Effects of Morphine and Alcohol on Functional Brain Connectivity During “Resting State”: A Placebo-Controlled Crossover Study in Healthy Young Men

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Abstract: A major challenge in central nervous system (CNS) drug research is to develop a generally applicable methodology for repeated measurements of drug effects on the entire CNS, without task-related interactions and *a priori* models. For this reason, data-driven resting-state fMRI methods are promising for pharmacological research. This study aimed to investigate whether different psychoactive substances cause drug-specific effects in functional brain connectivity during resting-state. In this double blind placebo-controlled (double dummy) crossover study, seven resting-state fMRI scans were obtained in 12 healthy young men in three different drug sessions (placebo, morphine and alcohol; randomized). Drugs were administered intravenously based on validated pharmacokinetic protocols to minimize the inter- and intra-subject variance in plasma drug concentrations. Dual-regression was used to estimate whole-brain resting-state connectivity in relation to eight well-characterized resting-state networks, for each data set. A mixed effects analysis of drug by time interactions revealed dissociable changes in both pharmacodynamics and functional connectivity resulting from alcohol and morphine. Post hoc analysis of regions of interest revealed adaptive network interactions in relation to pharmacokinetic and pharmacodynamic curves. Our results illustrate the applicability of resting-state functional brain connectivity in CNS drug research. *Hum Brain Mapp* 33:1003–1018, 2012. © 2011 Wiley Periodicals, Inc.

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INTRODUCTION

One of the major challenges of central nervous system (CNS) drug discovery is to develop a generally applicable methodology to demonstrate the effects of a compound on the brain across the different phases of drug development. An optimal methodology should be minimally invasive, repeatable, and able to identify overlapping and distinguishable effects of different CNS drugs with little reliance on *a priori* models for drug effects.

The advances in functional neuroimaging, especially positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), have opened important frontiers for CNS drug research. However, radiation dose restrictions in PET prevent repeated intrasubject measurements within short periods of time. This is a major limitation of PET because repeated measurements allow testing different drug compounds on the same individual and minimize the within-subject variances. More importantly, repeated neuroimaging acquisitions would allow the assessment of pharmacokinetic/pharmacodynamic (PK/PD) relationships, which form the scientific basis of modern drug development.

Compared with PET, blood-oxygen-level-dependent (BOLD) fMRI is noninvasive, and benefits from high temporal and spatial resolution. Until recently, fMRI was applicable in task-related designs, where pharmacologically induced changes in regional BOLD signal in response to a hypothesized CNS function (e.g., pain alleviation) were investigated. For examples see [Honey and Bullmore, 2004]. One of the major limitations of this approach was the difficulty in controlling for inter-subject variations in stimulus perception or performance in task-related experiments. In addition, fMRI tasks had to be chosen to activate regions of interest based on *a priori* hypotheses about the site of drug effect [Breiter et al., 1997]; or about the PK/PD profile [Stein et al., 1998], which in many cases are unknown.

A recent breakthrough in neuroimaging has resulted from discovering the complex (but consistent and reliable) functional architecture of brain activity from the BOLD fluctuations [Biswal et al., 2010; Bullmore and Sporns, 2009; Smith et al., 2009]. Today, a growing body of evidence suggests that the "resting-state" brain activity (i.e., spontaneous BOLD fluctuations in the absence of any specific stimuli) forms spatially correlated topographies, which represent functional connections that relate to, or even predict, emotional, and cognitive behavioral outcomes [Fox et al., 2007; Seeley et al., 2007] or clinical conditions [Greicius, 2008]. These resting-state networks (RSNs) represent distinct functional systems (e.g., motor, vision, attention, etc.), that are reliable and reproducible [Beckmann et al., 2005; Biswal et al., 2010; Damoiseaux

et al., 2006; Zuo et al., 2010]. Hence, if different drugs produce specific and detectable changes in the functional topography of these networks, then RSNs may become an important biomarker for CNS drug research.

In this study we investigate whether different psychoactive drug compounds (alcohol and morphine) elicit distinguishable changes in the functional topography of the RSNs. The RSN connectivity is determined in terms of similarity of temporal fluctuations in the whole brain in relation to eight networks of interest (NOI). These NOIs represent the most reliably and reproducibly detectable RSNs of functional significance (e.g. visual, somatosensory, motor, attention, working memory) [Beckmann et al., 2005; Damoiseaux et al., 2006]. Drugs are administered under PK-controlled infusion protocols. Seven resting-state fMRIs (RS-fMRI) and several PK and PD assessments are made at controlled intervals (see Fig. 1). We report drug-specific changes in the profile of RSN connectivity, and show their temporal relation to the profiles of drug concentrations and pharmacodynamic effects in areas where effects of morphine and alcohol are expected.

MATERIALS AND METHODS

Experimental Design

A schematic diagram of the study design and the analyses is provided in Figure 1. This study is randomized, double blind (i.e., both examiner and the participant are unaware of which drugs are given), and double dummy placebo-controlled (i.e., placebo was used for each treatment, consisting of infusion of the vehicle for treatment A in parallel to the administration of active treatment B or vice versa, and two vehicle infusions in the placebo session). Alcohol and morphine are two of the most commonly used substances in addiction and pain studies with similar analgesic and euphoric effects but they also result in different autonomic responses. In humans, alcohol exerts a wide range of psychoactive effects by interacting with GABAergic, dopaminergic, serotonergic and even opiate neurotransmitter systems [Koob et al., 1998]. In contrast, morphine targets specific receptors (μ -opioid), which in turn interact with many other neurotransmitter systems [Contet et al., 2004]. This within-subject design aimed to test whether drug-specific effects on the RSN connectivity would be detectable and whether their profile corresponded to the PK and PD profiles we measured. However, both alcohol and morphine have dose-dependent effects on the regional cerebral blood flow [Blaha et al., 2003; Wagner et al., 2001], and pharmacodynamic effects can significantly vary across individuals. Hence, we

Figure 1.

