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Pseudocontinuous arterial spin labeling reveals dissociable effects of morphine and alcohol on regional cerebral blood flow

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We have examined sensitivity and specificity of pseudocontinuous arterial spin labeling (PCASL) to detect global and regional changes in cerebral blood flow (CBF) in response to two different psychoactive drugs. We tested alcohol and morphine in a placebo-controlled, double-blind randomized study in 12 healthy young men. Drugs were administered intravenously. Validated pharmacokinetic protocols achieved minimal intersubject and intrasubject variance in plasma drug concentration. Permutation-based statistical testing of a mixed effect repeated measures model revealed a widespread increase in absolute CBF because of both morphine and alcohol. Conjunction analysis revealed overlapping effects of morphine and alcohol on absolute CBF in the left anterior cingulate, right hippocampus, right insula, and left primary sensorimotor areas. Effects of morphine and alcohol on relative CBF (obtained from z-normalization of absolute CBF maps) were significantly different in the left putamen, left frontoparietal network, cerebellum, and the brainstem. Corroborating previous PET results, our findings suggest that PCASL is a promising tool for central nervous system drug research.
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Introduction

One of the main objectives in central nervous system (CNS) drug development is to identify global and regional effects of drugs on the brain, and to establish a link between these factors and the clinical outcomes. Global change in cerebral blood flow (CBF) is an important marker of cerebral autoregulation (Heiss and Podreka, 1978), whereas the regional distribution of CBF might reflect drug effects on functional brain activity. Initially, ^{15}O-PET was used to detect drug effects on cerebral circulation (Ito *et al*, 1999; Volkow *et al*, 1988). However, replication and repetition of positron emission tomography (PET) studies is costly and not widely available. Besides cost and availability, drawbacks include radiation hazards that prohibit repeated measurements within the same subject over short periods of time. Yet, the ability to perform repeated measurements provides opportunity for better characterization of concentration-dependent drug effects and placebo by time interaction effects. Therefore, research in magnetic resonance imaging techniques for perfusion imaging, such as arterial spin labeling (ASL), aims to offer an advantageous alternative to PET for clinical pharmacology (Detre *et al*, 2009).

Perfusion imaging allows a quantitative measurement of the CBF based on the movement of magnetically labeled endogenous water molecules of the arterial blood. In the last few years, the introduction of clinical medium field magnetic resonance imaging scanners (3 T), background suppression, and improved labeling schemes based on a pulsed version of continuous ASL

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(pulsed- or pseudocontinuous ASL, PCASL) has led to maturation of ASL. Current implementations enable whole-brain perfusion imaging including the cerebellum at a resolution higher than $3 \times 3 \times 7\,mm^3$ within 5 minutes (Dai *et al*, 2008; van Osch *et al*, 2009). Recent studies have characterized the physiological basis of perfusion functional magnetic resonance imaging (fMRI) signal and have established its concordance with CBF measurement using oxygen-15 positron emission tomography (^{15}O-PET) (Bokkers *et al*, 2009; Detre *et al*, 2009).

In this study, we have examined the applicability of PCASL in pharmacological research by performing repeated PCASL measurements of the 'resting-state' perfusion in a randomized, placebo-controlled study of the effects of alcohol and morphine on CBF. The reason for choosing two substances is to examine PCASL's sensitivity beyond nonspecific changes in circulation that result from physiological effects of these drugs. For PCASL to be applicable for pharmacological research, it has to be sensitive to minute and regional changes in brain areas where the drugs act, above the general physiological effects.

As the first step in exploring the usefulness of PCASL for pharmacological CNS research, we chose alcohol and morphine. Alcohol and morphine are two well-characterized substances in psychoactive drug studies with both overlapping and distinct effects. Both alcohol (Blaha *et al*, 2003; Luksch *et al*, 2009; Sano *et al*, 1993) and morphine or similar opiate drugs are vasodilators, and increase global cerebral perfusion (Kofke *et al*, 2007; MacIntosh *et al*, 2008). Both alcohol and morphine induce euphoric and sedative effects. Several studies have shown that both alcohol and morphine alter brain activity in regions such as cingulate cortex, insula, somatosensory, and motor cortices, as well as hippocampus, basal ganglia, cerebellum, and brainstem, which are associated with behavioral and physiological effects of these drugs. (See Supplementary Table for reference to previous studies.) However, whereas morphine effects are primarily coupled to regional binding potential for μ-opioid receptors (Baumgartner *et al*, 2006; Zubieta *et al*, 2001), alcohol (ethanol) primarily targets a subunit of $GABA_A$ receptors (Hanchar *et al*, 2006), causes a global suppression of glucose metabolism (de Wit *et al*, 1990; Volkow *et al*, 2008), and affects widespread neuroendocrine and neurotransmitter systems throughout the brain, including dopaminergic, serotonergic, and even opiate neurotransmitter systems (Koob *et al*, 1998). This raises the question whether PCASL is able to identify overlapping and distinct drug effects that corroborate existing PET findings.

Another reason for starting with these substances is because we have developed and validated infusion protocols based on pharmacokinetic models for each of these drugs (Sarton *et al*, 2000; Zoethout *et al*, 2008), which enable us to achieve pseudo-steady plasma concentration levels of each drug for prolonged periods, thereby minimizing the intersubject and intrasubject pharmacokinetic variations and allowing for equilibration of drug concentrations in the brain. Thus, examining the effect of these drugs on regional cerebral perfusion in the same subject, without any task or perceptual condition, helps answer an important question about the substance specificity of the effects measured by PCASL. We examine drug effects on the global average CBF, and provide statistical maps of regional distribution of CBF across the brain (absolute CBF), and regional changes in relative CBF (rCBF; z-normalized to the global average). Although related, these various metrics provide complementary information about effects of drugs on systemic physiological effects (from global mean perfusion and absolute CBF distribution), and about interaction between different regions about global effects (from rCBF), which might be linked to adaptive brain function. These effects will be further examined in *post hoc* region of interest analyses to quantify the effects and illustrate the extent of between- and within-subject variances across time and experimental sessions.

Materials and Methods

Subjects

A total of 12 healthy male participants (age 18 to 40 years) volunteered for a randomized double-dummy, double-blind, placebo-controlled study involving three visits (each 1 week apart). Exclusion criteria included any kind of implants, pacemakers, or prothesis; any history of medical disorders that pose risk to subjects (e.g. opioid allergy, positive hepatitis B, C or HIV, cardiac or vascular disorder; asthma or pulmonary disease, major gastrointestinal abnormalities, peptic ulceration, hepatic, neurological, psychiatric, hematological, endocrine, renal, or major genitourinary disease) or jeopardize the aim of the study by introducing confounds (e.g. prevalence of illicit drug usage, daily consumption of more than four alcoholic beverages, cigarette smoking, heavy caffeine dependency, and irregular diurnal rhythm).

