* (2015)). The neighbourhood matrix was created in a data-driven manner by using wrappers to FieldTrip (Oostenveld *et al.* (2011) available in LIMO. As parameters, a threshold of 60 as Euclidian distance and a minimum number of channels set to 2 were considered optimal. All parameters (e.g. iterations of bootstrap, TFCE parameters etc.) were set according to Pernet *et al.* (2015). All two analyses used the same aforementioned set up. The direction of the nicotine effect was computed by following the procedure suggested in Douaud *et al.* (2013) which is appropriate for our design and the generation of box plots is the last step in the analysis. Briefly, the summary statistics image from the second-order level was used as mask for the Betas measured previously (first-order statistic) at those significant brain areas. These Betas were extracted per subject and per condition and then the median was computed along the single-subject trials. Finally these data were then plotted in a box plot.

3.3.11 Graphical output

To visualize the output of the statistical analysis, BrainNet Viewer toolbox (Xia *et al.* (2013) was employed. The same template (ICBM152) was used as a headmodel both for source reconstruction and for the graphical output. The location of the ROIs and the arrows presented in the figures are anatomically precise with respect to the computed source estimations.

3.4 Results

3.4.1 Subjective and physiological measures

As previously reported (Ranzi *et al.* (2016)), a Two-way ANOVA was performed with the between-subject factor drug (either placebo or nicotine) and the within-subject factor time (PRE and POST) both for cardiovascular measures and mood ratings.

We found a significant interaction between the two factors regarding systolic blood pressure ($F_{1,28} = 4.33, p < 0.047$), which meant a stronger decrease of systolic blood pressure under placebo as compared to nicotine. There was neither a significant main effect of drug nor a significant main effect of time. Regarding diastolic blood pressure and heart rate, no significant effects (neither main effects nor interaction) were found.

No significant interaction between the two factors on mood ratings with respect to alertness ($F_{1,28} = 0.844, p < 0.367$), contentedness ($F_{1,28} = 0.140, p < 0.720$) or calmness ($F_{1,28} = 0.170, p < 0.685$) were found. Neither a main effect of drug nor a main effect of time was found significant.

3.4.2 Connectivity analysis (rPDC)

To validate our connectivity analysis we compared connectivity detected by rPDC in the EC and EO condition using the data prior to the intervention (PRE) by a paired t-test. We found a significant increase (Fig. 5a) of rPDC connectivity within 10.5-12.4 Hz during EC with respect to EO (Fig. 5b) in the following direction: from left superior occipital gyrus to right superior frontal gyrus, to left middle frontal gyrus and to left superior frontal gyrus. Fig. 5b illustrates the significant increase in feedforward connectivity under EO as compared to EC. An increase of feedforward connectivity by comparing eyes-closed with respect to eyes-open was found also in previous study (Piantoni *et al.* (2013)).

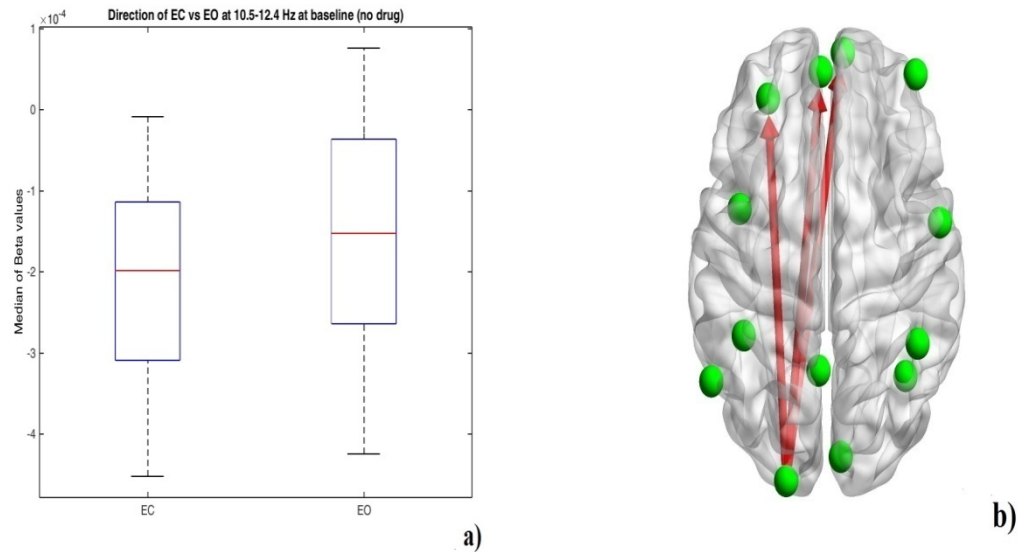


Figure 5: Methodological control using connectivity detected by rPDC within 10.5-12.4 Hz during EO with respect to EC. a, Significant increase of the EO forward connectivity with respect to EC is shown in the box plot. Data represented by the Betas after being masked by the max of TFCE scores. The x-axis refers to the condition of either EC or EO. The y-axis refers to median of Beta values in EC and EO, respectively. On each box plot, the central mark is the median, whereas the edges of the box are the 25th and 75th percentiles and the whiskers extend to the most extreme data points not considered outliers. b, Axial view of the feedforward connectivity. Red arrows indicate an increase of EO connectivity with respect to EC. The figure shows brain areas with maximum of TFCE scores. 13 ROIs analyzed are depicted in green. Nose at the top.

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To investigate the effects of nicotine on resting-state oscillations, we analyzed the between-subject factor drug (nicotine/placebo) PRE and POST. In the EC condition, we found a significant decrease of connectivity in the POST session under nicotine as compared to placebo within the frequency range of 10.5-12.4 Hz from right precentral gyrus to left precuneus, right angular gyrus, right cuneus and left superior occipital gyrus ($p < 0.0167$, FWE-corrected within each frequency band and Bonferroni-corrected for 3 frequency bands investigated; Fig. 6b). There was no significant effect in the PRE session. The box plot illustrates the difference between PRE and POST values under nicotine and placebo (Fig. 6a), with smaller differences under nicotine. No further

decreases or increases in connectivity were found neither for different frequency ranges nor during EO.