Schematic presentation of study design and data collection. Each subject participated in three randomized sessions. Both examiners and the subject were blind to which drug they received. The expected pharmacokinetic profiles for alcohol and morphine are plotted (1). The placebo infusion was conducted con-

currently with the drug infusion. After preprocessing (2) dual regression on all data sets to estimate whole-brain connectivity in relation to the eight template RSNs (3), followed by statistical testing (4). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

applied previously validated infusion regimens to achieve approximately steady state serum levels and controlled pharmacodynamic effects. The infusion protocols were based on validated PK models for each of these drugs [Sarton et al., 2000; Zoethout et al., 2008, 2009]. This allowed (1) minimizing the between- and within subject variations in plasma drug concentration and (2) maintaining each of these drugs' concentrations at a plateau level for 90 min (~60 min after the onset of experiment).

Subjects

Twelve healthy male subjects (age range, 18–40; BMI 18–26 kg/m²) were selected to participate in the neuroimaging study. (See supplemental material for exclusion criteria.) The study was approved by the Medical Ethics

Review Board of Leiden University Medical Centre. Both oral and written informed consents were obtained from all participating subjects. All studies were performed in compliance with the law on clinical trials of the Netherlands (WMO). Each participant was scanned on three separate days, at least seven days apart. Subjects fasted for at least 4 h prior to the start of a study day. After arrival at the hospital a light standardized meal was provided. On each study day, negative scores on alcohol breath tests and a urinary drug screen for amphetamines, cocaine, morphine, and Δ9-Tetrahydrocannabinol (THC) were required. Three intravenous cannulas (one for morphine or placebo administration, one for alcohol or placebo administration and one for blood sampling) were placed in veins of the arms. Before the start of drug or placebo administration, a baseline resting-state scan was made and two baseline

measurements of each visual analogue scale (VAS) were taken. After the last scan, the intravenous cannulas were removed and a second meal was provided.

Morphine Infusion Protocols

In order to reach stable serum levels of morphine (~80 nmol/L) an initial bolus of 100 µg/kg/h was infused during 1 min; followed by a continuous infusion of 30 µg/kg/h for 2.5 h. A prior morphine study using an identical infusion paradigm [Sarton et al., 2000] showed that this infusion regimen could be safely applied, without the occurrence of major side effects. This dose was also associated with significant pharmacodynamic CNS effects. The total amount of morphine administered during one occasion using this dosage scheme, was approximately 14.5 mg for an average weighted male subject, infused over a time period of 2.5 h. This is considered to be a safe and rational dose, since it is within the therapeutic range of morphine (i.e., 2.5 mg–15 mg in 4–5 mL in 4–5 min intravenously, for acute pain). To determine the plasma concentration of morphine, venous blood was collected in 5 mL plain tubes (Becton and Dickinson Franklin Lakes, NJ, USA). Blood samples were taken at 0, 15, 30, 50, 60, 90, 120, 150, 180, 210, and 270 min after the start of the placebo or drug administration. All samples were centrifuged for 10 min at 2000g between 30 and 45 min after collection. Plasma samples were stored at –21°C. Plasma concentrations of morphine were determined using liquid chromatography with tandem mass spectrometry [Sarton et al., 2000].

Alcohol Infusion Protocol

Alcohol concentrations were controlled based on an intravenous alcohol clamping paradigm using ethanol 10% in glucose 5% [O'Connor et al., 1998]. We aimed to maintain alcohol levels at 600 mg/L, which barely exceeds the legal limits for driving in the Netherlands (approximately equivalent to two glasses of wine). The alcohol clamp has previously been well-tolerated at this serum level and produced statistically significant pharmacodynamic CNS effects [Zoethout et al., 2009]. Infusion rates required to maintain stable alcohol levels were computed by a nonblind staff member without any other involvement in the study, based on measurements of breath alcohol (BrAC) at 5-min intervals between 0 and 30 min, at 10-min intervals between minutes 30 and 60, and 30-min intervals between minutes 60 and 300 after the start of the placebo or drug administration. The alcohol placebo condition consisted of a sham-procedure using a glucose 5% solution, including computer-driven adaptations of infusion rates and BrAC measurements.

Neuroimaging Acquisition and Processing Protocols

A 3T Achieva scanner (Philips Medical System, Best, The Netherlands) was used for image acquisition. For each subject we obtained 21 resting-state T2*-weighted acquisitions

(gradient echo EPI with parameters set to a TR = 2180 ms, TE = 30 ms, flip angle = 80; 64 × 64 × 38 isotropic resolution 3.44 mm, 220 frames, 8 min) and a T1-weighted high resolution scan for anatomical registration. During scanning, a pulse oximeter (INVIVO MRI 4500, Siemens Healthcare, Germany) was used to monitor heart rate and oxygen saturation. The scanner's flexible pressure belt was used to record the respiratory signals. These RS-fMRI data were preprocessed using the standard procedure including motion correction, brain extraction, Gaussian smoothing with a 5 mm FWHM kernel, mean-based intensity normalization of all volumes by the same factor (i.e., 4D grand-mean), and high-pass temporal filtering (FWHM = 100 s). After preprocessing, the functional scans were affine-registered to an MNI152 standard space (Montreal Neurological Institute, Montreal, QC, Canada). In all stages Functional Magnetic Resonance Imaging of the Brain (FMRIB) Software Library (FSL 4.0, Oxford, UK; www.fmrrib.ox.ac.uk/fsl) was used.

Assessment of Drug Effects on RSN Connectivity

We defined RSN connectivity in terms of the similarity of the BOLD fluctuations in each brain voxel in relation to characteristic fluctuation in eight predefined networks of interest (NOI). These networks are obtained from a weighted mask of networks that are most reliably (and reproducibly) identified from a model-free analysis of the spatio-temporal structure of the resting-state BOLD fluctuations [Beckmann et al., 2005]. These template NOIs include over 80% of the total brain volume and comprise: medial and lateral visual systems (NOIs 1 and 2, respectively), auditory and somatosensory system (NOI3), sensorimotor system (NOI4), the default mode network (NOI5), executive salience network (NOI6), and visual-spatial and working memory networks (NOIs 7 and 8, which represent almost mirrored networks). Each NOI represents shared neurophysiological fluctuations of the anatomical locations it includes.

