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Ketamine interactions with biomarkers of stress: A randomized placebo-controlled repeated measures resting-state fMRI and PCASL pilot study in healthy men

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ABSTRACT

Ketamine, an NMDA receptor antagonist, is increasingly used to study the link between glutamatergic signaling dysregulation and mood and chronic pain disorders. Glutamatergic neurotransmission and stress corticosteroids (cortisol in human) are critical for Ca^{2+} mediated neuroplasticity and behavioral adaptation. The mechanisms of action of glutamatergic neurotransmission and stress corticosteroids on the NMDA-receptors of the hippocampus have been long investigated in animals, but given little attention in human studies. In this randomized single-blinded placebo-controlled crossover study (12 healthy young men), five sets of resting-state fMRI (RSFMRI), pseudocontinuous arterial spin labeling (PCASL), and corresponding salivary cortisol samples were acquired over 4 h, at given intervals under pharmacokinetically-controlled infusion of subanesthetic ketamine (20 & 40 mg/70 kg/h). An identical procedure was repeated under a sham placebo condition. Differences in the profile of ketamine versus placebo effect over time were examined. Compared to placebo, ketamine mimicked a stress-like response (increased cortisol, reduced calmness and alertness, and impaired working memory). Ketamine effects on the brain included a transient prefrontal hyperperfusion and a dose-related reduction of relative hippocampal perfusion, plus emerging hyperconnectivity between the hippocampus and the occipital, cingulate, precuneal, cerebellar and basal ganglia regions. The spatiotemporal profiles of ketamine effects on different hippocampal subnetworks suggest a topographically dissociable change in corticohippocampal functional connectivity. We discuss our findings in the context of the negative feedback inhibition theory of the hippocampal stress-control. This pilot study provides a methodological framework for multimodal functional neuroimaging under resting-state conditions, which may be generalized for translational studies of glutamatergic- or stress-related etiology of neuropsychiatric disorders.

Introduction

Ketamine, an antagonist of the N-methyl-D-aspartate (NMDA) receptor has long been used as a dissociative anesthetic, but more recently the potential therapeutic benefits of ketamine in various chronic pain disorders has come to the fore (Hocking and Cousins, 2003; Niesters et al., 2013; Sigtermans et al., 2010). Today, ketamine is also used as an experimental probe to study glutamatergic signaling dysregulation in depression (Diamond et al., 2014; Galvez et al., 2014; Murrough et al., 2013; Niciu et al., 2014; Zarate et al., 2013a) and post-traumatic stress disorder (Chambers et al., 1999; Feder et al., 2014).

Many of the conditions that are affected or treated by ketamine also correlate with dysregulation of the stress system. In fact, the role of stress hormones in triggering glutamatergic signaling deficiencies has been noted (Popoli et al., 2012), and a handful of studies have shown that the administration of NMDA-receptor inhibiting drugs increases cortisol

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levels (Hergovich et al., 2001; Krystal et al., 1994; van Berckel et al., 1998). From a biological perspective, stress is defined as a generalized adaptive response of an organism to a real or perceived demand for increased metabolic resources (Selye, 1951). A stress response follows the activation of the hypothalamic pituitary adrenal (HPA) axis and is marked by the release of adrenal corticosteroids that cross the blood brain barrier and act on target receptors in the hippocampus (McEwen et al., 1968) and in the prefrontal cortex (Diorio et al., 1993). The hippocampus has the highest affinity for stress hormones (glucocorticoid and mineralocorticoid receptors), and is important for coping with stress through 'negative feedback' inhibition (De Kloet et al., 1998; Herman et al., 2005; McEwen, 2006). The mechanisms of stress adaptation are not fully uncovered yet. According to a widely accepted model, the glutamatergic hippocampal inputs innervate the GABAergic neurons in the paraventricular nucleus (PVN) of the hypothalamus (Herman and Mueller, 2006; Radley and Sawchenko, 2011). If this connection is interrupted (pharmacologically or due to lesion), then the PVN becomes disinhibited and a neuroendocrine signaling cascade is triggered. The stress hormones then modulate the Ca^{2+} influx at their receptor site (Groeneweg et al., 2011), and likely re-establish the inhibitory influence of the hippocampus on the PVN.