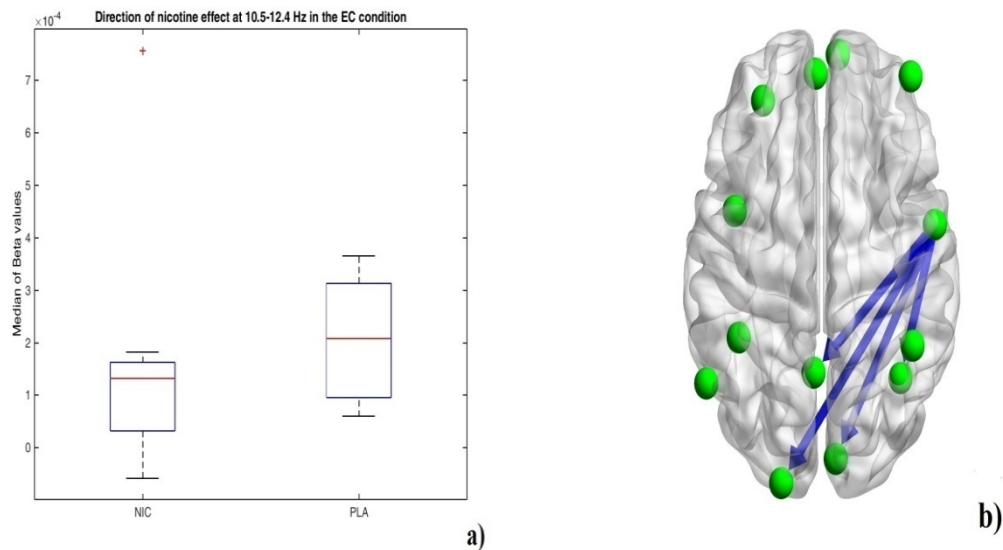


Figure 6: Nicotine-induced decrease of connectivity detected by rPDC within 10.5-12.4 Hz during EC. a, Direction of the nicotine effect on 10.5-12.4 Hz in the EC condition on rPDC connectivity. The box plot shows a significant decrease from 10.5 to 12.4 Hz of NIC with respect to PLA. The x-axis refers to the condition of either NIC or PLA. The y-axis refers to median of Beta values in NIC and PLA, respectively. On each box plot, the central mark is the median, whereas the edges of the box are the 25th and 75th percentiles and the whiskers extend to the most extreme data points not considered outliers. Outliers are marked with a red cross. b, Decrease (blue arrows) of rPDC connectivity induced by nicotine within 10.5-12.4 Hz during EC (unpaired T-test). The figure shows brain areas FWE-corrected cluster-based results at $p < 0.0167$ (Bonferroni-corrected for 3 frequency bands investigated). 13 ROIs analyzed in green. Nose at the top.

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3.4.3 Phase-amplitude CFC analysis (VL)

To validate our VL analysis we compared phase-amplitude CFC detected by VL in EC condition in all subjects prior to placebo or drug application with a one sample t-test. All ROIs yielded statistically significant results. We found the most significant VL values (namely, max TFCE scores; e.g. TFCE score 0.00895 in Table 4) within 8-13 Hz

in left precuneus, left inferior parietal gyrus, right angular gyrus and right cuneus (Fig. 7). The pattern shown in Fig. 7 resembles the increase of phase-amplitude CFC found occipitally previously by one sample t-test during eyes-closed (Roux *et al.*, 2013).

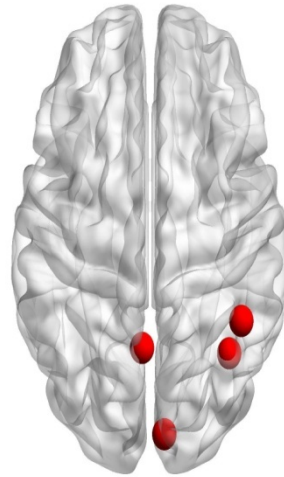


Figure 7: Methodological control using phase-amplitude CFC detected by VL during EC at baseline (PRE). Plot of the max TFCE scores computed within 8-13 Hz during EC. Nose at the top. For actual TFCE scores see Table 4.

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Table 4: Anatomical labels and corresponding TFCE scores obtained by using medians of VL values computed within 8-13 Hz during EC.

<i>Anatomical label (AAL) of ROIs</i>	<i>TFCE scores</i>
right superior frontal gyrus	0.00686
left middle frontal gyrus	0.00686
right middle frontal gyrus	0.00544
left superior frontal gyrus	0.00686
left precentral gyrus	0.00001
right precentral gyrus	0.00544
left inferior parietal gyrus	0.00544
left precuneus	0.00895
left inferior parietal gyrus	0.00895
right angular gyrus	0.00895
left angular gyrus	0.00544
right cuneus	0.00895
left superior occipital gyrus	0.00727

Note. Data were taken from the session prior (PRE) to drug/placebo administration. In Fig. 7 the only the max of TFCE scores are shown.

To investigate the effects of nicotine on phase-amplitude CFC, we analyzed the between-subject factor drug (nicotine/placebo) PRE and POST. In the EC condition, we found a significant increase of VL values from POST session under nicotine as compared to placebo within the frequency range of 10.5-18.4 Hz (α_2 and β_1) in left precuneus, right cuneus and left superior occipital gyrus ($p < 0.03$, FWE-corrected; Fig. 8b). There was no significant effect in the PRE session. The box plot illustrates the difference between PRE and POST values under nicotine and placebo (Fig. 8a), with larger differences under nicotine. No further significant decreases or increases in VL values were found neither for different frequency ranges nor during EO.