Drug Infusion

All drug and placebo sessions were randomized. During each visit, subjects experienced identical experimental procedures but in each session, different drug compounds were administered. Placebo occasions consisted of a sham procedure using a glucose 5% solution, including computer-driven adaptations of infusion rates and breath alcohol measurements.

We used a breath alcohol clamping method paradigm that provides accurate stable levels of alcohol (O'Connor *et al*, 1998), as previously shown (Zoethout *et al*, 2008). We aimed for maintaining alcohol levels at 0.6 g/L for 90 minutes. Alcohol concentrations were controlled based on an intravenous ethanol clamping paradigm using ethanol 10% in glucose 5% (Zoethout *et al*, 2008). To minimize infusion pain, alcohol placebo occasions

consisted of a sham procedure using a glucose 5% solution, including computer-driven adaptations of infusion rates and breath alcohol measurements. 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 at 5 minute intervals between 0 and 30 minutes, at 10 minute intervals between minutes 30 and 60, and 30 minute intervals between minutes 60 and 300 after the start of the placebo or drug administration.

Morphine infusion was conducted according to pharmacokinetic models established earlier (Sarton *et al*, 2000). To reach stable serum levels of morphine (80 nmol/L), an initial bolus of 100 μg/kg/h was infused during 1 minute, followed by a continuous infusion of 30 μg/kg/h for 2.5 hours. Total volume of morphine infusion was $\sim$14.5 mg—a safe dose within the therapeutic range of intravenous morphine for acute pain. To determine the plasma concentration of morphine, venous blood was collected in 5 mL plain tubes (Becton and Dickinson and company, Franklin Lakes, NJ, USA). Blood samples were taken at 0, 15, 30, 50, 60, 90, 120, 150, 180, 210 and 270 minutes after the start of the placebo or drug administration. All samples were centrifuged for 10 minutes at 2000 G between 30 and 45 minutes after collection. Next, plasma samples were stored at -21°C. Plasma concentrations of morphine were determined using liquid chromatography with tandem mass spectrometry (Sarton *et al*, 2000).

Pharmacodynamic Assessments

Computerized visual analog scales (VASs) were used to determine whether drugs induced measurable subjective CNS effects. All VASs were performed once at baseline and were repeated at 30, 60, 90, and 120 minutes after the start of infusion. 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. We also used VAS nausea and VAS feeling drunk, each consisting of a single scale in which the extreme left side (0 mm) corresponds to, for instance, 'not nauseous at all' and the extreme right side (100 mm) to 'maximum nauseous'. Subjects were asked to indicate a single point on the scale, reflecting their amount of nausea.

Physiological data were measured during scanning using the standard scanner equipment. We averaged the heart rate over the period of each scan ($\sim$4 minutes). The respiratory signal over the scanning period was Fourier –transformed, and the highest harmonic was used as representative of the average respiration rate during the scan.

Image Acquisition and Processing

A 3T Achieva scanner (Philips Medical System, Best, the Netherlands) was used for image acquisition. The PCASL acquisitions were part of a larger resting-state fMRI study that will be reported elsewhere (Khalili-Mahani *et al*, 2011).

The CBF was measured using PCASL (Dai *et al*, 2008; van Osch *et al*, 2009) immediately before and 120 minutes after drug injection began. A total of 30 pairs of perfusion-weighted and control scans (single shot echo planar imaging (EPI), 17 slices of 7 mm with an in-plane resolution of $3 \times 3\,\text{mm}^2$, sensitivity-encoded (SENSE) factor 2.5, time of echo (TE) = 13.9 ms at a delay of 1525 ms, slice time 35 ms) were obtained (total scan time of 4 minutes 10 seconds). Data for each subject was inspected visually to rule out deleterious intraacquisition motion artifacts. For each subject, we obtained six PCASL data sets ($\text{Placebo}_{\text{pre}}$, $\text{Placebo}_{\text{post}}$, $\text{Morphine}_{\text{pre}}$, $\text{Morphine}_{\text{post}}$, $\text{Alcohol}_{\text{pre}}$, and $\text{Alcohol}_{\text{post}}$). For each set, voxelwise CBF was computed using

$$\text{CBF}(x,y,z) = \frac{\sum_{t=1}^{N}\left(S_{\text{control}}(x,y,z,t) - S_{\text{label}}(x,y,z,t)\right)}{N} \times \frac{1}{\text{lab efficiency} \times M_0 \times T_1\text{blood} \times \lambda} \times e^{\left(\frac{\text{delay}+\text{slice time}(z-0.5)}{T_1\text{blood}} + \frac{\text{TE}}{T_2}\right)}$$

where $N = 30$, $\lambda = 0.76$, lab efficiency = 0.85, TE = 13.9 ms, $T_2 = 50$ ms, and T_1blood = 1680 ms. These computations were performed using MATLAB R2009.a (Mathworks). Data orientation was preserved by using a MATLAB nifti-reader tool (http://www.rotman-baycrest.on.ca/~jimmy/NIfTI/, Rotman Research Institute, Toronto, ON, Canada).

Having computed CBF in native space for each subject, we spatially standardized them to the MNI152 template (Montreal Neurological Institute, Montreal, QC, Canada) to be able to do group-level statistical inference testing. Spatial standardization involved generating an unbiased CBF template for every subject by first, a rigid body registration using FMRIB's Linear Image Registration Tool (FLIRT, with 6 degrees of freedom, based on reducing the least square cost function, and resampling with trilinear interpolation) of each image to the other 5 images in the series; and next, generating the template by averaging the 30 resulting images. This subject template was then registered to the MNI152 template (Montreal Neurological Institute) using FLIRT, with six degrees of freedom, based on reducing the least square cost function, and under sampled to 2 mm isotropic resolution with trilinear interpolation. The resulting transformation matrix was used to align all individual CBF maps to the MNI152 space. A 5 mm blurring kernel was used to smooth the resulting CBF maps. The resulting absolute CBF maps were masked with an eroded standard MNI152 brain mask.

After spatial standardization, we defined three metrics: global mean CBF (averaged over the spatially registered brain volume), absolute CBF (which is the spatially normalized CBF maps obtained above), and rCBF maps (obtained by voxelwise z-transformation of each one of the 72 absolute CBF maps with respect to its own global mean and its own standard deviation (s.d.). This produced normalized CBF maps whose global mean and s.d. (computed across the whole-brain volume) were 0 and 1, respectively. The areas of high Z amplitude (whether positive or negative) indicate regions where CBF exceeds

relative to global mean CBF. Results from absolute CBF and rCBF maps are complimentary: the first test allows localizing areas where highest changes in CBF (while accounting for repetitions across time and subject) occur; and the latter allows identifying changes in perfusion distribution across the brain in relation to global mean CBF. Statistical analysis was performed on these absolute and rCBF maps. In all stages, Functional Magnetic Resonance Imaging of the Brain (FMRIB) Software Library (FSL 4.0, Oxford, UK; http://www.fmrib.ox.ac.uk/fsl) was used.