In the hippocampus, excitatory neurotransmissions and neuroplasticity depend on the Ca^{2+} influx, mediated through NMDA-receptor controlled ionic channels (Bekkers and Stevens, 1989). Glucocorticoid actions in the hippocampus are directly linked to NMDA receptor function (Armanini et al. 1990; McEwen, 1999). Animal studies suggest that stress hormones can exacerbate or enhance the (mal)adaptive effects of NMDA neurotransmission on learning and adaptation, although the effects may be dose- or context dependent (Chaouloff et al., 2007; Maggio and Segal, 2007; Martin et al., 2009; Martin and Wellman, 2011). However, less attention is given to how NMDA receptor inhibition might influence hippocampal function and neuroendocrine stress response. Given the role of the neuroendocrine system in neurodevelopment and neurodegeneration during lifetime (McEwen and Morrison, 2013; Meaney et al., 2007; Oitzl et al., 2010), and the interindividual differences in stress-resiliency/vulnerability (Lupien et al., 2009), a basic understanding of the impact of NMDA receptor blockade on the stress system is critical. Developing methods for probing the interactions between these systems is potentially important for developing precise clinical treatments, as well as for avoiding potential side effects of glucocorticoid/glutamatergic toxicity.

In this randomized, placebo controlled resting-state fMRI and PCASL study under pharmacokinetically controlled ketamine administrations, we investigated the effect of selective NMDA receptor blockade on some of the biomarkers of stress, such as cortisol response, cognitive function, cerebral blood flow and corticohippocampal connectivity.

Materials and methods

Subjects

Twelve healthy male volunteers (age 19–36; body mass index: 21–27 kg/m^2) were recruited to participate in the study, after approval by the local ethics committee. The study was registered in the Dutch Trial Register under number NTR2717.[i] Oral and written informed consent was obtained from all participants. Prior to participation, all subjects received a physical examination. Exclusion criteria for participation were: age <18 years or >45 years; a medical disease such as renal, liver, cardiac, vascular (including hypertension) or infectious disease; presence or history of a neurological and psychiatric disease (e.g., increased cranial pressure, epilepsy, psychosis, depression, or anxiety disorders); glaucoma; obesity (body mass index >30); history of chronic alcohol or drug abuse; use of central-acting medication; presence of metal implants (e.g. pacemaker, hip/knee prosthesis, cochlear implants, vessel clips); and claustrophobia.

Study design

The data for this study were collected with the objective to test the relation between NMDA-receptor inhibition and HPA axis response, as an independent part of a larger project investigating the neural correlates of the analgesic effects of ketamine on acute perception of brief heat stimuli (Niesters et al., 2012). The study had a single blinded, randomized, placebo-controlled crossover design with two occasions, drug and placebo (at least 1 week between sessions). In order to avoid circadian variations, the study began at the same time for all subjects for both sessions, at 8:30 AM. The subjects were asked to refrain from eating and drinking coffee before and during the experiment. A schematic diagram of data acquisition is plotted in Fig. 1. The details of each measurement follow.

Cortisol measurement

Cortisol can be non-invasively measured from the saliva, and is a robust index of interindividual variations in adaptive HPA axis response to acute and chronic stressors (Dickerson and Kemeny, 2004; Hellhammer et al., 2009; Kirschbaum and Hellhammer, 1989).

Salivary cortisol was obtained from passive drool, collected in 2 ml *SaliCaps* (IBL, Hamburg, Germany) upon the subject's arrival at 8:30 AM (t = −90 min), right after the baseline MRI (t = −30 min), before the start of infusion (t = 0), before the second and third encoding tests (t = 30 and 90 min), after the third and the fourth scans (t = 130 and 170 min), and after the recognition task (t = 200 min). Saliva samples were frozen and stored at −20 °C until analysis. After thawing, SaliCaps were centrifuged at 3000 rpm for 5 min, which resulted in a clear supernatant of low viscosity. Salivary concentrations were measured using commercially available chemiluminescence-immunoassay with high sensitivity (IBL International, Hamburg, Germany). The intra- and inter-assay coefficients for cortisol were below 8%. (The assaying was performed by Prof. Kirschbaum's laboratory at Technical University of Dresden (http://biopsychologie.tu-dresden.de).)

Saliva samplings were done at the same time as the scans. In order to obtain a measure of cortisol variations during each scanning session, we computed the area under the curve of cortisol with respect to ground ($AUC_{cortisol}$), by first interpolating the profile of all cortisol readouts during each experimental visit, and then integrating the amount of cortisol available during 20 min before to 20 min after each scan.