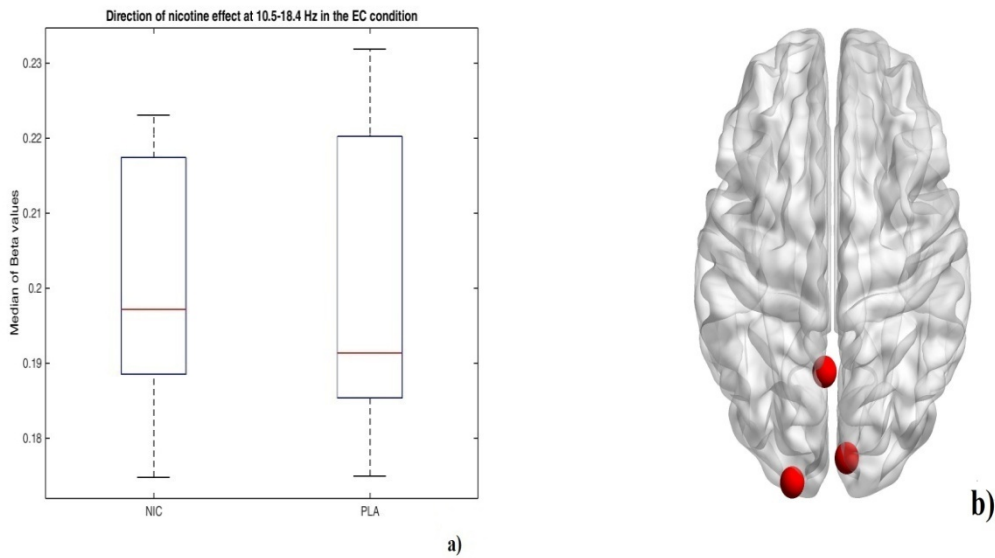


Figure 8: Nicotine-induced increase of phase-amplitude CFC detected by VL within 10.5-18.4 Hz during EC. a, Direction of the nicotine effect at 10.5-18.4 Hz in the EC condition measured by VL. The box plot shows a significant increase in the frequency bands from 10.5 to 18.4 Hz of NIC with respect to PLA. The x-axis refers to the condition of either NIC or PLA. The y-axis refers to the median of Beta values in NIC and PLA, respectively. On each box plot, the central mark is the median, whereas the edges of the box are the 25th and 75th percentiles and the whiskers extend to the most extreme data points not considered outliers. b, Increase (red ROIs) of VL values induced by nicotine within 10.5-18.4 Hz during EC. The results are FWE-corrected cluster-based results at $p < 0.03$ (unpaired T-test). Nose at the top.

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3.5 Discussion

The main goal of the article was to detect nicotine-induced modulations of connectivity (rPDC algorithm) which should reflect nicotine-induced modifications of vigilance. Additionally, the nicotine-induced modulations of phase-amplitude CFC (VL algorithm) were also addressed. The oscillatory brain activity was originally measured by scalp EEG during resting-state and thirteen ROIs belonging to a resting-state network were extracted.

About the methodological control of our rPDC-pipeline, the increase of power within 10.5-12.4 Hz during EO with respect to EC (Fig. 5a and Fig. 5b) is in line with previous findings that reported eyes-open connectivity in the forward direction to be greater compared to eyes-closed (Piantoni *et al.* (2013)). As a caveat there are differences between the present article and Piantoni *et al.* (2013): the frequency range; algorithm used for computing connectivity, and the number and location of the estimated sources. Nonetheless, the authors believe that the pattern emerged in the present study looked similar to the discovery mentioned in the abovementioned article. Therefore, our rPDC-pipeline was considered validated.

Regarding the results of the connectivity analysis (rPDC), a nicotine-induced decrease of connectivity was found during EC within the frequency range of 10.5-12.4 Hz, mainly in parietal and occipital regions. Prior studies suggest that feedforward connectivity - from back to front - is related to increases in vigilance (Maksimow *et al.* (2014), Piantoni *et al.* (2013)), whereas feedback connectivity - from front to back - is increased with a decrease of vigilance (Maksimow *et al.* (2014)). In a nutshell, the two patterns of connectivity are anti-correlated and both indicate the on-line level of vigilance (Maksimow *et al.* (2014)). In the present study, we have demonstrated how nicotine can decrease the feedback connectivity during EC. We suggest that the increase of feedback connectivity in PLA during EC (Fig. 6b) was due to natural tiredness which characterizes the PLA_EC_POST versus the PLA_EC_PRE period. Therefore nicotine seems to be able to counteract the natural increase of feedback connectivity, hence counteracting sedation. In conclusion, nicotine seems to increase vigilance by dampening feedback connectivity.

Increases in phase-amplitude CFC in our methodological control under EC resemble findings in Osipova *et al.* (2008) and Roux *et al.* (2013). Using MEG, they showed the strongest phase-amplitude CFC between the phase of alpha and the amplitude of gamma during eyes-closed being located in parieto-occipital brain areas. A caveat is the fact that the above paper computed low and high gamma (up to 70 Hz), whereas in the present paper only low gamma (up to 40 Hz) is computed. A minor caveat regards some technicalities which differ between the present paper and the aforementioned paper (e.g. different length of EC). VL showed the strongest phase-amplitude CFC at alpha range (8-13 Hz) parieto-occipally (Fig. 7). Therefore we considered VL-pipeline validated.

Regarding the results of the phase-amplitude CFC analysis (VL), a nicotine-induced increase of VL values was found parieto-occipally at 10.5-18.5 Hz during EC.

Regarding the interpretation of results of our VL analysis, Blain-Moraes *et al.* (2015) found a parietal decrease of coupling during eyes-closed when subjects became sedated after the administration of an anaesthetic. Although they used a different phase-amplitude CFC algorithm (Canolty's Modulation Index, Canolty *et al.* (2006)), we conclude that their measure is nonetheless comparable with our VL estimation. Therefore our nicotine-induced increase of VL phase-amplitude CFC during EC could indicate that nicotine actually increased vigilance. Corroborating the validity of our VL phase-amplitude CFC results, the effect of nicotine was found occipito-parietally despite the fact that nicotine-induced changes in power spectrum were found frontally (Ranzi *et al.* (2016)). Further, we conducted a Kullback-Leibler divergence analysis (not published) and we found comparable results with our VL analysis. In other words, we think that our results from VL analysis are unlikely to be false positives, rather the actual nicotine effect on phase-amplitude CFC. In conclusion, the nicotine-induced increase of VL estimates could indicate an increase of vigilance.

Now we will compare the results from the two analyses. Overall, a direct comparison is not possible, since they reflect potentially different neurophysiological phenomena (i.e. connectivity measures more linked with vigilance modulations). Further, the frequency range where the effect was found, differed in the three analyses as well (10.5-12.4 Hz for rPDC; 10.5-18.4 Hz for VL;). Nonetheless, there were some commonalities among the two analyses. For example, both analyses showed a significant effect during EC condition but not during EO condition. This was surprising since EO data had a twice as good SNR (namely, a twice as long time-series) as the EC condition. A possible explanation for such a result could be attributed to a ceiling effect during EO condition. In other words, it seems that the EC condition induces a decrease in vigilance (Olbrich *et al.* (2009)), whereas the psychostimulant effect of nicotine could effectively counteract this drop of vigilance. Instead, during EO the brain activity is possibly already in a state of high vigilance.