To measure connectivity, we used the dual-regression method [Beckmann et al., 2009]. Briefly, dual-regression is based on first extracting the temporal pattern of resting-state signal fluctuations within an RSN for each resting fMRI dataset; and next regressing these "fitted time courses" against fluctuations in the entire brain. Dual-regression analysis generates statistical maps of z-scores that represent connectivity to the given NOI. In other words, the higher the absolute value of the z-score, the stronger the connectivity to an NOI. These statistical maps can then be used in voxel-wise mixed model analyses of complex experimental designs to obtain a statistical representation of where the drug interactions with any particular network connectivity are significant. The statistical sensitivity of the dual-regression method has been successfully demonstrated in a study that reported distinct differences in brain connectivity in young carriers of the APOE4 gene [Filipini et al., 2009].

Here, dual-regression resulted in 252 statistical parametric maps of whole brain connectivity to each of NOIs (12 subjects $\times$ 7 RSfMRIs $\times$ 3 treatments $\times$ 8NOIs) (See Figure 1). For each NOI, the respective RSN connectivity maps were entered in a mixed-effect generalized linear model (GLM) to identify which brain regions and which networks were most significantly affected by the drug $\times$ time interactions. We used fixed factors treatment and time, and random factor subject. Particularly, we tested the difference between morphine-placebo and alcohol-placebo, while accounting for the variance across time (six post-injection time points vs. the first preinfusion). The effects of morphine versus placebo; alcohol versus placebo, each of the time points 2 to 7 versus the pre-drug time point, and an intercept for each subject were modeled as covariates.

Pharmacologically induced variations in heart rate and respiration rate were expected. Previous studies have shown that both heart rate [Chang et al., 2009; Shmueli et al., 2007] and respiration [Birn et al., 2008; Chang and Glover, 2009b; Wise et al., 2004] fluctuations introduce variance in the resting-state BOLD signal. We controlled for global effects of respiration and heart rate by examining the linear association of average heart rate and average respiration rate with resting-state connectivity of each network. We also tested separate models, with and without respiration and heart rates as covariates to examine the robustness of detected effects to nuisance variables.

Permutation-based statistical inference [Nichols and Holmes, 2002] (5,000 permutation tests) was used. Statistical significance was set at $P < 0.05$, after cluster-based correction for family wise errors (based on the null distribution of the max cluster size across the image). In all stages FSL 4.0 was used.

Pharmacodynamic Assessment

Computerised Visual Analogue Scales (VAS) were used to measure subjective CNS effects of drugs at baseline and repeated at 30, 60, 90, 120, 150, 180, and 210 min after infusion started. The assessments were performed outside the scanner.

The VAS Bond and Lader [Bond and Lader, 1974] was used for the subjective assessment of the state of mind at that moment. Three factors corresponding to "alertness," "mood," and "calmness" can be derived from the VAS Bond and Lader. The VAS Bond and Lader scores are expressed in millimeter (mm), in which 50 mm indicates a normal feeling.

An adapted version of the VAS Bowdle [Bowdle et al., 1998] was used for subjective assessment of psychedelic effects. From the VAS Bowdle, three factors corresponding to "internal perception" and "external perception" (two modalities of psychedelic effects) and "feeling high" can be derived. "External perception" consist of the VAS scores changing of body parts, changes of surrounding, altered passing of time, difficulty controlling thought, changes in color intensity and changes in sound intensity. It indicates a misperception of an external stimulus or a change in the

awareness of the subject's surroundings. "Internal perception" reflects inner feelings that do not correspond with the reality, e.g. hearing of unrealistic voices or sounds, unrealistic thoughts, paranoid feelings and anxious feelings. "Feeling high" is a separate item of the VAS Bowdle. The minimum score for the VAS Bowdle (absence of psychedelic effects) is 0 mm. We also used VAS nausea [Mearadji et al., 1998] and VAS alcohol effect (intoxication), each consisting of a single scale in which the extreme left side (0 mm) corresponds to "not nauseous/drunk at all" and the extreme right side (100 mm) to "maximum nauseous/drunk".

Repeatedly measured pharmacodynamic and physiological data were compared with a mixed model analysis of variance with fixed factors treatment, period, time, and treatment by time and random factor subject, subject by treatment and subject by time and the average prevalue (average over all measurements at or before time = 0) as covariate (SAS for windows V9.1.2 ; SAS Institute, Inc., Cary, NC). Images were generated using Prism 5, Graph-Pad Software Inc., La Jolla, CA).

RESULTS

Controlled Pharmacokinetic Profiles

To reduce inter- and intrasubject variability, we examined variations in resting-state connectivity under controlled pseudo-steady state plasma drug concentrations, using a target-controlled infusion regimen for morphine and an intravenous clamp for alcohol. Figure 2 illustrates the concentrations over time for each subject. Sixty minutes after the start of the infusion at the onset of RS-fMRI 2, the between-subject averaged ($\pm$ SD) morphine level was 67.17 ± 10.22 (nmol/L), which remained in the same range in the following RS-fMRIs 3, 4, and 5 (68.63 ± 8.0 ; 66.88 ± 5.8 ; 68.04 ± 8.8 , respectively). Average alcohol concentrations were also stable, with low between-subject variability at RS-fMRI 2, 3, 4, and 5 (0.60 ± 0.05 , 0.60 ± 0.056 ; 0.58 ± 0.069 , and 0.63 ± 0.038 (g/L), respectively.)

Pharmacodynamic Effects of Morphine and Alcohol

Figure 3 and Table I summarize the pharmacodynamic effects.

Morphine significantly reduced the respiration and heart rates and increased calmness and sensation of nausea compared with placebo. Alcohol significantly increased the heart rate and feelings of intoxication. Other effects did not reach statistical significance.

Effects of Physiological Factors on RSN connectivity

Figure 4 shows overlaid F-stat maps for each network, at $P < 0.05$ (cluster corrected). Simple regression shows significant correlation between respiration rate and

Figure 2.