Statistical Analysis

Repeatedly measured pharmacodynamic data (3 treatments $\times$ 5 times $\times$ 12 subjects) were compared with a mixed model analysis of variance with fixed factors treatment, 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, Cary, NC, USA). Related graphs of these data were drawn with Prism 4.0 (Graphpad Software, La Jolla, CA, USA).

To avoid assumptions about normal distribution of the data, analysis of variance for repeated measures of global mean CBF (3 treatments $\times$ 2 times $\times$ 12 subjects) was conducted nonparametrically using Friedman's test with Dunn's correction for multiple comparisons.

Regional changes in CBF were determined nonparametrically. Permutation-based statistical inference (Nichols and Holmes, 2002; 5000 permutation tests) was used in a triple *t*-test, examining effect of each drug over time, compared with placebo, on absolute and rCBF. Statistical significance was set at $P < 0.05$, after cluster-based correction for familywise errors (based on the null distribution of the max cluster size with cluster-defining threshold of $t = 3.2$, across the entire brain) (Worsley *et al*, 1992).

In the Supplementary Materials, we also provide a description of the effects associated with post versus pre (importantly placebo) at uncorrected *P*-values < 0.05.

Region of Interest Quantification of CBF Changes

To quantify CBF changes under each treatment within statistically significant clusters, we computed the difference of the ratio of absolute CBF within the region of interest (ROI) about global mean at each time point: ΔCBF_{ROI} = absolute $CBF_{ROI\ (post)}$ − absolute $CBF_{ROI\ (pre)}$ and $\Delta rCBF_{ROI}$ = (absolute $CBF_{ROI\ (post)}$/global CBF_{post}) − (absolute $CBF_{ROI\ (pre)}$/global CBF_{pre}). These computations provide a numerical estimate of the CBF effect sizes that satisfied the criteria for statistical significance of the tested model, and help understanding and interpretation of the regional effects.

Effects of Physiological Factors on CBF Maps

It has been shown that altered respiration because of opiate treatment leads to a hypercapnic-related increase in CBF (MacIntosh *et al*, 2008; Pattinson *et al*, 2007). Also, heart rate increase because of alcohol might be associated with vasoreactive changes that alter the arterial blood flow velocity (Blaha *et al*, 2003). Because respiration (after morphine administration) and heart rate (after alcohol and placebo) were significantly affected by treatment, their effect on global mean and regional absolute CBF was examined.

First, regression analysis was performed to determine how much of variation in the global mean CBF was explained by heart and respiration rates. Second, effects of respiration and heart rate variations on the topography of the absolute CBF changes were tested by including a demeaned vector of both respiration and heart rate in the statistical models tested above.

Results

Pharmacokinetic Profiles

Figure 1 illustrates the individual pharmacokinetic profiles. At the time of posttreatment scan, average morphine levels were at 68.04 ± 8.8 nmol/L and average alcohol levels were at 0.63 ± 0.038 (g/L).

Pharmacodynamic Effects

Table 1 summarizes the results of the mixed model analysis of variance of the pharmacodynamic effects.

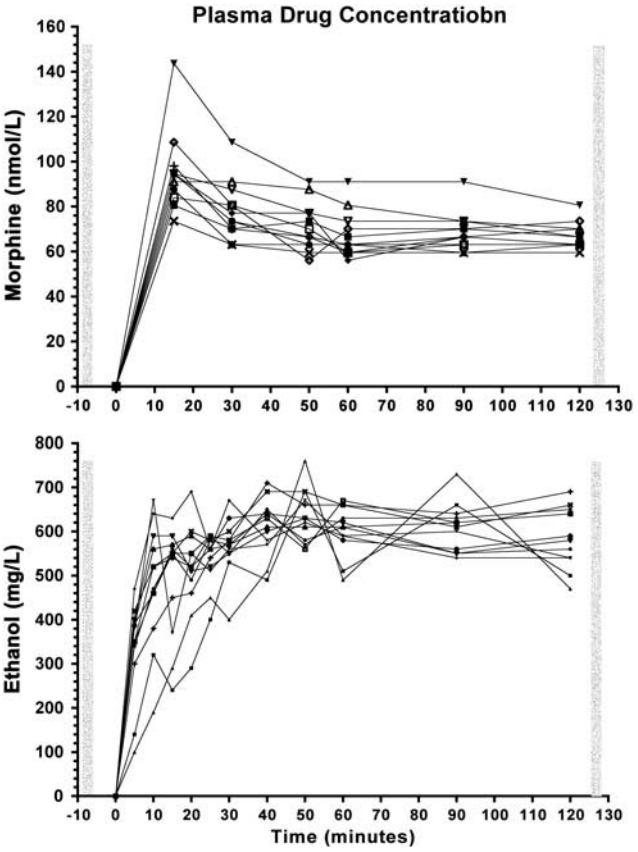

Figure 1 Pharmacokinetic profiles in the 12 individuals. Plasma concentration of morphine (top) and alcohol (bottom). The vertical bars correspond to when the pseudocontinuous arterial spin labeling images were acquired.

Table 1 Pharmacodynamic effects

Parameter	*LS means*			*Treatment* P-*value*	*LS mean contrast 95% CI (lower, upper)*		
	Placebo	*Alcohol*	*Morphine*		*Alcohol vs placebo*	*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) $P=0.324$	−5.0 (−10.3, 0.4) $P=0.069$	−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) $P=0.262$	1.7 (−3.4, 6.7) $P=0.498$
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) $P=0.020$
Heart rate (bpm)	55.5	60.7	51.7	0.0004	5.2 (1.4, 9.0) $P=0.010$	−3.8 (−7.5, −0.0) $P=0.049$	−9.0 (−12.7, −5.2) $P<0.0001$
Respiration rate (m)	16.6	16.6	13.0	<0.0001	0.0 (−1.4, 1.4) $P=0.966$	−3.6 (−5.0, −2.2) $P<0.0001$	−3.6 (−5.0, −2.2) $P<0.0001$
Cerebral blood flow (ml/100 ml tissue/min)	9.09	10.46	11.59	<0.0001	1.37 (0.56, 2.18) $P=0.002$	2.49 (1.69, 3.30) $P<0.0001$	1.13 (0.32, 1.94) $P=0.009$

CI, confidence interval; LS, least square; VAS, visual analog scale.