As a last point, note that the five general limitations mentioned in Ranzi *et al.* (2016) apply also to the present analyses (e.g. lack of blood sample; using standardized frequency ranges etc.).

In conclusion, brain activity is changed by nicotine during eyes-closed, but not during eyes-open. The connectivity analysis (rPDC) showed a parieto-occipital decrease of connectivity at 10.5-12.4 Hz during eyes-closed condition. Instead, the phase-amplitude CFC analysis (VL) showed a parieto-occipital increase of phase-amplitude

CFC at 10.5-18.4 Hz during eyes-closed condition. Particularly for the connectivity analysis, previous literature confirmed a relationship between the increase of feedback connectivity and the decrease of vigilance. What was shown here was a nicotine-induced decrease of feedback connectivity. Altogether it seems that nicotine favours a brain state that is characterized by an increase of vigilance.

3.6 Acknowledgments

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3.7 Supplementary material: Preventing false positives, a checklist

In what follows, we present a checklist extracted from Aru *et al.* (2015) with the aim of avoiding errors and pitfalls of interpretation. Although the checklist originates from a method review on CFC, we think it may also apply (at least partly) to the directed connectivity analysis. Namely, the nine-items checklist was considered valid for all both rPDC and VL analyses.

First, the presence of oscillations in the alpha frequency have already been well-documented in eyes-closed (Bazanov and Vernon (2014)) and eyes-open activity (Haegens *et al.* (2014)). In a spectral analysis of the same dataset (Ranzi *et al.* (2016)) it was found that these three lfo bands used here, indeed cover a dominant oscillatory component which is a prerequisite for a meaningful analysis. Second, we selected fixed bandwidths in order to standardize the procedure (Jobert *et al.* (2012)) and knowing a priori the frequency ranges where nicotine has an effect (Bowers *et al.* (2015) and Fisher *et al.* (2012)). Specifically for phase-amplitude CFC, the hfo band was selected as the range from 30-40 Hz. While the bandwidth of 10 Hz is less than twice the lfo (mathematically, $10 \text{ Hz} < (2 \times \text{lfo})$), it means that the bandwidth is too narrow to capture the side peaks. Such situation was described as a significant source of false negatives (Aru *et al.* (2015)). Nonetheless it will at least not lead too false positives. Third, regarding the interpretation of instantaneous phase, such problem affects only phase-amplitude CFC estimation (VL) but not connectivity estimation (rPDC). We found that the extracted lfo phases were meaningful since we consistently observed a monotonic growth with time. Fourth, regarding precision by snipping of 0.5 (head and tail) as described in paragraph 2.9 we automatically checked for edge effects. We have also computed the data without snipping and we found no essential changes of the value statistics. Fifth, since resting-state time-series are considered weakly non-linear with respect to evoked activity (Stam (2005)) and since we used only resting-state data, we considered it not necessary to test for non-linearities. Sixth, non-stationarities in the source reconstructed time-series have been forcibly eliminated, with the averaging of all voxels belonging to a specific ROI (Brookes *et al.* (2014)). See paragraph 2.7 for details. Therefore our 13 ROI's time-series have been forced to be stationary. Seventh, regarding temporal structure we look at changes between at 1 min vs at 7 min for PRE_NIC and PRE_PLA during EC. We snipped 10 secs at the beginning and at the end of 1 min and of

7 minutes, having two epochs per subject of 60-sec each. We then plot box plots of the sources where we found a nicotine effect in the VL analysis. The box plot showed no evident temporal difference between min 1 vs min 7. Therefore, we think that the temporal structure should have not contributed to the nicotine-induced significant changes found in our VL analysis. Eighth, regarding the surrogate method used, we are confident that the bootstrap method and permutation-based methods in general should control quite well against false positives both in connectivity (Schoffelen and Gross (2009)) and in phase-amplitude coupling (van Driel *et al.* (2015) analyses. Ninth, regarding the specificity of effects it is clear that nicotine does change the power spectrum in both eyes-closed (Bowers *et al.* (2015)) and eyes-open (Fisher *et al.* (2012)) activity. Therefore the generation of false positives in rPDC and VL caused by power changes is possible. For rPDC the renormalization allows more robustness against changes in power (Schelter *et al.* (2009)). As explained in the Introduction, for VL the 2% cut-off guarantees that VL estimates values are robust against power changes. In conclusion, we think that both rPDC and VL analyses fulfilled current standards necessary for mitigating the presence of false positives.

Chapter 4: General discussion

4.1 Potentials of non-invasive neuroimaging and pharmacology

In the first part (Chapter 1) I presented the motivation why it is important to bring neuroimaging at the attention of the CNS pharmacology community. Such increase awareness should improve the way we currently understand CNS pharmacology.

First I proposed an historical framework of the improvements which took place within CNS pharmacology. The scientific discipline started in the 1950 by systematically looking at signs and symptoms of the effect of CNS drugs on humans. Then the discipline began to incorporate the advances in animal models: it was the time of big discoveries in CNS pharmacology lead by biochemistry and by genetics. The current state of CNS pharmacology relates with the leading role overtaken by non-invasive neuroimaging. So far no new terminology has been developed. Therefore I preferred to name the field at the intersection between CNS pharmacology and non-invasive neuroimaging as simply “pharmacology and neuroimaging”.

Second I listed six main sources of problems which are currently affecting CNS pharmacology and neuroimaging. I dealt with each one separately. Although the six sources of problems could be extended to the biological sciences as a whole, I particularly emphasized their role for CNS pharmacology. I attempted to delineate potential remedies for each source of problem. Lastly, I mentioned the remedies whenever we implemented them in our three EEG analyses about nicotine in the brain.

I then expanded further the topic of non-invasive neuroimaging and early drug development. We have to recall that around 2010 there was a trend of pharmaceutical industries to abort neuroscientific projects due to their intrinsic excessive costs, high-failure rates and high-risk investments (Abbott (2011)). However, there is a recent attempt to reverse previous skepticism of big pharmaceutical industries towards the development of CNS drugs. Indeed, there are attempts to motivate pharmaceutical industries in doing research by granting them special patenting issues for CNS drugs (Choi *et al.* (2014)). Further, private-public partnerships seem to be at the horizon with the aim of sharing risks (Wegener and Rujescu (2013)). In general, there is also some research that quantified financially the decreased costs for the industries if they would include neuroimaging as a standard tool in early drug development (e.g. Iannetti and Wise (2007)). In conclusion, since the potential ability of neuroimaging to decrease research costs, early drug development seems to be the immediate consequence of the combination

of CNS pharmacology and neuroimaging. Therefore an industrial implementation of neuroimaging is considered realistic and holds promises in the forthcoming future.