Pharmacokinetic effects: (a) Plasma morphine concentrations for each subject; (b) Breath alcohol readouts. The bars illustrate when the RS-fMRI acquisition took place.

connectivity patterns of NOI4, NOI5, NOI6, and NOI7. In contrast, heart rate correlated with connectivity of several areas including the cerebellum, the brainstem, and the amygdala in relation to NOI3. Overlapping heart rate modulation of connectivity of a region close to major arteries (and extending to the amygdala) in relation to NOI 4, NOI5, NOI6, and NOI8 was also present. However, when effects of treatment by time were modeled in the GLM, the patterns of correlation between physiological factors and connectivity were diminished. Including respiration and heart rate, the topography of morphine effects were changed (Supporting Information Fig.-a), but alcohol effects were unchanged (Supporting Information Fig.-b). With the exception of the default mode network, it seems drug effects on resting-state connectivity were independent of global physiological variations of heart rate and respiratory depression (due to morphine).

Interactions of Morphine and Alcohol with RSN Connectivity

Considering the location of regions where including physiological effects in the statistical model resulted in significant differences (white matter and a zone peripheral to the default mode network in relation to respiration), we expect these effect are non-neuronal and related to global cerebrovascular responses, potentially resulting from a hypercapnic condition due to respiratory depression [Patkinson et al., 2007], or physiological noise [Birn et al., 2008]. Therefore, for the purpose of this study, we have reported drug by time interactions while including these variables in the GLM, to avoid such confounds.

Figure 5 illustrates statistical maps (F -test; cluster P -values < 0.05 , corrected) of areas where connectivity in relation to a given NOI (in red) was significantly different due to morphine (green) and due to alcohol (blue) over time. Details in terms of cluster size, cluster P -value and the t -value of the peak of the cluster are provided in Table II.

Morphine

Effects of morphine on RSN connectivity over time (compared to placebo) were significant and dissociable in different NOIs. The affected areas include prefrontal regions (subgenual ACC, medial prefrontal and basolateral prefrontal regions), posterior parietal areas (precuneus, posterior cingulate), medial temporal regions (amygdala and the hippocampal), primary sensory, primary motor, basal ganglia, and cerebellum. The most extensive effects of morphine were observed in NOI4 (primary sensory motor network). Within the total areas affected (52.6 ml of brain volume) 39% of the effect was in the bilateral somatosensory areas (inside NOI4), but the rest of the effects were distal to NOI4 (26% in the limbic system and basal ganglia, 16% in the primary motor, 13% in the cerebellum, and 10% in the visual area). The next most extensive effects were in the executive salience network, NOI6 (15.13 mL, 73% of which was prefrontal area that lies inside NOI6).

Alcohol

Compared with morphine, effects of alcohol on RSN connectivity were more limited. The most extensive effects of alcohol were observed in NOI3 (15.15 mL of brain volume), where 62% of the change was in the posterior parietal cortex and the rest in the cerebellum and brainstem (which are outside NOI3). Small changes in connectivity of dorsocaudal ACC and precentral gyrus to NOI4 were present as well (4.52 mL) and in a small area (2.46 mL) inside NOI1.

Overlap

In NOIs 1, 2, 3, 5, 6, and 7, the intersection of the significant clusters of each drug's effects did not reveal overlap, suggesting topographic differences in each drug effect on

Figure 3.

Pharmacodynamic effects over time (mean ± SEM). See Table I for details of main effects.

TABLE I. Pharmacodynamic effects

Parameter	Least square means				Treatment P-value		(mean, 95% CI, P) Contrasts		
	Placebo	Alcohol	Morphine		Morphine	Alcohol	Morphine vs. Placebo	Morphine vs. Alcohol	
VAS Alcohol effects (mm)	1.7	19.1	7.2	0.0096	17.4 (6.7, 28.2) $P = 0.003$	5.5 (-5.1, 16.2) $P = 0.291$	-11.9 (-22.7, -1.1) $P = 0.032$		
VAS Alertness (mm)	48.6	46.0	43.7	0.1843	-2.6 (-8.0, 2.8) $P = 0.3236$	-5.0 (-10.3, 0.4) $P = 0.0696$	-2.3 (-7.7, 3.0) $P = 0.374$		
VAS Calmness (mm)	53.4	58.2	60.6	0.0428	4.7 (-1.5, 11.0) $P = 0.132$	7.2 (1.6, 12.7) $P = 0.014$	2.5 (-3.3, 8.2) $P = 0.386$		
VAS Mood (mm)	51.7	52.8	54.4	0.5171	1.1 (-4.1, 6.2) $P = 0.662$	2.8 (-2.2, 7.8) $P = 0.2618$	1.7 (-3.4, 6.7) $P = 0.4982$		
VAS Nausea (mm)	3.7	3.0	18.6	0.0320	-0.6 (-13.5, 12.2) $P = 0.918$	14.9 (2.1, 27.8) $P = 0.025$	15.6 (2.8, 28.4) $P = 0.020$		
VAS External log (mm)	0.371	0.398	0.406	0.4601	0.027 (-0.035, 0.089) $P = 0.365$	0.035 (-0.026, 0.097) $P = 0.241$	0.008 (-0.054, 0.071) $P = 0.783$		
VAS Internal log (mm)	0.359	0.366	0.382	0.4592	0.007 (-0.033, 0.046) $P = 0.725$	0.023 (-0.017, 0.062) $P = 0.236$	0.016 (-0.024, 0.056) $P = 0.393$		
VAS feeling high log (mm)	0.467	0.575	0.635	0.5275	0.107 (-0.202, 0.417) $P = 0.477$	0.168 (-0.141, 0.476) $P = 0.271$	0.060 (-0.251, 0.371) $P = 0.691$		
Heart beats (per minute)	55	61	52	0.0004	5.2 (1.4, 9.0) $P = 0.009$	-3.8 (-7.5, 0) $P = 0.049$	-9.0 (-12.7, -5.2) $P < 0.0001$		
Respiration (per minute)	16.6	16.6	13	<0.0001	0.0 (-1.4, 1.4) $P = 0.966$	-3.6 (-5, -2.2) $P < 0.0001$	-3.6 (-5, -2.2) $P < 0.0001$

Figure 4.