Intravenous ketamine administration

Upon arrival, subjects were given two intravenous lines (in separate arms), one for drug infusion and one for blood sampling. Subjects received S(+)-ketamine (Ketanest-S, Pfizer BV, Capelle a/d IJssel, The Netherlands) on one occasion and placebo (NaCl 0.9%) on the other. Ketamine infusion followed a previously established pharmacokinetic model to minimize the between-subject variability in plasma concentration of ketamine (Dahan et al., 2011). The timing of the experiment was set with respect to the start of infusion time, t = 0. Subjects received a low dose of ketamine (20 mg/70 kg/h) for the first 60 min, followed by a high dose (40 mg/70 kg/h) for another 60 min. After these two infusion hours drug administration was terminated. See Niesters et al. (2012) for details on the measurement of plasma concentrations of ketamine. See Fig. 1 for the timeline of blood sampling. See Inline Supplementary Table S1 for plasma concentrations of ketamine.

Inline Supplementary Table S1 can be found online at http://dx.doi.org/10.1016/j.neuroimage.2014.12.050.

Memory assessment

The timeline of memory assessment is shown in Fig. 1. We used a picture-encoding/recognition paradigm that has been shown to activate

[i] Post-hoc.

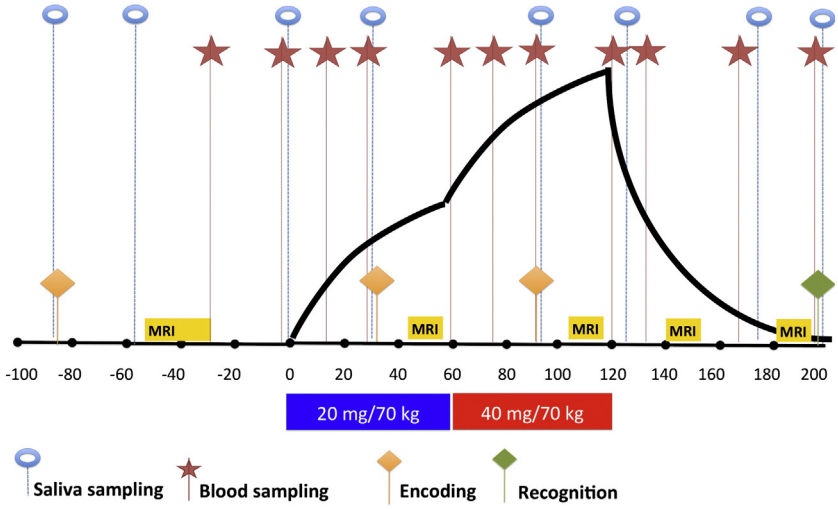

Fig. 1. Schematic presentation of study design with respect to the expected pharmacodynamic profile of ketamine (solid black line). This procedure was performed identically for ketamine and sham placebo sessions, which were randomized. The study was single-blinded (the participant).

the hippocampal formation (Stern et al., 1996), with a significant association with the HPA axis function (Khalili-Mahani et al., 2010).

The encoding task involved viewing 50 complex black and white pictures of random objects or non-specific urban scenes. Subjects had to press a "recognize" button when they saw a picture for the second or third time. Thirty of the pictures were shown twice in the encode session. The rest of the pictures were shown only once during the encode session. Each picture remained on the screen for 5 s. Within 25 to 75 s after the first viewing, the subjects saw the pictures again and had to press a button if the picture was familiar to them, and do nothing if it was novel. If the subjects did not press any buttons on a previously shown picture, the program recorded a "miss". If a button was pressed on a picture that was not shown before, the program recorded a "false alarm". The subjects performed three sets of this encoding task per visit (with different pictures in each set and each visit).

At the end of the experiment, the subjects were presented a recognition test, consisting of 60 pictures, 30 of which were novel, and the other 30 were seen in one of the three previous encoding sessions in the same visit. Similar to the encoding session, subjects had to press a button when they recognized a picture they had seen in the encoding sessions. The stimuli were programmed with E-Prime (Psychology Software Tools Inc., Pittsburgh).

Mood assessment

We used the Bond and Lader visual analog scale (Bond and Lader, 1974) at minutes (0, 30, 90, 120, 160, 200). The Bond and Lader scales are commonly used in pharmacological studies; and are calculated from sixteen 100 mm visual analog scales. The endpoints are set at antonymous word pairs such as 'alert–drowsy', 'well coordinated–clumsy', 'mentally slow–quick witted' and 'incompetent–proficient'. The study participant's task is to make a mark on each scale at the point that best describes how they currently feel considering that the two anchors reflect the greatest extent they have ever experienced each state. Responses from these 16 scales are then scored to yield three main factors of alertness (alert, strong, clear-headed, coordinated, energetic, quick-witted, attentive, proficient, interested), contentment (contented, happy, amicable, gregarious, tranquil), and calmness (calm, relaxed). Bond and Lader visual analog scale (VAS) were presented at the same time as saliva sampling. A high score indicates impairment.