In chapter 2 and 3 I have presented the results of three EEG analyses: Chapter 2 (CSD analysis); Chapter 3 (rPDC and VL analyses). Those two chapters represent real-life implementations of neuroimaging and pharmacology. The *research question* was whether nicotine improves vigilance. Our experimental design was appropriate for a pharmacological study since it has faithfully followed the principles presented both in Chapter 1 and in already published guidelines (Jobert *et al.* (2012)). Therefore we think that our experimental design was robust, in the sense that all potential confounders have been controlled. In a nutshell, we believe that the results of our three EEG analyses are unlikely to be biased. Indeed, we used the highest standards in terms of both experimental design and statistics.

In Chapter 4 I will further strengthen the topic regarding the potential that neuroimaging could have for CNS research. I will first show how the results of our three EEG analyses converged. I will then provide a framework in order to interpret and understand the results achieved regarding the acute effect of nicotine. Later I will provide a broader framework and I will integrate our results with what is known so far about the effect of nicotine in the brain. Then I will suggest which of the three EEG analyses undertaken seems to have more chances to be used. Indeed, I will evaluate the three analyses using as criteria the simplicity of use and the existence of previous literature supporting its use as a biomarker candidate. I will then present some improvements which can increase the performances of a CNS experiment. I will then show the current hurdles which affect CNS experiments. Lastly I will summarize the whole dissertation, focusing on the key points emerged.

4.2 Convergence of results among the three analyses

Overall, I presented three EEG analyses (CSD, rPDC and VL) which results converged. They showed that nicotine does increase the neural correlates of vigilance. Further, all analyses showed that the nicotine effect is particularly strong during EC. Instead during EO we found a weaker effect with respect to EC. I hypothesized that the reason for such a difference in strength could be due to a *ceiling effect*: during EO the subject is already in an awake state, therefore the wakefulness-promoting effect of nicotine should be minimal. Instead, during EC the subject is forced to move into a drowsier brain state

(Olbrich *et al.* (2009) and Putilov and Donskaya (2014)), thus the effect of nicotine is likely to be more evident.

In simple terms, we confirmed what was already demonstrated behaviorally: nicotine does increase vigilance (Gilbert *et al.* (2000)). This is particularly intriguing since it shows how behavioral and neuroimaging do converge in terms of results. It seems like that the use of different methods and the convergence of results resulted in an increase of coherent knowledge how nicotine works. Although some problems with replication of results still exist (Chapter 1), at least from an EEG points of view it seems that some concordance in the results is found. In other words, at least using the same method (EEG) but different techniques (CSD, VL and rPDC) we discovered a common ground in terms of results. In paragraph 4.4 I will discuss more how different methods (e.g. fMRI and EEG) showed also convergence regarding the acute effect of nicotine. In general, convergence of results help us to gain a comprehensive understanding how the CNS drug works, possibly unraveling important aspects about its mechanisms of action.

4.3 Interpretation of results

The results of our three analyses suggest that the cognitive enhancing properties of nicotine are possibly due to its ability to decrease drowsiness. We generated such interpretation by matching behavioral results (Gilbert *et al.* (2000)) present already in the literature. As follows I will present the steps which help us to come to such conclusion.

First, we found proofs in the literature showing how nicotine improves vigilance (Gilbert *et al.* (2000)). Then, we found a recent meta-analysis confirming the cognitive enhancing properties of acute nicotine administration (Heishman *et al.* (2010)). Note the nicotine improvement of cognitive performance appears to be smaller with respect to other psychostimulants (caffeine), but still significant (Gilbert *et al.* (2000)).

Our interpretation that nicotine exerts its cognitive enhancing properties by decreasing drowsiness is in line with a previous study on psychostimulants (Husain and Mehta (2011)): the cognitive enhancing properties of psychostimulants seem particularly effective in sleep-deprived subjects. In other words, the ability to hinder drowsiness could be at the core of the psychostimulants-induced cognitive enhancement. This is what was suggested by studies using modafinil as cognitive enhancer (Minzenberg and Carter (2008)): sleep-deprived subjects taking modafinil have possibly better cognitive improvements with respect to not sleep-deprived subjects.

In conclusion, it seems that the cognitive enhancement properties of nicotine - which were measured in the study of Gilbert *et al.* (2000) - could be largely due to its ability to decrease natural drowsiness. Nonetheless, it is still a matter of debate if true cognitive enhancement could be separated by wakefulness/arousal (Berridge and Arnsten (2013)).

4.4 Convergence of results with previous literature

I have already discussed in paragraph 2.5 that our CSD results (Ranzi *et al.* (2016)) during EO were able to replicate previous results (Fisher *et al.* (2012)). Indeed. Both papers show that - during eyes-open fixating on a cross - nicotine impacts the left frontal cortex only.

Moreover our CSD results (Ranzi *et al.* (2016)) matched quite well a recent meta-analysis about fMRI and nicotine (Sutherland *et al.* (2015)). Unfortunately they combined eyes-closed, eyes-open and task-dependent BOLD activation. Further, they merged the data from both smokers and non-smokers. Nevertheless, Fig. 3B in Sutherland *et al.* (2015) shows a strikingly convergence with our CSD results (Chapter 2). They found that anterior cingulate gyrus and left dorsolateral prefrontal cortex manifested an increased of BOLD activation. These two areas match the effect of nicotine I showed in Fig. 2 and Fig. 3 in Chapter 2 of the present dissertation. Further, the direction of the effect seems to converge given that BOLD signal and alpha activity appear to be anti-correlated according to Sadaghiani *et al.* (2010): when alpha goes down (our nicotine effect) the BOLD signal should increase (Sutherland *et al.* (2015)). Further, the nicotine-induced decrease of alpha power fronto-centrally during EC discovered in our CSD analysis makes sense according to the general agreement about the inhibitory role of alpha (van Dijk *et al.* (2008)). Therefore, I can speculate that nicotine likely improves attention/vigilance by decreasing the alpha brain oscillatory activity. In conclusion, even if the techniques used were either different (fMRI, Sutherland *et al.* (2015)) or the same (EEG, Fisher *et al.* (2012)), our results seem to replicate quite well previous literature. Reminding what I said in paragraph 1.4.4 the activation of left frontal cortex and anterior cingulate cortex after acute nicotine administration are a perfect example of biomarkers candidates. This means that each time we administered nicotine - during eyes-open or eyes-closed -, the two areas should be activated (by BOLD fMRI). Such consistent

pattern corresponds to “common signature/biomarker candidate” described in paragraph 1.4.4.