Overlaying maps of voxel-wise regression of respiration (top) and heart rate (bottom) with connectivity to the 8 template networks (F -statistics; cluster corrected $P < 0.05$). See supplemental Figure for effect of including heart rate and respiration in GLM for testing treatment by time interactions.

these functional networks. Only a small (42 voxels = 0.34 mL) overlap was observed in connectivity of the cerebellum to NOI4 and in connectivity of the superior frontal gyrus to NOI8 (52 voxels = 0.416 mL).

Post hoc Examination of Selected ROI Responses

The purpose of the post-hoc analysis is to demonstrate the range and profile of changes in connectivity in some exemplar ROIs in relation to PK and PD profiles. These ROIs were chosen from NOIs that were most prominently affected by alcohol (NOI3) or morphine (NOI4).

In the sensorimotor network (NOI4), where the negative hippocampal connectivity emerged (Fig. 6a), the hippocampal connectivity in relation to NOI4 was small in alcohol and placebo conditions (95% CI: -1.176 to -0.1104 and -1.230 to 0.09146 ; respectively). By contrast, morphine infusion increased the absolute value of hippocampal connectivity to NOI4 (95% CI: -4.309 to -3.043). We note that

connectivity is defined in terms of linear fitting of spontaneous fluctuations at any given region to the “specific” fluctuations within the entire network (i.e., the weighted average time course of all voxels). Therefore, the negative z -score suggests a distinct inverse relationship in terms of simple oscillations also a phase shift of the fluctuations of the hippocampus with respect to the sensorimotor network. By contrast, the connection of the central gyrus to NOI4, which was similar prior to both infusions, remained stable for alcohol and placebo (95% CI: 8.08 – 10.45 ; and 8.34 – 10.33 , respectively) but became stronger after morphine infusion (95% CI: 11.79 – 13.20) (Fig. 6b).

Figure 6c illustrates that alcohol significantly increased the connectivity within NOI1 within the first 90 min of infusion (95% CI: 0.36 – 2.22). By contrast, in the brainstem (Fig. 6d) morphine and placebo showed an increase in negative connectivity to NOI3 (95% CI: -0.97 to -2.26 and -0.53 to -2.37 ; respectively), whereas this probably adaptive effect was not present with alcohol (95% CI: -0.07 to 0.87).

Figure 5.

Overlaying maps of drug by time interactions with connectivity to the eight template networks (F -statistics voxel-wise $P < 0.0001$; cluster corrected $P < 0.05$; average physiological rates included as covariates). The NOIs are represented in pale red, morphine effects in green and alcohol effects in blue. Table II for the effects details. WM, working memory; DVS, dorsal visual stream.

DISCUSSION

Effects of morphine and alcohol on changes in the RSN connectivity were measured at several time points (together with the PD and PK profiles of each drug). We detected effects where the within-subject variation in pre-infusion connectivity in relation to a given NOI was minimal, whereas the difference in RSN connectivity (in relation to placebo) increased over the next six time-points—after plasma concentrations became stable and during the recovery phase. Drugs produced distinct CNS and clinical responses in terms of PD effects, physiological responses, and resting-state connectivity.

The question of the neurological substrates of resting-state BOLD fluctuations is still an open one. The BOLD signal originates from changes in cerebral blood flow, cerebral metabolism and oxygen extraction. At the present state of knowledge, interpretations of BOLD resting-state connectivity are tentative; although evidence for electrical [Britz et al., 2010; Mantini et al., 2007] and arterial perfusion [Chuang et al., 2008] bases of functional connectivity in some of the putative resting-state networks is already available. Notwithstanding a definite biological interpretation of connectivity, we detected effects in most brain regions where changes in receptor binding, cerebral blood flow, or function in response to these specific substances

TABLE II. Details of drug effects on functional connectivity (F-tests; at cluster corrected threshold $P < 0.05$)