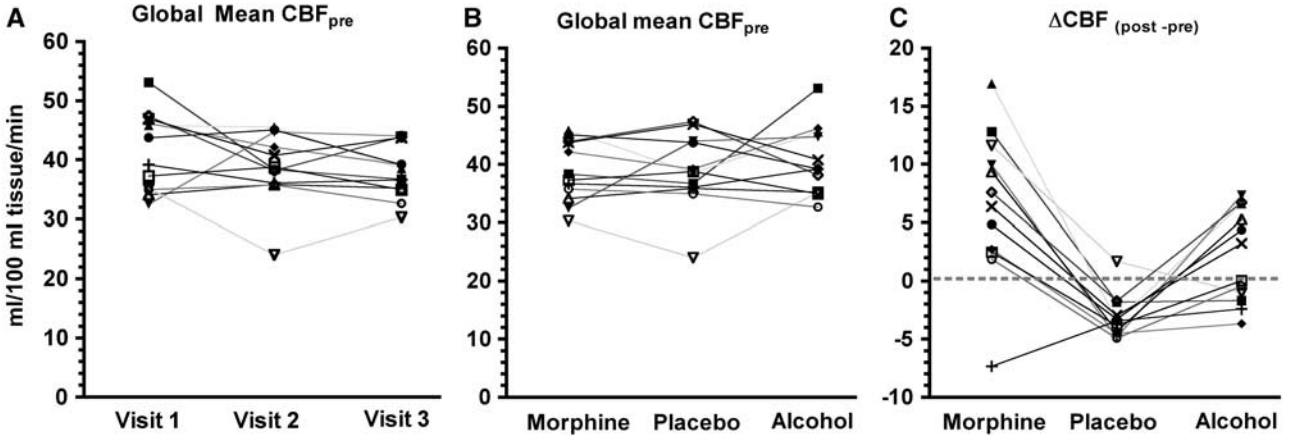

Figure 2 Interindividual variations in pretreatment and posttreatment global mean cerebral blood flow (CBF). (**A**) Effects of visit on the baseline global mean CBF are not significant; (**B**) effects of session on baseline global mean CBF are not significant; and (**C**) the treatment effect on CBF change from baseline is significant and a large degree of interindividual variance is present after the alcohol treatment.

Compared with placebo, morphine treatment increased calmness and feeling of nausea, and reduced respiration rate and heart rate. There was a trend for reduced alertness after morphine administration compared with placebo. Compared with placebo, alcohol treatment increased the drunkenness feeling and heart rate. Compared with baseline, heart rate significantly decreased after placebo treatment.

Effects of Drug on Global and Regional CBF

Effects on global CBF: As the significant decrease in heart rate after placebo might be related to experiencing stress because of novelty and unpredictability of the experiment, we first ensured that there was no significant effects of visit order ($S(12,3)=0.67$, $P>0.7$, Figure 2A) versus treatment session ($S(12,3)=0.167$, $P>0.9$, Figure 2B) on baseline (i.e., preinfusion) average CBF values.

Figure 2C illustrates intersubject variations in ΔCBF = global mean CBF_{post} − global mean CBF_{pre}. Friedman's test revealed a significant drug by time interaction effect on ΔCBF ($S(12,3)=16.7$, $P<0.0005$). Morphine treatment increased CBF in 11/12 subjects (95% confidence interval (CI): 4.7 to 11.1/100 ml tissue/min), and placebo treatment decreased CBF in 11/12 subjects (95% CI: −2.8 to −4.3/100 ml tissue/min); CBF increase after alcohol was present in only half of the subjects (95% CI: 2.5 to 7.1/100 ml tissue/min) and the other half showed a mild decrease (95% CI: −0.3 to −3.4/100 ml tissue/min).

Regional effects of morphine and alcohol on absolute and relative CBF: Spatial distribution of increase in absolute CBF because of morphine and alcohol is illustrated in Figures 3A and 3B. Maps show the *t*-values of comparing $\text{Drug}_{\text{post}-\text{pre}}$ versus $\text{Placebo}_{\text{post}-\text{pre}}$ within the significant clusters (corrected $P<0.05$) for each drug. These tests indicate a broad increase in the absolute CBF across the brain. Because of the widespread effect of these drugs on perfusion, our cluster correction criteria did not form anatomically distinct regions when examining the drug effect on absolute CBF. Therefore, we applied a stringent criterion of voxelwise-corrected $P<0.05$ to illustrate brain regions where increase in absolute CBF was most predominant. These peak location and cluster sizes are listed in Table 2. Results from a simple comparison of after time points versus before time points for each of the drugs and the placebo sessions are presented in the Supplementary Figure.

The most significant effects (voxelwise-corrected $P<0.05$) of morphine treatment on the absolute CBF were observed in the pregenual anterior cingulate cortex (ACC), brainstem, cerebellum, and right operculum (Figure 3A). Significant rCBF increases were detected in the brainstem and cerebellum, and significant rCBF decreases were in the putamen, precentral gyrus, angular cortex, precuneus, temporooccipital, and frontoparietal regions (not shown in the figures).

The most significant effects (voxelwise-corrected $P<0.05$) of alcohol treatment on the absolute CBF were observed in the precentral gyrus, occipital pole, bilateral hippocampus, and posterior cingulated cortex (near juxtapositional lobule) (Figure 3B). The rCBF did not show a significant alcohol effect.

To examine where effects of morphine and alcohol overlap, a conjunction analysis was performed on