Such a “common signature” could be used for “*reversed engineering*” purposes: administering a recently discovered compound and seeing that the same areas get activated by BOLD fMRI should give us the chance to formulate an educated guess. Particularly, we can generate a reasonable expectation that the compound under investigation has similarities with nicotine. Therefore we could expect some ability of the compound to counteract drowsiness. Further, we could also expect that the ability of EEG to detect pharmacodynamics should discern whether or not an unknown compound belongs to a specific neurotransmitter system. For example, Reeves *et al.* (2002) found that the administration of donepezil decreased the alpha activity frontally. As a short digression, donepezil is an acetylcholinesterase inhibitor drug which triggers the acetylcholinergic system and it is considered a cognitive enhancer. In other words, it seems that we can generalize the above “common signature” (e.g. decrease of frontal alpha) to a specific neurotransmitter system (e.g. acetylcholine) and to a specific function (e.g. cognitive enhancement).

Although some problems with replication of results still exist (see Chapter 1), it seems by all means possible to find a “common thread” across different neuroimaging techniques and incrementally increase the pharmacological knowledge. Such an increase of knowledge should unravel important mechanisms of action how a specific CNS drug works in the brain.

4.5 Discussion about the EEG techniques used

As neuroimaging techniques, I preferred to use EEG instead of fMRI, since it has better sensitivity to vigilance modifications. As said in Chapter 1, oscillations correlates very well with drowsiness, thus EEG was considered appropriate. In Chapter 2 and 3 I presented three EEG analyses which unravel different aspects how nicotine works in the brain of healthy male non-smokers.

As a rule of thumb, the use of multiple techniques/methods should be endorsed in order to provide different layers of knowledge how the specific molecule works in the brain. Such a process has been referred in an industrial setting as *drug profiling*: it is generally better to collect as much information as possible how a specific molecule works in the brain. Such a gathering of qualitatively different pieces of information should

improve the general understanding of the mechanisms of action of the specific CNS drug under investigation.

Idiosyncrasies could also appear by using different techniques for the drug profiling process. Nevertheless, as time goes by we should incrementally understand better the mechanisms of action of a specific CNS drug. Therefore, it should become easier in the future to find convergence between results generated by the use of different techniques. If this would happen, than we should make sense of disparate pieces of information that we have in these days. Such a fragmentation could be generated by measuring the effect of a specific CNS drug by different techniques. In conclusion, I believe that future holds promises in finding a general and comprehensive framework regarding the understanding how specific CNS drugs work in the brain.

In terms of the type of the EEG analyses chosen CSD, rPDC and VL have been used to track the nicotine-induced modulation of vigilance. I now briefly remind the discussion had in paragraph 1.7 about the rationale behind the choice of CSD, rPDC and VL techniques. CSD, rPDC and VL were used because previous literature (Olbrich *et al.* (2009), Maksimow *et al.* (2014) and Blain-Moraes *et al.* (2015), respectively) indicated that such techniques are sensitive enough for detecting online modulations of vigilance. Indeed, they correspond to *three different biomarkers of drowsiness*. Therefore they represent the ideal benchmark for investigating whether nicotine could counteract the effect of natural drowsiness. Note that drowsiness and its biomarkers can occur within few minutes just by closing our eyes (Olbrich *et al.* (2009)). Then we tested whether nicotine could possibly change such biomarkers.

In simple words, the *rationale* behind the benchmarking of the nicotine effect consisted in three steps. The first step was to select the most sensitive techniques which are able to detect natural drowsiness. I will describe the three biomarkers of drowsiness which we selected. The second step was to induce a temporary state of drowsiness by asking the subjects to close their eyes. The third step was to study how an investigational molecule could depart from (e.g. psychostimulants) or enhance (e.g. sedatives) a state of drowsiness.

The three biomarkers of drowsiness were: anteriorization of alpha - see later - (for CSD analysis); decrease of forward connectivity and increase of backward connectivity (for rPDC analysis) and decrease of phase-amplitude coupling (for VL analysis).

As follows I will explain in detail the three biomarkers of drowsiness chosen. We used CSD analysis because its sensitivity to detect the phenomenon of *anteriorization of*

alpha (Olbrich *et al.* (2009)). Anteriorization of alpha is a physiological phenomenon that occurs when we close our eyes: alpha power seems “to migrate” from occipital region to frontal region within few minutes. When such a migration occurs, it means that the subject gets drowsy.

Regarding connectivity measures instead, we found out that a *directed connectivity* measure (rPDC) is also sensitive to an online decrease of vigilance. Such a decrease of vigilance correlates strongly with the pharmacologically-induced drowsiness (Maksimow *et al.* (2014)). These authors were able to discover a specific direction of the effect: when we are pharmacologically drowsy - by bolus dose of the anesthetic propofol - then the forward connectivity increases, whereas the backward connectivity decreases. Lastly, it is interesting to remember that the two types of connectivity are anti-correlated (Maksimow *et al.* (2014) and Piantoni *et al.* (2013)): when forward connectivity increase, backward connectivity decrease.

Regarding VL, we found in the literature that *cross-frequency coupling* (known also as simply phase-amplitude coupling) is influenced by drowsiness (Blain-Moraes *et al.* (2015)). Specifically, they tracked the effect of the anesthetic sevoflurane detected along time, and they found that a decrease of phase-amplitude coupling – namely, the coupling between the phase of low-frequency (1 Hz) with respect to the amplitude of alpha (10 Hz) - correlates with a decrease of vigilance.

As follows, I will present personal recommendations about the best techniques to be used for forthcoming research. The criteria for considering some techniques as better as others depends on the strength of the biomarkers used. Such strength depends on its simplicity as well as on the abundance of previous literature which is able to link clearly a specific biomarker with behavior. Therefore the stronger the agreement in the neuroscientific community, the more solid the biomarker is considered. Likewise, the more replications found in the literature about a specific biomarker, a better trust of the validity of such biomarker is reachable. As follows I will suggest two techniques which are particularly sensitive in detecting modulations of drowsiness.