	Morphine vs. Placebo					Ethanol vs. Placebo				
	Location	Cluster size voxels	Cluster peak			Location	Cluster size voxels	Cluster peak		
			t	x	y z			t	x	y z
NOI1: Medial Visual Network										
Includes, calcarine, inferior precuneus, and primary visual cortex and relays visual input through thalamus to primary visual area.	R cerebellum	149 ^{***}	4.77	27	28 16	R visual	140 ^{***}	4.78	35	27 41
	L cerebellum	101 ^{**}	4.8	64	30 15	L visual	100 ^{**}	5.57	52	26 46
	R hippocampus	65 ^{**}	5.05	33	50 29	L parietal lobule	67 ^{**}	-4.91	59	31 56
	R S frontal	87 ^{**}	5.87	37	88 50					
	Precentral gyrus	26 [*]	4.88	44	53 71					
NOI2: Lateral Visual Network										
Includes lateral occipital cortex (superior and fusiform areas) and is involved in visual spatial attention.	R occipital V5	91 ^{**}	5.52	23	26 40	R cuneus	56 ^{**}	4.9	22	33 21
	L occipital V4	70 ^{**}	4.87	57	20 29	Precuneus	24 [*]	4.45	46	32 43
	L posterior insula	45 [*]	-5.24	65	51 45					
	R S frontal	33 [*]	-4.83	38	88 51					
NOI3: Auditory and Somatosensory Network										
Includes superior temporal cortex, insula, operculum, dorsocaudal ACC, somatosensory cortices and bilateral thalamus. ^{††}	L precuneus	348 ^{***}	-5.78	45	29 53	Bilateral PCC	728 ^{***}	5.3	45	50 59
	R paracingulate	322 ^{***}	-4.96	44	67 60	R S parietal lobule	310 ^{***}	5.86	27	39 58
	R cerebellum	96 ^{**}	-4.57	39	45 26	L cerebellum	167 ^{***}	4.97	49	42 25
	L cerebellum (tonsil)	87 ^{**}	-5.31	54	42 13	Bilateral culmen	153 ^{***}	5.73	45	37 15
	L cerebellum (culmen)	74 ^{**}	-4.59	50	44 25	R cerebellum	144 ^{***}	5.03	41	42 25
	L amygdala	63 ^{**}	-4.6	54	62 25	Brainstem	144 ^{**}	6.29	43	54 23
	R amygdala	40 [*]	-4.28	35	64 25	L supramarginal gyrus	109 ^{**}	5.61	68	45 59
	L frontal pole	46 [*]	-4.75	57	86 52	L precuneus	45 [*]	4.92	47	35 41
	L S occipital	38 [*]	-4.77	56	26 62	R supramarginal gyrus	38 [*]	5.44	18	45 59
	R S occipital	44 [*]	-4.37	37	26 58	L OFC	34 [*]	5.16	70	76 32
	R postcentral	53 [*]	-4.58	18	59 57	R ACC	22 [*]	4.8	39	86 36
NOI4: Sensorimotor Network Includes pre- and postcentral somatosensory somatomotor areas.										
	L primary somatosensory	1774 ^{***}	7.52	65	51 65	R cerebellum/fusiform	223 ^{***}	-5.74	27	40 23
	L hippocampus	1679 ^{***†}	-7.61	55	54 29	L dorsocaudal ACC	132 ^{**}	-4.02	39	61 59
	R hippocampus		-6.06	32	56 29	R I parietal lobule	112 ^{**}	-4.79	19	47 49
	L putamen		-4.3	58	58 41	R precentral R	98 ^{**}	-5.3	49	65 56
	L amygdala		-6.27	37	61 27					
	R amygdala		-6.02	52	61 28					
	L caudate		-4.9	52	64 47					
	Bi primary motor	1001 ^{***}	7.18	45	52 66					
	L cerebellum VI	851 ^{***†}	-7.95	52	30 27					
	Vermis		-5.37	45	29 28					
	L cerebellum VI		-5.3	39	30 28					
	R primary somatosensory	722 ^{***}	5.7	25	51 62					
	R putamen	668 ^{***†}	-6.28	32	58 41					
	R caudate		-5.53	39	64 46					
	L occipital fusiform	494 ^{***}	6.11	54	19 31					
	R occipital fusiform	314 ^{***}	6.01	33	23 30					

TABLE II. (Continued)

	Morphine vs. Placebo				Ethanol vs. Placebo					
	Location	Cluster size voxels	Cluster peak			Location	Cluster size voxels	Cluster peak		
			t	x	y z			t	x	y z
NOI5: Default Mode Network Includes rostral medial PFC and precuneal and PCC areas. This network is linked to readiness for adaptive response to cognitive and emotional tasks.	L posterior insula	122 **	5.39	63	52 45					
	R posterior insula	84 **	5.12	23	56 44					
	Thalamus	87 **	-4.64	45	52 41					
	Brainstem	95 **	5.25	49	47 16					
	Paracingulate gyrus	61 *	5.53	47	80 50					
NOI6: Executive Salience Network Includes medial and inferior PFC. Regions in this network are implicated in executive control, attention and emotional response to pain.	L subgenual ACC	40*	-5.27	47	76 31					
	L middle frontal	40*	-4.81	61	76 59					
	PCC	37*	-4.53	45	40 48					
	ACC	490***	-6.47	45	85 41					
NOI7: Left Dorsal Visual Stream Network: Includes Broca area 44, frontal pole, dorsolateral PFC and parietal lobule. Involved in working memory, visual spatial processing and language.	L frontal pole	392***	-6.31	61	94 36					
	R frontal pole	350***	-6.65	30	89 50					
	R primary motor	212***	-5.7	39	49 69					
	L insula	115**	-6.13	63	71 33					
	Bilateral thalamus	101**	-5.42	45	62 42					
	R lingual gyrus	63**	-5.32	46	18 32					
	R I parietal lobule	50*	-4.82	21	39 51					
	L OFC	42*	-5.43	54	71 27					
	L primary motor	40*	-5.49	50	49 70					
	L lingual	36*	-4.99	52	30 36					
	L S frontal	31*	4.83	46	91 45					
	R frontal pole	81**	-5.56	25	86 48					
R middle frontal	55**	-4.8	32	73 63						
NOI8: Right Dorsal Visual Stream Network Includes homologous regions as NOI7, plus right paracingulate gyrus, left PCC and left medial PFC.	L S frontal	270***	-5.16	56	75 58	L S frontal	79**	-5.64	55	74 59
	L basolateral PFC	243***	-5.22	67	84 34					
	L S parietal lobule	160***	-5.36	62	35 60					
	Paracingulate, medial PFC	144***	-5.29	47	80 56					
	R primary somatosensory	63**	-4.83	21	46 63					
	L S middle frontal	52**	-5.5	28	67 63					
	R thalamus	35*	-4.71	40	53 42					

Cluster significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.Voxel dimension is $2 \text{ mm} \times 2 \text{ mm} \times 2 \text{ mm}$ (voxel volume 0.008 mL). S, superior; I, inferior; L, left; R, Right. If no lateralization specified, the effect is in the midline region. ACC, anterior cingulate cortex; OFC, orbitofrontal cortex; PCC, posterior cingulate cortex; PFC, prefrontal cortex. These clusters included high peaks in several nuclei.