To monitor autonomic changes, we also monitored heart rate during scan.

MRI acquisitions

Neuroimaging was performed on a 3-Tesla Achieva Scanner (Philips Medical System, Best, The Netherlands) at fixed time points (t = −30, 45, 105, 140, 170 min; drug infusion started at t = 0). For practical reasons, the subjects were taken out of the scanner by rolling the scanning bed outside of the scanning room while they remained in the supine position. This was to ensure the participants' comfort, and to avoid potential abrupt changes in blood flow related to getting up and walking. Achieva scanners are equipped with a SMART scan protocol that allows repositioning the field of view (FOV) based on the first scan of a subject. In this manner, we avoided between-scan variations in selection of FOV. The neuroimaging protocol included a high resolution T1-weighted scan (TR/TE = 9.7/6.5 ms, flip angle = 8°, 256 × 256 × 140, 2 mm isotropic, 4 min) at the first time point, and one RS-fMRI time series (each consisting of 220 T2*-weighted whole-brain volumes obtained with a gradient echo planar with TR/TE = 2180/30 ms, flip angle = 80°; 64 × 64 × 38, 3.44 mm isotropic resolution, 8 min) at each of the five time points as shown in Fig. 1.

RS-fMRI provides data that reflect fluctuations in the blood oxygen-level dependent (BOLD) signal over time, but does not provide an absolute and quantitative measure of cerebral function. Therefore, each RS-fMRI scan was followed by a pseudo-continuous arterial spin labeling (PCASL) acquisition in order to quantify regional cerebral blood flow during the course of the experiment. Each PCASL consisted of 30 pairs of perfusion weighted and control scans (single shot EPI, 17 slices of 7 mm with an in-plane resolution of 3 × 3 mm^2, SENSE factor 2.5, TE = 13.9 ms at a delay of 1525 ms, slice time 35 ms, labeling duration τ = 1650 ms, background inversion pulses at 1680 ms and 2760 ms after start of labeling) were obtained (total scan time of 4 min 10 s). For PCASL calibration, an additional M_0 scan was collected using a proton density scan with the same readout as PCASL acquisition. A high resolution T2*-weighted EPI (~30 s) was acquired at the end of each repeated MRI in order to facilitate registering the RS-fMRI data to the anatomical image.

During scanning, heart rate was monitored with an MRI-compatible pulse oximeter; and respiratory rate was recorded and registered using a flexible pressure belt (Philips Medical System, Best, The Netherlands).

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These data were analyzed as described previously (Khalili-Mahani et al., 2013).

Neuroimaging analyses

RS-fMRI preprocessing

Standard pre-processing was performed on the RS-fMRI data using MELODIC 3.1 (FSL4.1, FMRIB, Oxford) including motion correction, brain extraction, 5-mm FWHM Gaussian smoothing, variance normalization and high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting with FWHM = 100 s frequency roll-off) in order to remove low-frequency drifts. Then RS-fMRI data were normalized to a standard space by registering the middle RS-fMRI volume first to the high resolution T2*-weighted image, next to the subject's anatomical T1-weighted image, and finally to the MNI152 standard template. The resulting transformation matrix was used to map the RS-fMRI data into standard space.

Functional connectivity of the hippocampus

The hippocampus is a heterogeneous and complex structure, with regionally different metabolic and cytoarchitectural configurations that make each subsection specialized for function, and susceptible to different neurophysiological risk factors (Small et al., 2011). Attention to functional differentiation along the long axis of the hippocampus is growing in neuroimaging studies (Maruszak and Thuret, 2014; Poppenk et al., 2013; Zarei et al., 2013). The information flow between the hippocampus and other brain areas is organized in anterior-medial and posterior-lateral gradient. Therefore, we investigated whether different hippocampal subregions (e.g. head, tail and body) could serve as different pharmacodynamic endpoints for ketamine action.