I would personally recommend at first CSD (namely, the anteriorization of alpha), specifically during eyes-closed. Indeed, there is plenty of literature showing the existence of the phenomenon of anteriorization of alpha (e.g. Olbrich *et al.* (2009)). Such a phenomenon correlates strongly with drowsiness. Therefore, the anteriorization of alpha can be considered one of the strongest biomarker of drowsiness. This one should be used as first-line assessment tool of the pharmacological activity of a CNS drug.

As a second choice I would opt for some connectivity measures which work well in tracking modifications of drowsiness in real-time. Indeed, in the literature there are plenty of examples which are showing both the existence of two connectivity patterns (either forward or backward connectivity) and how they correlate with drowsiness (Piantoni *et al.* (2013)). These two patterns are anti-correlated (Maksimow *et al.* (2014)): when one goes up the other goes down. Lastly, an increase of backward connectivity accompanied by a decrease of forward connectivity are both sensitive measures to a pharmacologically-induced drowsiness (Maksimow *et al.* (2014)).

The last techniques I would recommend to use for tracking pharmacologically-induced modulations of drowsiness is VL. Indeed, there is up to now scarce literature showing a clear link between cross-frequency coupling and drowsiness.

In conclusion, in order to track pharmacologically-induced vigilance modulation I would start with using CSD technique during eyes-closed condition. Indeed, CSD during eyes-closed offers at the moment the simplest biomarker for tracking drowsiness (“anteriorization of alpha”). The “anteriorization of alpha” should be best captured when the EEG power spectrum becomes also spatially localized. Therefore techniques like “EEG scalp topography” and “EEG source reconstruction” are advisable. In simple words, “anteriorization of alpha” cannot be fully captured by single-electrode recording. Nonetheless, drowsiness is a pervasive brain state that can be detected also by measuring the power spectrum at a single-electrode level. Indeed, Putilov and Donskaya (2014) showed that the “attenuation of alpha” (another - simpler - variant of “anteriorization of alpha”) that occurs when we close our eyes can be measured by a single occipital scalp electrode. The attenuation of alpha is another valid biomarker of drowsiness. The CSD method we used in Chapter 1, it is just the evolution of the power spectrum measured at a single electrode, but now brought to a three dimensional level via source reconstruction algorithms. In fact, CSD source reconstruction methods allows computing the power spectrum in different location within the brain.

4.6 Improvements for pharmacological research

What I consider paramount for future pharmacological research is to collect blood samples. Such a procedure has at least two advantages:

- 1) it allows to check on-line the pharmacokinetics. In other words, the subject’s absorption of the drug is measured objectively. Indeed, there are individualized

absorption ratios and they can affect the experimental results. Particularly interesting are correlational analyses (e.g. generalised semi-linear canonical correlation analysis, GSLCCA) which are able to correlate blood concentration of the investigational drug with the effect of the drug in the brain (Brain *et al.* (2014) and Diniz *et al.* (2016)). In such an approach, pharmacokinetics (i.e. blood level of the drug) and pharmacodynamics (e.g. neuroimaging output) would be correlated in a more realistic way. In such a way is possible to obtain advanced *PK/PD models* (pharmacokinetics, PK; pharmacodynamics, PD) which are crucial for testing the efficacy of a drug.

Another simple consequence of measuring the pharmacokinetics is the possibility of *discarding the subjects* who did not reach the expected absorption of the drug in the system. Those subjects are in fact to be considered generally as “non-responders”.

2) collecting blood samples it allows also to screen the subjects regarding *polypharmacy*. Indeed, the experimenter does not want any other CNS drug in the system apart the one under investigation.

In general, such a procedure of collecting blood sample would allow to strengthen the results and decrease noise in the dataset. Indeed, it allows to *stratify* the data set by discarding either the non-responders (e.g. little amount of drug in the blood) or the “contaminated” subjects (e.g. polypharmacy). In conclusion, thanks to the collection of blood samples it is possible to have an objective measure of both the actual level of drug in the individual’s system and a proof that other chemicals are not present in the system. In conclusion, blood sampling alone should improve a lot the consistency of an experiment: by reducing the variability in the sample of subjects, more replicable results are expected.

Another potential improvement would be designing a specific session where behavioral measurements would be collected. Such behavioral assessment would be important for tracking vigilance modulations, eventually. Such session should be separated from the other two resting-states (either EO or EC), otherwise we would threaten the very definition of resting-state. A good example is Khodayari-Rostamabad *et al.* (2015): the authors were able to run a vigilance task during eyes-closed activity. They used acoustic continuous stimuli towards which the subjects should guess which direction they come from (either left or right ear) by simple reaction time task (pressing a button).

Another improvement amenable for future pharmacological research is combining two different neuroimaging techniques together. For example, recently simultaneous recording of either EEG and fMRI or EEG and MEG are becoming more utilized in

neuroscientific research. Regarding the pharmacological CNS research there are already some articles showing the use of simultaneous EEG-fMRI at the advantage of CNS pharmacology (e.g. Warbrick *et al.* (2012)). Instead, some applications of simultaneous EEG-MEG to CNS pharmacology are still missing.

Combining two neuroimaging techniques together should capitalize in the wealth of results that can be obtained regarding the drug profiling process for a specific CNS drug. Indeed, the rule-of-thumb is to collect as many data as possible regarding the activity of a CNS drug in order to have a complete picture how that molecule works in the brain.

From a neuroimaging perspective, it is true that different neuroimaging techniques have different sensitivity to different pharmacological agents. As first example I mention the strong sensitivity of EEG to remifentanyl (an anesthetic) in detecting anesthetic-induced drowsiness (Khodayari-Rostamabad *et al.* (2015)). Further, an fMRI study using anfenitanil (anesthetic with similar chemical structure as remifentanyl) showed better sensitivity with respect to EEG in localizing the effect of such compound in the brain (Oertel *et al.* (2008)). As a second example I mention articles regarding the effect of antidepressants which can be detected both by fMRI (Bredt *et al.* (2015)) and by EEG (Koo *et al.* (2015)). A more relevant example for the present dissertation is nicotine, whose central effects were captured both by fMRI (Sutherland *et al.* (2015)) and by EEG (Fisher *et al.* (2012)), as discussed above. In conclusion, there is room for using simultaneous neuroimaging techniques in pharmacological CNS drug research. Current CNS pharmacology should capitalize on our modern ability to acquire different levels of understanding how the specific CNS drug works, thanks to the usage of simultaneous neuroimaging techniques.