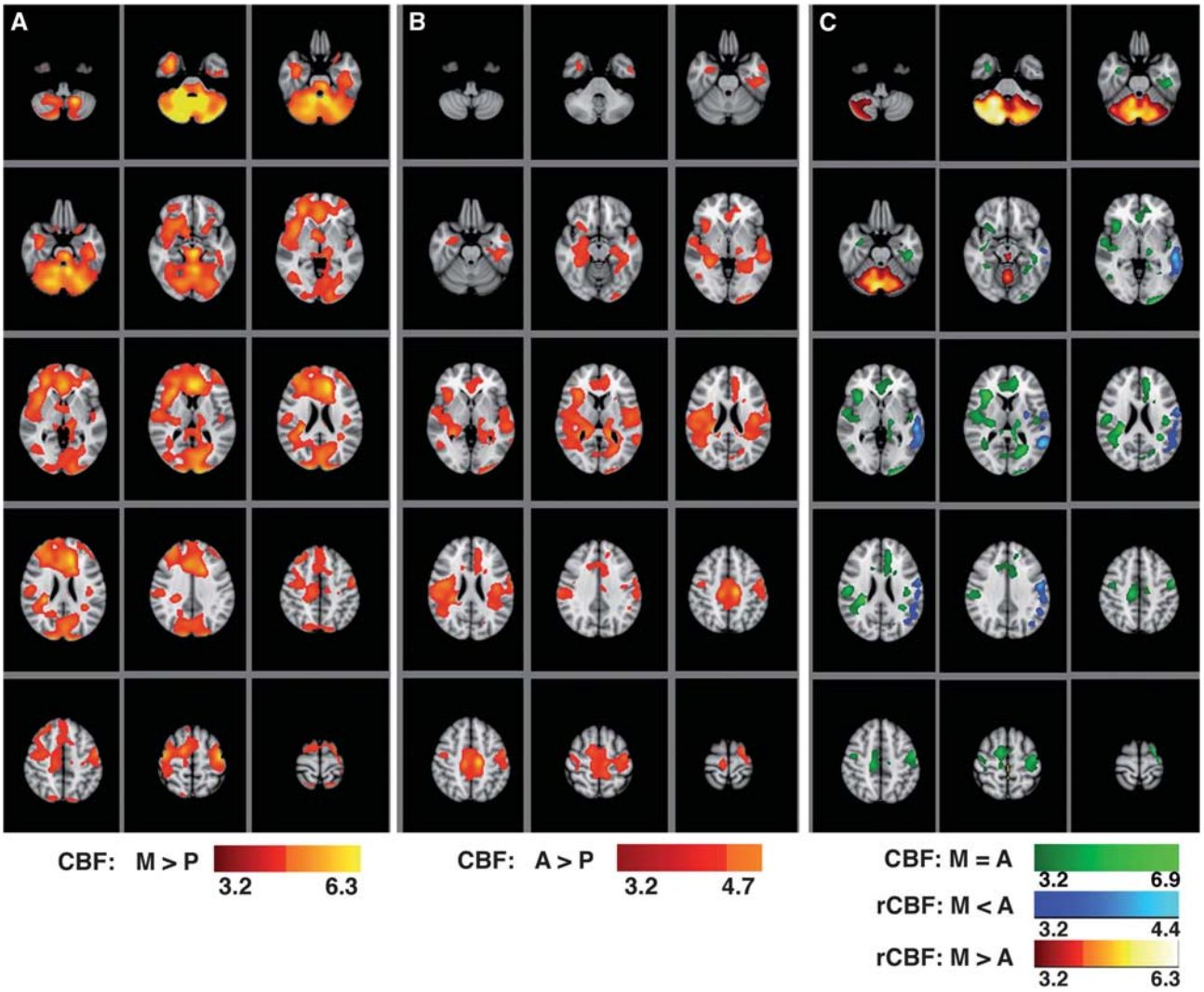

Figure 3 Statistical maps of regional cerebral blood flow (CBF) variations: (**A**) CBF increase because of morphine; (**B**) CBF increase because of alcohol; (**C**) comparison of morphine and alcohol effects: overlapping increase in absolute CBF depicted in green; differences in rCBF, while accounting for the placebo treatment, are depicted in hot and cool colors. See Figure 4 for quantitative illustration.

Table 2 Summary of the effect size, cluster size, and MNI coordinates of brain locations where the highest (voxelwise-corrected $P < 0.05$) increase in absolute CBF and significant differences in rCBF were observed

Structure	t-*value*	*No. of voxels* $2 \times 2 \times 2\,mm^3$	*x*	*y*	*z*
$CBF_{\text{Morphine (post−pre)}} > CBF_{\text{Placebo (post−pre)}}$					
Cerebellum (IX, tonsil)	5.74	3041	50	36	19
Pregenual ACC	5.31	1534	52	78	45
Right frontal operculum	5.37	529	26	73	42
Left cerebellum crus	5.03	133	58	21	15
Right temporal pole	5.12	119	28	65	16
Brainstem, pons	4.96	159	47	51	30
$CBF_{\text{Alcohol (post−pre)}} > CBF_{\text{Placebo (post−pre)}}$					
Left precentral	4.75	100	63	55	69
Left fusiform	4.89	75	69	51	22
Right hippocampus	4.74	72	30	42	36
Left premotor	4.94	52	57	62	70
Left cingulate cortex	4.70	50	52	52	60
Left occipital pole	4.82	45	61	15	35
Right premotor	4.60	36	39	63	67
Left prim. somatosensory	4.60	15	73	59	58
$rCBF_{\text{Morphine (post−pre)}} > rCBF_{\text{Alcohol (post−pre)}}$					
Cerebellum	6.77	11473	29	25	11
ACC	4.53	582	47	83	39
$rCBF_{\text{Morphine (post−pre)}} < rCBF_{\text{Alcohol (post−pre)}}$					
Left primary motor, somatosensory, and lateral occipital	5.07	2141	72	62	44
Left occipitotemporal	5.20	1257	67	32	37
Right hippocampus	5.99	1085	29	53	27
Left hippocampus	5.07	619	63	45	30
Left putamen	4.14	83	59	61	40
Left superior temporal	4.20	69	73	49	34
Right lateral occipital	4.02	62	22	31	48

ACC, anterior cingulate cortex; CBF, cerebral blood flow; MNI, Montreal Neurological Institute; rCBF, relative CBF.

maps for Figures 3A and 3B to identify areas that satisfied the criterion of $t > 3.2$ and formed clusters at $P < 0.05$, depicted in green in Figure 3C. The absolute CBF was commonly increased in the precentral, medial occipital, cingulate, and opercular cortices.

Differences in rCBF changes of $\text{Morphine}_{\text{post−pre}}$ versus $\text{Alcohol}_{\text{post−pre}}$ were tested in a paired *t*-test (5000 permutations), whereas the subtraction effect of $\text{Placebo}_{\text{post−pre}}$ was included as a covariate. The most significant differences were in the cerebellum and the brainstem (morphine > alcohol, depicted in hot colors), and in the precuneus, primary sensory, and primary motor and occipitotemporal cortices (morphine < alcohol, depicted in blue colors, Figure 3C).

Quantitative changes in absolute and relative CBF in ROIs: Figure 4 illustrates the quantitative CBF values in ROIs obtained from Figure 3C.

These results illustrate the spatial heterogeneity, and underline the sensitivity of the statistical modeling.

The lowest variance because of treatment in ΔCBF (2.3%) was in the left putamen, although the main effects of treatment (morphine versus placebo, alcohol versus placebo, or morphine versus alcohol) were not significant. The main effect in the left putamen was because of morphine-induced reduction of ΔrCBF from 2.5% to 15% of the global mean CBF (95% CI).

The highest variance because of treatment in ΔCBF (11.4%) was in the anterior cingulate cortex, where ΔCBF was increased between 13 and 29/100 ml tissue/min after morphine; and between 2 and 19/100 ml tissue/min after alcohol.

The highest variance because of treatment in ΔrCBF (55%) was in the right hippocampus, where morphine reduced ΔrCBF within a 95% CI of 6% to 13%; and alcohol increased it within a 95% CI of 5% to 18% of the global mean CBF. A similar effect was also observed in the left frontoparietal network. Opposite effects of morphine and alcohol on ΔrCBF were also present in the cerebellum, where morphine increased ΔrCBF by up to 7% and alcohol reduced it between 3% and 10%.