Evidence for converging circuitry of auditory and somatosensory function is available (e.g. Rodgers et al, Cerebral Cortex, 2008; Bizley et al, Cerebral Cortex, 2007; Fu et al., Journal of Neurosci, 2003; Foxe et al., Journal of Neurophysiology, 2002.). Considering the prevalence of drug effects on somatosensation, we refer to NOI3 as somatosensory network.

are previously reported. For instance, PET studies with opiodergic radiotracers have shown that the ACC, opercular/insular cortex, thalamus, amygdala and putamen (the medial parts of the pain system) have the highest, and the primary somatosensory, sensorimotor areas (the lateral parts of the pain system) [Baumgartner et al., 2006; Jones

et al., 1991; Zubieta et al., 2001] and occipital areas [Sadzot et al., 1991] to have the lowest binding potentials. As Table II shows, we have detected predominant morphine effects both in areas that, according to PET studies, have low opioid binding potentials (such as primary sensorimotor connectivity to NOI4), and areas that have high opiodergic binding potentials (such as the ACC connectivity to NOI6). This observation precludes interpretation of the effects simply as an outcome of metabolic modulation at the site of receptor action. However, it is possible that the detected effects reflect interactions between these different regions in terms of functional adaptation. Future studies of combined RSfMRI and opiodergic PET studies can shed a light on this central question.

Conversely, we have been able to demonstrate different connectivity effects resulting from morphine and alcohol, which have different sites of action. Unlike morphine, CNS effects of alcohol are primarily mediated via GABA_A receptors, which are expressed across the brain, although different parts of the brain have higher affinity for different subunits [Kumar et al., 2009]. Presently, there are no radiotracer studies to have illustrated alcohol-induced variances in regional GABA_A binding potentials in humans. The most direct in vivo evidence for the effect of alcohol on cerebral activation is observed in terms of reduced global metabolism [de Wit et al., 1990; Volkow et al., 2008; Volkow et al., 2006; Wang et al., 2000], with more relative decreases in the occipital cortex [Schreckenberger et al., 2004; Volkow et al., 2008; Wang et al., 2000] and the cerebellum [Volkow et al., 2008; Wang et al., 2000], and relative increases in the brainstem, striatum and the ACC [Schreckenberger et al., 2004]. Also, a radiotracer study of a GABA_A ligand ([¹¹C],Ro 15-4513, which has preferred binding potential for ethanol [Hancher et al., 2006]) in monkeys indicates that the ACC, insula and the limbic system have the highest, and the occipital cortex and the cerebellum have the lowest binding potential for [¹¹C],Ro 15-4513 [Maeda et al., 2003]. Here, we observed that alcohol affected the connectivity of the visual cortex (in relation to NOI1), the cerebellum (in relation to NOI3 and NOI4), the ACC (in relation to NOI3 and NOI4) and the brainstem (in relation to NOI3). Surprisingly, the most extensive effect of alcohol was in changing the connectivity of PCC (in relation to NOI3). It is known that alcohol effects can vary with dosage [Blaha et al., 2003; de Wit

Figure 6.

Profile of change in resting-state connectivity in select ROIs. Statistical parametric maps (*t*-statistics, cluster corrected $P < 0.05$) reflect the amplitude of drug-induced changes in functional connectivity (drug versus placebo over time). Morphine leads to increased negative connectivity of the hippocampus (a), and positive connectivity of the central gyri (b) in relation to the sensory-motor network (NOI4). Alcohol enhances the intraconnectivity of the visual network (c) and disrupts adaptive connectivity of brainstem (d). In the ROI diagrams, red corresponds to placebo, green to morphine, and blue to alcohol.

et al., 1990; Gordon et al., 1995; Luksch et al., 2009; Sano et al., 1993], and they may depend on interindividual differences in regular alcohol consumption. The lesser extent of detected alcohol effects in our study can be hypothetically attributed to these sources of variance and future studies need to address the issue more closely.

With the exception of a small overlap in the cerebellum (in relation to NOI4) and the superior frontal gyrus (in relation to NOI8), drug effects on connectivity were distinct. Similarly, overlap in pharmacodynamic effects was limited. Alcohol significantly increased a feeling of drunkenness and morphine significantly increased nauseous sensation. The CNS effects expected to be common in both drugs such as calmness, alertness, and mood were not significant for alcohol (although calmness was significantly increased by morphine). We cannot conclude that alcohol and morphine do not interact on common brain circuitry. In this experiment, the VAS scores indicate little overlap in terms of subjective changes in mood and feelings induced by alcohol and morphine. However, alcohol (ethanol) was administered at relatively low levels (0.6 g/L), whereas morphine was given in a therapeutically relevant dose (14.5 mg) with considerable functional impact. It is likely that higher doses of alcohol intoxication that result in stronger cognitive and affective responses, will elicit more pronounced changes in local functional connectivity. Presently, without extensive psychometric tests the behavioral correlates of the observed CNS effects cannot be discussed. More precise recordings of certain aspects of emotion and cognition would have enabled us to study the functional significance of RSN connectivity changes. In this experiment, we omitted cognitive testing between different resting-state measurements to avoid introducing performance-related confounds. In fact, our study underlines an important advantage of the resting-state pharmacofMRI (RS-PhfMRI). Significant changes were detected in regions that are expected to be associated with the CNS effects of these drugs; without *a priori* models and performance- or task-related confounds.

It is crucial to note the effects of global physiological variations due to morphine and alcohol. Previous studies have shown that respiratory depression due to opioidergic drugs generates hypercapnic conditions that increases cerebral blood flow [MacIntosh et al., 2008], and causes focal BOLD signal reduction in response to hypercapnic condition in the sensorimotor brain regions [Pattinson et al., 2007]. On the other hand, it has been shown that BOLD fluctuation correlating with respiration and heart rate can compromise delineation of the default mode network [Chang et al., 2009; Chang and Glover 2009b]. Nevertheless, the impact of physiological noise on BOLD is nearly global and including the timecourse of physiological fluctuations do not drastically change the results of connectivity estimation [Birn et al., 2006; Chang and Glover 2009a]. We observed a significant respiratory depression after morphine injection and an increase in heart rate due to alcohol. Our study does not address effect of physiological