4.7 Caveats with current pharmacological research

Unfortunately, there are still insurmountable problems which are intrinsic with a pharmacological experiment. The first problem is how to effectively control for the placebo effect. Indeed, many CNS drugs (e.g. alcohol) have an immediate impact in the subject's mood and perception. This cannot be unnoticed. Therefore both the subject and the experimenter "know" whether the subject has taken the actual drug. Instead, there are other CNS drugs which effect is delayed in time and not clearly noticeable neither by the

subject nor by the experimenter (e.g. antidepressants). These last type of compounds suit better undergoing a *placebo-controlled blinding procedure*.

Second and third problems regard the lack of objective measures for two further confounders which can play a role in biasing the experiment: *sleep deprivation* and *withdrawal syndrome*. With sleep deprivation I mean the lack of good quality sleep which can bias the behavioural and neuroimaging endpoints (Samann *et al.* (2010)). Indeed, in sleep deprived subjects homeostatic brain processes can be detected both by EEG (Knoblauch *et al.* (2002)) and by fMRI (Klumpers *et al.* (2015)). Withdrawal from some chemicals can also be a source of bias because it can impact neural activity as well (Chu *et al.* (2015)). Lastly, I do recognize that the most frequent biases in neuroimaging studies are sleep deprivation and simultaneous administration of other chemicals (polypharmacy). Instead, neural changes due to withdrawal from substances is unlikely to be observed.

Expanding the second problem, the lack of objective markers of sleep deprivation could be an issue with techniques like EEG and MEG which are sensitive to changes in the brain oscillatory activity. Instead, fMRI and PET are less burdened by such problem. The partial solution regarding the assessment of sleep deprivation is to ask the subject via a questionnaire how much he/she slept during the previous night. The experimenter must rely on those subjective measures of sleep deprivation. Another suggestion to mitigate the problem of understating sleep deprivation is recording a bigger sample of subjects. In this way, the effect of outliers present in the sample should be minimized.

As third problem we have a lack of objective markers regarding an ongoing withdrawal syndrome. With full-blown withdrawal syndrome we have a situation where several symptoms are clearly observable by a trained experimenter and detected by neuroimaging techniques as well (e.g. withdrawal syndrome from opioids in Chu *et al.* (2015)). If such situation would be observed, obviously the subject will be excluded from the data analysis. However there are small symptoms of withdrawal syndrome which are not readily observed by the experimenter. For example, even if it looks surprising withdrawal syndrome from caffeine deprivation could be an issue according to some authors (James and Rogers (2005)) because it can bias the experimental results. Unfortunately, an objective measure of ongoing withdrawal symptoms is not achieved neither by a blood collection nor by neuroimaging nor by behavioral measurements. So the experimenter should rely only on self-reports coming directly from the subject. The partial remedy to such a problem would be increasing the sample: such a confounder should then be mitigated.

4.8 Future research

I now point out which could be the potential improvements, having as starting point the experimental design used.

As a first improvement, it would be useful to include women in the sample. This means obviously doubling the sample of subjects. Nonetheless the advantage would be to generalize the effect of a drug to the whole population.

As a second improvement, it would be interesting to quadruple the amount of subjects in order to have at least 28 subjects per group (e.g. 28 males under nicotine, 28 males under placebo and so forth). Above such threshold strongest statistical power should be achieved (Pernet *et al.* (2011)).

As a third improvement, it would be to run source reconstruction using single-subject MRI image. This should decrease the localization error intrinsic in the source reconstruction methods (Akalin Acar and Makeig (2013)). Lastly, it will be interesting to run a simultaneous EEG/fMRI recording, in order to further identify common brain areas activated by the CNS drug. Regarding nicotine, simultaneous EEG/fMRI should provide results that replicate previous findings (Sutherland *et al.* (2015)). Specifically regarding nicotine, a simultaneous EEG/MEG recording could be done since at the moment such studies are not existent. MEG could be considered complementary to EEG, since its sensitivity to magnetic field generated by the brain which cannot be captured by EEG alone (Ahlfors *et al.* (2010)).

As a fourth improvement, I have already mentioned the collection of blood samples as key to generate accurate PK/PD models. Please see paragraph 4.6 about the advantages of collecting blood samples in a pharmacological research.

4.9 Overall conclusion

I stressed throughout the dissertation that neuroimaging is currently shaping CNS pharmacology. I strongly support the idea that we are into a revolutionary period for CNS pharmacology. Indeed, there is now a huge opportunity of looking at the effect of CNS drugs on-line and non-invasively in humans. Such opportunity was not possible before. Particularly for EEG, there is now a large amount of quantitative EEG techniques that can be readily used in the early drug development of new CNS drugs (Diniz *et al.* (2016)), thus meeting the needs of pharmaceutical industries. In conclusion, neuroimaging

deserves in these day a lot of attention since it seems to be a revolutionary and powerful tool for providing new understanding of CNS pharmacology.

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Author Contributions:

P.R. designed the research, collected, computed and analyzed the data. C.M.T. and C.S.H. supervised the project. P.R., C.M.T. and C.S.H. wrote the manuscript. All authors have read and approved the final manuscript.

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P.R. designed the research, computed the source reconstructed time-series, computed the statistical inference and interpreted the results. J.A.F. computed rPDC and VL values and interpreted the results. C.M.T. and C.S.H. supervised the project. P.R., J.A.F., C.M.T. and C.S.H. wrote the manuscript. All authors have read and approved the final manuscript.

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Declaration

I have completed the work independently and used only the indicated facilities. This dissertation is my own work. All the sources of information have been acknowledged by means of complete references.

The dissertation as a whole or in parts has not been submitted to assessment in a doctoral procedure at another university.

This dissertation has neither as a whole nor as a part been published apart from those parts where this is explicitly indicated.

I am aware of the guidelines of good scientific practice of the Carl von Ossietzky University Oldenburg and I observed them when preparing this dissertation.

I confirm that I have not availed myself of any commercial placement or consulting services in connection with my promotion procedure.

Paolo Ranzi

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