Effects of Physiological Factors on Global and Regional CBF

Because respiration (after morphine treatment) and heart rate (after alcohol and placebo) were significantly affected by treatment, their effect on variations on global and regional CBF was examined. It has

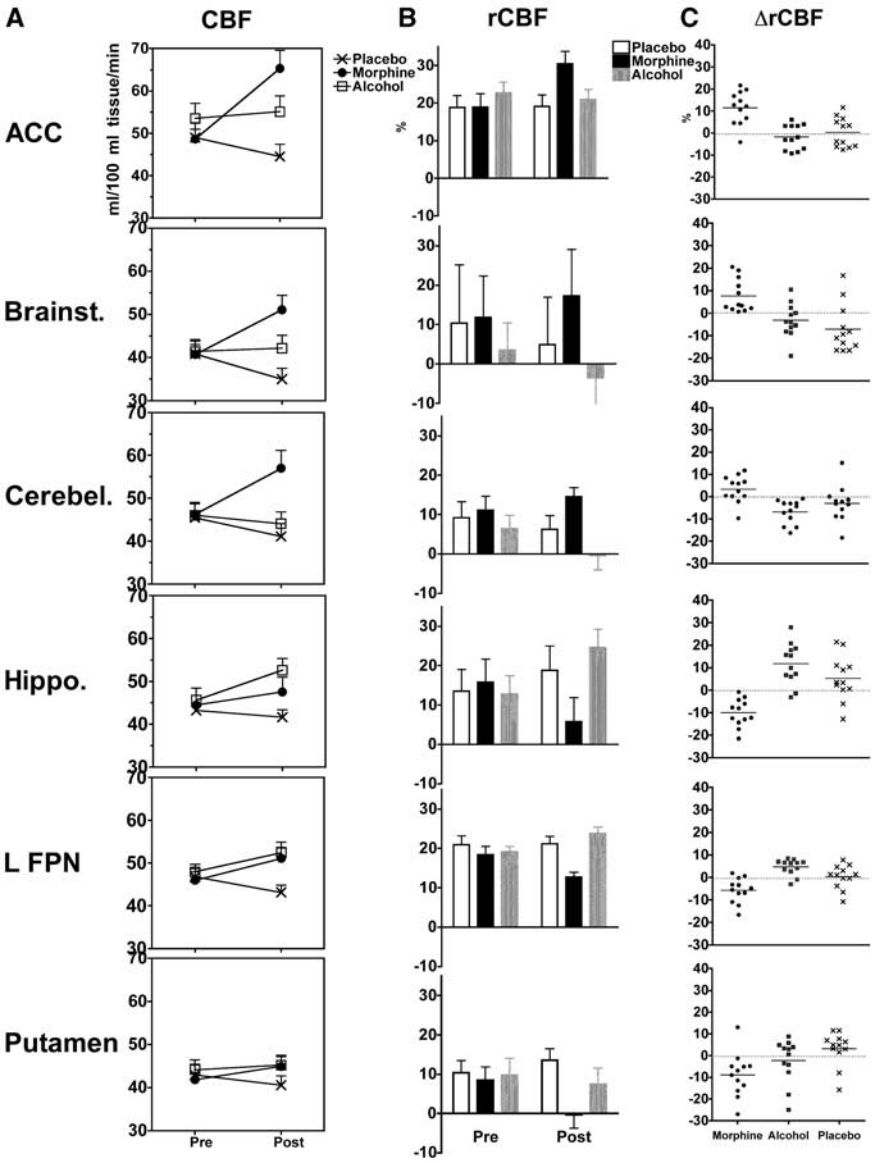

Figure 4 Quantitative illustration of the statistically significant effects detected in ROIs: (**A**) absolute cerebral blood flow (CBF), (**B**) relative CBF values, and (**C**) percentage of relative change with treatment.

been shown that altered respiration because of opiate treatment can cause a hypercapnic-induced increase in CBF (MacIntosh *et al*, 2008; Pattinson *et al*, 2007). Also, heart rate increase because of alcohol might be associated with vasoreactive changes that alter the arterial blood flow velocity (Blaha *et al*, 2003).

Linear regression analysis indicates that lowering of respiration rate after treatment is associated with an increase in global CBF (slope: -0.2361 ± 0.08444; $r = 0.432$, $P < 0.01$). Effects of heart rate variation on global CBF were more heterogeneous for different treatments, and no significant linear relationship was present ($P > 0.3$) (Figure 5A).

The extent of absolute CBF changes because of morphine became smaller, if average physiological variables were included in the model (Figure 5B), but no effect on the CBF changes because of alcohol was observed (Figure 5C). Exclusion of the heart rate covariate from the model did not change the effects (data not shown).

Main effects of respiration and heart rate (while effects of treatment and time are modeled) do not satisfy any of the statistical criteria after correction for multiple comparisons.

Placebo Effects Over Time

Our statistical tests did not reveal any relation between the order of the visits, or treatments in preinfusion global CBF averages (see above). However, as Figure 2C indicates, there is a small but consistent decrease in global CBF (average 2.5/100 ml tissue/min) in all but one subject. Using a paired *t*-test, $\text{Placebo}_{\text{post}}$ and

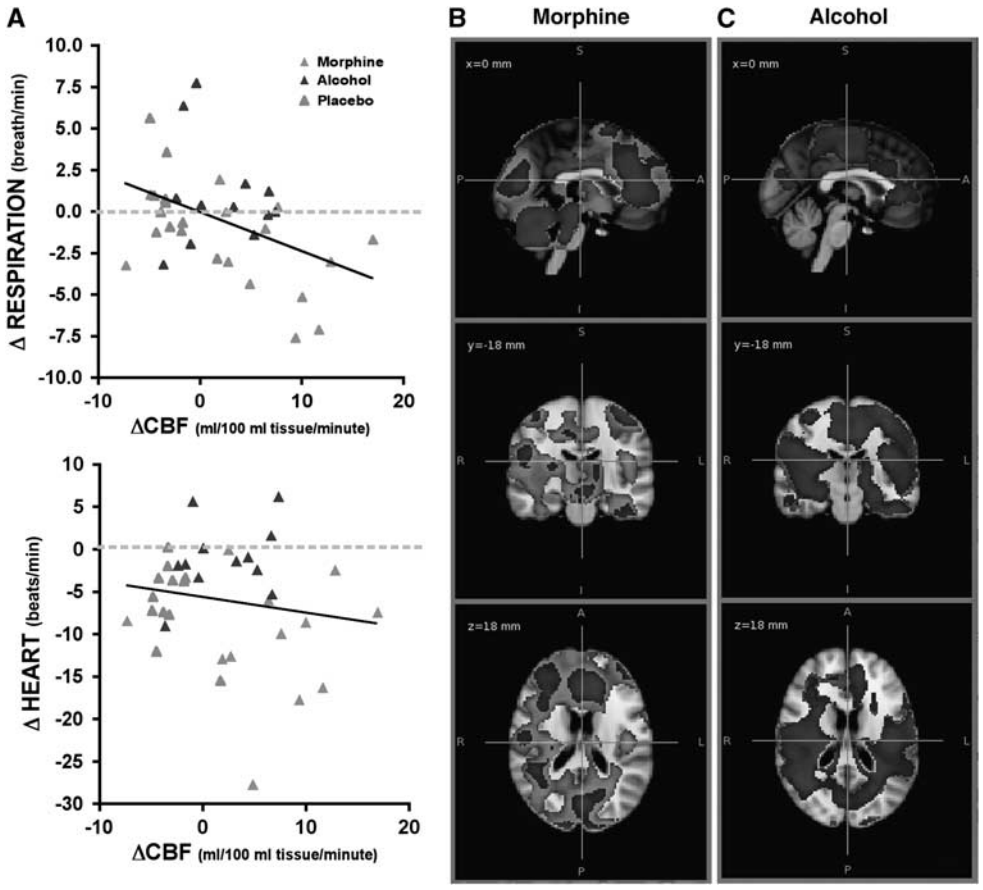

Figure 5 Significant effect of respiration on cerebral blood flow (CBF). (**A**) A significant inverse linear relationship was present between global changes in CBF and respiration rate, but not heart rate; (**B**) including respiration and heart rates as covariates in the general linear model reduces the extent of morphine effects on absolute CBF compared with placebo (**B**), but has no effect on alcohol effects on absolute CBF compared with placebo (**C**). Red clusters correspond to drug effects without physiological covariates in the model. Blue clusters correspond to drug effects after including physiological covariates. For color figure see html version.