variables in dual regression outcome, but we have examined the effects of overall physiological variations (in terms of average respiration and average heart rates per each subjects per each session) on resting-state connectivity, and have shown that inclusion of physiological covariates in a repeated measures model does not change the main effects of the drug by time interaction. Interestingly, including respiration and heart rate in the model removes emerging connections from white matter to NOI4, and reduces the extent of morphine effect on connections to NOI5, the default mode network, whose fluctuations seem to overlap in frequency with spontaneous BOLD changes in this network [Birn et al., 2006; Wise et al., 2004]. It should be noted that our framework is based on examining connectivity to eight NOI templates corresponding to the most consistently determined RSNs. These template networks are initially obtained from independent component analyses (ICA). It has been suggested that the default mode network obtained from ICA might not be fully respiration-proof [Birn et al., 2008]. It is therefore striking that modeling a global measure of respiration change at the group level analysis would demonstrate an effect expected from more careful analysis of the respiration spectra [Birn et al., 2006; Wise et al., 2004]. We cannot preclude the possibility that variation in the temporal physiological profiles have a stronger explanatory power on effects of some of these networks, such as NOI4. Our aim in this study was to show that RS-fMRI is a sensitive and applicable method for detecting drug effects in the brain. Future studies that aim to validate RS-fMRI in relation to specific metabolic effects of especially opioidergic drugs should make precise recordings of variables such as end-tidal CO₂ or arterial CO₂ tension during the RS-fMRI session and incorporate them in such analyses.

It is worth mentioning that, without considering the time and the session effects in the model, we have detected significant associations between the heart rate and connectivity of several regions outside of NOI3 in relation to this network, in addition to changes in connectivity of a region near major arteries (circle of Willis) that also extends to the amygdala, in relation to NOI3, NOI4, NOI5, NOI6, and NOI8. By contrast, respiration changes affected connectivity to NOI4, NOI5, NOI6, and NOI7. An understanding of the neural or vascular substrates of physiological modulation is potentially crucial to interpretation of the neurological substrate of resting-state BOLD connectivity. Especially in pharmacological studies, the autonomic responses to the CNS drug are central to the research and these questions should be carefully considered in the study design. For instance, heart rate modulated the connectivity of amygdala to NOI4 (sensorimotor), NOI5 (default mode network), NOI6 (salience executive), and NOI8 (right dorsomedial visual stream). This observation is striking, since amygdalar association with visual-emotional [Critchley et al., 2005; van Marle et al., 2009] or executive-motor [Napadow et al., 2008] modulation of the heart rate is previously reported. Regardless of neurobiological interpretations, our findings illustrate that different

NOIs vary in degree of susceptibility to physiological modulation; and highlight a need for considering different statistical models to ensure important results are not obscured.

Our study overcomes some limitations in earlier pharmacological fMRI studies, and poses challenging questions. We have successfully detected effects reported in previous studies of morphine and alcohol-without subjectively delineating an ROI necessary for commonly used seed-based RS-fMRI analysis [Anand et al., 2007; Hong et al., 2009; Kelly et al., 2009; Rack-Gomer et al., 2009]. It may be argued that predefined NOIs limit the scope of observations by restricting the criterion of connectivity to temporal similarity with a given NOI. However, these networks were chosen on the basis of previous studies that robustly reproduced them in entirely different population [Beckmann et al., 2005; Damoiseaux et al., 2006]. By choosing these networks we avoided typical ambiguities associated with ICA approach that depends on choosing the model order, or on subjective selection of similar or relevant components for further analysis. Nevertheless, we have detected significant changes in connectivity of regions such as the cerebellum and the hippocampus that are outside the reference NOIs. Therefore this method seems to be robust and less limiting than alternative ICA or ROI methods in detecting regions where the drug effects may not be expected. New approaches such as eigenvector centrality mapping (ECM) [Lohmann et al., 2010] might bring us closer to the goal of total model-free analyses, but such methods are computationally more costly than dual-regression using predefined NOI templates, as we did. It would be interesting to compare results from ECM analysis and our proposed methodology.

An important advantage of resting-state connectivity, compared to other (more quantitative) neuroimaging methods is that it reveals the complexity of the temporal profile of drug effects in each region or interest. For instance, no significant connection is present between the hippocampus and the sensorimotor network (NOI4) under placebo or alcohol conditions, but during morphine infusion, a significant negative connectivity emerges. At the same time, morphine strengthens connectivity within some regions of the NOI4. To date, the issue of negative connectivity remains a topic of debate [Murphy et al., 2009]. Several hypothetical explanations are possible: It may be argued that global vascular effects of morphine (especially due to respiratory depression) would give rise to the observed negative connectivity demonstrated in the hippocampus. However, these particular emerging negative correlations are anatomically confined to bilateral hippocampus, and although we cannot comment on the neurophysiological basis of the effect, they are unlikely to be non-specific artifacts. Another possibility is that reduction of physiological noise (e.g., due to respiratory depression due to morphine) would enhance negative correlations [Fox et al., 2009]. Alternatively, the negative connectivity in the hippocampus may correspond to a delayed, and perhaps adaptive function of this structure

[Bast 2007; Khalili-Mahani et al., 2010]. We remind that these particular effects were not affected by inclusion of respiration and heart rate averages in the group-level statistical model. Such interregional and internetwork heterogeneity of effects raises questions about compensatory and adaptive mechanisms that interact with the drug (which reflects interactions of different brain regions with each other); and underlines the added advantage of resting-state connectivity analysis compared to other neuroimaging modalities.

In conclusion, we have shown that drug-class specific effects on dynamics of brain function at resting-state can be detected even without *a priori* models. An important advantage of with RS-PhfMRI is that it is not confounded by differences in alertness or test strategies and other factors that influence the performance and outcomes of task-induced fMRI, and demonstrates effects even if no appropriate task is available. This study was designed to exhibit limited pharmacokinetic variability, and was hence unsuitable to investigate concentration-effect relationships, although the time profiles of the MRI-effects roughly followed those of the clinical responses. This study demonstrates that RS-fMRI could be useful for “finger-printing” different drug actions within the same individual’s brain. Notwithstanding the exploratory nature of this study, and the inability to characterize the neurophysiological substrates of resting-state BOLD fluctuations, many of our results correspond to findings from previous quantitative PET studies. Because RS-fMRI is noninvasive, widely available and frequently repeatable, it may become an important biomarker for CNS-drug development, able to distinguish detailed drug-induced response patterns in healthy subjects and patients.

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