$Placebo_{pre}$ were contrasted without including the data from either of the drug sessions. Results did not satisfy the significance condition for multiple comparisons (neither voxelwise nor cluster correction). Using a paired *t*-test (degrees of freedom = 11), $Placebo_{post}$ and $Placebo_{pre}$ were contrasted without including the data from either of the drug sessions. Results did not satisfy the significance condition for multiple comparisons (neither voxelwise nor cluster correction). However, to provide a preliminary explanation for the heterogeneity of regional effects that were revealed in the later quantitative region of interest analyses, these uncorrected statistical maps, thresholded at $P < 0.05$ (uncorrected) for paired *t*-tests of absolute CBF and rCBF changes in $Placebo_{post}$ versus $Placebo_{pre}$; $Morphine_{post}$ versus $Morphine_{pre}$; and $Alcohol_{post}$ versus $Alcohol_{pre}$, are illustrated in the Supplementary Figure.

Discussion

Our results indicate that PCASL is sensitive to detecting drug-specific regional and quantifiable changes in cerebral perfusion. Importantly, we show that the loci of the most significant effects survive after controlling for physiological covariates, such as respiration depression and heart rate, which generate global effects on cerebral perfusion. Our *post hoc* quantitative analysis demonstrates how complimentary information can be derived from statistical mapping of absolute and relative changes in CBF. We also highlight the within-subject stability of global CBF averages before treatment and the importance of accounting for placebo effects in interpretation of the findings.

The primary objective of this study was methodological. Improved signal to noise ratio, improved tagging efficiency, and higher spatial resolution afforded by PCASL make it a desirable quantitative and noninvasive tool in early phases of pharmacological CNS research. An ideal pharmacological tool would be independent of any *a priori* assumptions about the drug effect and would be robust to scanning artifacts in repeated measure studies, and to systemic physiological changes, even if these are drug induced. Here, we minimally preprocessed the data and used permutation testing that is independent of assumptions about normal distribution of

the data. Criteria for statistical significance and correction for multiple comparisons were also set independent of any region of interest or *a priori* expectations. Effects of visit order or treatment on pretreatment global average of CBF were not statistically significant, suggesting that CBF is a stable within-subject variable. Because our statistical tests were performed nonparametrically, we did not exclude outliers. Regardless of this 'crude' methodology, our analysis revealed significant effects in several brain regions that were previously reported in the literature and will be discussed shortly.

Before interpreting neurological significance of our findings, several methodological aspects of our findings must be considered.

First, we detected highly localized effects within anatomical areas, such as putamen (where rCBF changes because of morphine were less than placebo) and hippocampus (where absolute CBF changes because of alcohol were greater than placebo, and rCBF effects of morphine and alcohol were different). This observation is noteworthy because it shows sensitivity and anatomical specificity of PCASL to measuring variations in subcortical perfusion. Secondly, *post hoc* analysis of the effect in these regions underlines regional heterogeneity in how the cerebral perfusion changes. For example, conjunction analysis on statistical parametric maps indicates a similar increase in the absolute CBF in the right hippocampus because of both morphine and alcohol. In fact, quantitative ROI analysis confirms that whereas the CBF values in this region are not significantly different across treatments and subjects, a significant increase of up to 12 and 17/100 ml tissue/min compared with placebo emerges after morphine and alcohol treatments, respectively. However, contrasting rCBF effects of morphine and alcohol treatment shows a consistent decrease in rCBF of the right hippocampus because of morphine and a consistent increase of rCBF because of alcohol (this effect was not significant when compared with placebo), showing a significant difference in regional effect of each drug in comparison to the rest of the brain. However, although a significant drug by time interaction in the putamen is detected, the main change after alcohol or morphine is not significantly large. In fact, effects in the left putamen come from statistical testing of the rCBF maps. This underlines the complementary information that statistical parametric mapping on absolute CBF maps and rCBF maps provide.

Another important reason for examining the rCBF maps is that the variance explained by them is directly related to systemic physiological effects of drugs on cerebral autoregulation (e.g., respiration frequency) that affects perfusion. Here, the average respiration rate explained 18% of variance in global perfusion. Although we did not have data for end-tidal CO2, or arterial CO2 tension, our results are consistent with previous reports of a hypercapnia-induced increase in CBF related to respiratory depression caused by opioidergic drugs (MacIntosh *et al*, 2008; Pattinson *et al*, 2007). As expected, inclusion of respiration rates in our statistical model reduced the spread of the observed effect on absolute CBF maps, without diminishing the significance of the peak effects of morphine. Obviously, such a correlated physiological factor also confounds rCBF effects (where the rCBF maps are obtained by normalization of absolute CBF maps to global mean CBF), which needs to be considered in interpretation of distinct effects in rCBF maps after morphine treatment in comparison with placebo and alcohol treatments. For example, the observed differences in effects of morphine and alcohol on the rCBF of the putamen were more prominent on the left side (Figure 3C, blue color). Pattinson and colleagues have shown that putamen and left sensory motor areas have an important role in motor control of respiration, irrespective of the pharmacological effect of an opioidergic drug (Pattinson *et al*, 2009). However, MacIntosh and colleagues (MacIntosh *et al*, 2008) have shown that hypercapnia because of opioidergic respiratory depression is associated with reduced arterial transit time in the left putamen (and insula), which they interpreted as the possible outcome of higher arteriolar density in these regions. Although our current data cannot substantiate either interpretation, the anatomical specificity of the treatment effects on rCBF maps (also observed in the hippocampus, brainstem, and cerebellum, which are important structures for adaptation) suggests that this metric (rCBF) can salvage important information about the neuronal substrates of the global physiological effects of the drug. Therefore, these various CBF metrics may help future validation studies that aim to establish a direct link between changes in CBF and other factors, such as receptor activation, or event-related potentials.

Given these methodological considerations, how do our PCASL observations compare with previous findings regarding the effects of these drugs?

For morphine, we observed the highest increases in absolute CBF in the ACC, right operculum, brainstem, and the cerebellum. PET studies with opioidergic radiotracers have shown that the ACC, opercular/insular cortex, thalamus, amygdala, and putamen (the medial parts of the pain system) have the highest (Baumgartner *et al*, 2006; Jones *et al*, 1991; Zubieta *et al*, 2001) and that 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) have the lowest binding potentials. It has also been shown that the cerebellum has spatially differential binding potential for different subtypes of opioid receptors (Schadrack *et al*, 1999). Moreover, previous perfusion studies with opioid drugs, such as hydromorphone (Schlaepfer *et al*, 1998), remifentanil (Kofke *et al*, 2007; Petrovic *et al*, 2002; Wagner *et al*, 2007), and fentanyl

(Casey *et al*, 2000), corroborate our finding of regional increase in the CBF of these regions. Notably, a bilateral reduction in rCBF because of morphine was present in the primary sensory; primary motor and occipitotemporal cortex, bilateral putamen, and the right hippocampal area. Considering that the putamen has opioidergic binding potentials comparable to the ACC (Baumgartner *et al*, 2006), it is surprising that the rCBF in this region decreases similar to the primary sensorimotor and occipital regions with lower binding potentials. Because a similar observation is reported in a pulsed arterial spin labeling (PASL) study with remifentanil and controlled respiration (Wise *et al*, 2010), it may be worth noting that a simple paired *t*-test (albeit uncorrected for multiple comparisons—see Supplementary Figure) reveals an anatomically well-characterized bilateral increase in rCBF of $Placebo_{post}$ versus $Placebo_{pre}$, and a less extensive and more right-lateralized decrease in rCBF of $Morphine_{post}$ versus $Morphine_{pre}$ and $Alcohol_{post}$ versus $Alcohol_{pre}$. Although not satisfying our criteria of statistical significance, they hint at an inverse relation between rCBF changes in the putamen and the global average changes in absolute CBF. Whether this effect results from true cerebral activity or from regional differences in the vascular response of the basal ganglia deserves to be investigated.

For alcohol, the strongest effect on the absolute CBF was in the primary sensory and primary motor regions (more on the left side), as well as the left temporal pole and bilateral hippocampus, where a strong increase in absolute CBF of alcohol versus placebo manifested. Alcohol is a depressant of cerebral metabolism (de Wit *et al*, 1990; Volkow *et al*, 2006, 2008; Wang *et al*, 2000) and it impairs memory, visual, and motor coordination, which seems contradictory with regional increases in the CBF. However, frontal and temporal increase, and cerebellar decrease in absolute CBF are also observed in earlier placebo-controlled O^{15} PET perfusion studies (Boecker *et al*, 1996; Volkow *et al*, 1988). Moreover, depressant effects of alcohol are not ubiquitous. When normalizing to the global metabolism, relative metabolic increases in the left temporal lobe (Wang *et al*, 2000) and the 'reward' centers of the brain (Volkow *et al*, 2008) are also reported. Therefore, increased CBF in the hippocampus and sensory and motor areas is plausibly related to disinhibitory effects of alcohol on related functions (Rose and Duka, 2007). Interestingly, *post hoc* examination of the cerebellar region suggests a mild but consistent decrease of rCBF in the cerebellum (95% CI 3.5% to 10%) in all subjects. This is consistent with previous reports that alcohol reduces both the CBF (Boecker *et al*, 1996; Volkow *et al*, 1988) and glucose metabolism (de Wit *et al*, 1990; Volkow *et al*, 2008; Zhu *et al*, 2004) in the cerebellum. However, in the same region, the rCBF was also reduced after placebo treatment, perhaps relating to the role of cerebellum in adaptation to environmental stimuli (such as scanner noise) over time (Timmann *et al*, 1998).

A unique feature of this study is that effects of morphine and alcohol are examined in a within-subject crossover and placebo-controlled study, allowing inferences about common or divergent effects of these drugs. Here, we observed a mild and consistent reduction of the global CBF (a least 2.5/100 mL tissue/min) in the placebo session. Although this did not produce statistically significant regional effects, the rCBF changes because of placebo condition were not similar in all brain regions; for instance, ΔrCBF was positive in the putamen and negative in the brainstem of most of the participants (see Figure 4C, also the Supplementary Figure for uncorrected *t*-statistic maps of comparison $Placebo_{post}$ versus $Placebo_{pre}$). This observation raises the possibility that psychophysiological factors such as subject fatigue contributed to this effect, thus emphasizing the importance of crossover experimental design. By controlling for such placebo effects, we were able to show similar drug effects on absolute CBF increase in the cingulate cortex, medial occipital cortex, right insula, and bilateral operculum, perhaps reflecting common action of drugs on opioidergic receptors (Tiihonen *et al*, 1994). Interestingly, different effects of morphine and alcohol were found on rCBF of the sensorimotor system (comprised of both primary sensory and primary motor areas, as well as left basal ganglia and the cerebellum). The overlaps and differences may relate to similar effects of alcohol and morphine on feeling of calmness and alertness, and different effects on respiration, heart rate, sensation of nausea or intoxication. In the absence of more extensive psychometric tests, we are not able to show the behavioral correlates of absolute or rCBF changes. However, our findings encourage future hypotheses-driven tests specifically examining drug effects on functional activity of structures such as the hippocampus, the ACC, or the cerebellum.

In summary, we have illustrated that PCASL is able to reveal most of the effects of alcohol and opioids that were previously observed with PET studies using receptor-specific, CBF, or metabolic ligands. Research and development of drug-specific radiotracer ligands for PET continues to provide essential understanding of how different psychoactive substances interact with neurotransmitter signaling pathways. However, initial phases of drug development require a cost-efficient, repeatable, and generally applicable measurement tool that allows quantifiable measurement of regional changes in cerebral physiology. Methodologically, PCASL has higher spatial resolution and is considerably simpler to use in a research setting than ^{15}O-PET. With simple preprocessing steps, and without *a priori* statistical assumptions, we illustrated anatomically specific drug effects in a group analysis of repeated measurements of PCASL and quantified these effects in ROI analyses. Considering the relatively few

existing pharmacological studies, and the diversity of applied methodologies, we refrain from drawing conclusions about the advantageous sensitivity of this method compared with others. However, we have emphasized the strength of this method in anatomical delineation of effects in subcortical regions such as the putamen or the hippocampus. To be able to concretely establish the advantages of this methodology over PET, validation studies under similar experimental conditions must follow. We remind that the sensitivity of our analysis can be further improved. Currently, we have set the statistical significance criteria as in blood oxygen level-dependent studies, which did not reveal significant effects in some of the regions where, nevertheless, the *post hoc* analyses showed quantifiable effects. Establishing PCASL-measured voxel spread-point functions that determine the dependency of neighboring voxels and the number of resolution elements can increase statistical sensitivity of this technique. Nonetheless, correspondence of our findings to previously reported imaging effects of alcohol and morphine suggests the promising potential of PCASL in CNS drug research.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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