Considering the small sample size (12 healthy young male subjects), and the low resolution of the EPI acquisition, we used FMRIB's Harvard–Oxford probability template for the left and right hippocampi. To be conservative, the template hippocampus was generated from voxels that has a higher than 50% probability. This template mask was then segmented into three anatomical subsections, head (voxel y = 39 to 45), body (y = 45 to 52) and tail (y = 52 to 60), roughly corresponding to the criteria described by Malykhin et al. (2010). The volumes of the hippocampal head, body and tail were 325, 162 and 60 voxels at 2 mm isotropic resolution, respectively. We also performed a manual segmentation of the hippocampus after registration to standard space, in order to ensure that the boundaries of the template subsegments fell inside the anatomical boundaries of the hippocampus proper (see Inline Supplementary Fig. S1).

Inline Supplementary Fig. S1 can be found online at http://dx.doi.org/10.1016/j.neuroimage.2014.12.050.

To obtain hippocampal functional networks, the average time series data within each hippocampal segment were extracted from the preprocessed functional data sets (see above). For each of the RS-fMRI data, multiple regression analysis was performed using FSL FEAT. For each hippocampal side a general linear model (GLM) was set up with time series of the three segments of each hippocampus as covariates, and the time series of the WM and CSF as nuisance factors. This analysis produced subject-level z-score maps for voxels predicted by each regressor.

Head motion (Yan et al., 2013) and physiological noise (Birn, 2012) are potentially detrimental to estimating functional connectivity in RSfMRI experiments, and although several promising solutions have been offered, there is no consensus on the optimal correction. In our previous placebo-controlled repeated measures pharmacological studies, we have shown that although correction alters the regional variance in resting-state connectivity, it does not change the statistical outcome, particularly if there is no significant effect on noise factors (Khalili-Mahani et al., 2013). Here, we did not observe any drug-induced change in any of physiological factors. We also performed the standard motion correction, by aligning each EPI frame to the middle frame, but the range of motion was less than 0.5 mm and no spikes were detected during quality control of motion correction log files for each dataset. Therefore, to keep models parsimonious, motion parameters were not included in the estimation of connectivity. Instead, we included average time series data within the deep white matter (FMRIB's WM template with probability >90%, eroded), and CSF (FMRIB's ventricle template with probability >90%, eroded) as nuisance variables to account for non-specific or physiological variations. No global mean regression was performed.

PCASL preprocessing

Data for each subject was inspected visually to rule out deleterious intra-acquisition motion artifacts. For each PCASL set, voxelwise CBF was quantified as described in van Osch et al. (2009). The equilibrium magnetization, M_0, was obtained for each subject, by averaging the signal within an eroded CSF mask in the ventricles of the proton density scan corresponding to each imaging session. These computations were performed using MATLAB R2009.a (Mathworks, Inc.). Data orientation was preserved by using a MATLAB nifti-reader tool (http://www.rotman-baycrest.on.ca/~jimmy/NIfTI/, Rotman Research Institute, Toronto, Canada).

Having computed CBF in native space for each subject, we spatially standardized them to the MNI152 template (Montreal Neurological Institute, Montreal, QC, Canada) using rigid body registration using FMRIB's Linear Image Registration Tool (FLIRT, with 6 degrees of freedom, based on mutual information to each subject's high resolution T1). Transformation matrices used to register subject T1 images to the MNI152 template were then used to put quantitative CBF maps in standard space. A 5 mm FWHM isotropic blurring kernel was used to smooth the resulting CBF maps.

Table 1
Changes in regional CBF with respect to global average CBF ($p < 0.05$ corrected).

	Voxels	p	Peak			Brain regions in the cluster
			X	Y	Z	
Ketamine > Placebo	14,228	<0.02	37	73	24	Bilateral medial and dorsolateral prefrontal cortex, orbitofrontal cortex
	163	<0.02	73	73	50	Left middle frontal gyrus
	116	<0.02	67	61	66	"
	71	<0.02	62	74	41	Left operculum
	8	<0.04	24	70	61	Right operculum
Ketamine < Placebo	13,056	<0.02	33	27	7	Cerebellum, occipital fusiform, cuneus, right hippocampus, right putamen
	1146	<0.04	21	62	49	Post-central gyrus
	736	<0.02	64	57	52	Precentral gyrus
	277	<0.04	50	61	50	Dorsocaudal ACC
	141	<0.04	59	62	31	Left putamen

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Statistical analysis

Drug effects on brain activity

Regional ketamine effects in the brain (HC connectivity and CBF) were inferred by permutation testing (n = 5000) of a mixed GLM, with fixed factors treatment and time, and random factor subject. Drug effects on brain connectivity were assessed by means of permutation testing in order to avoid strong assumptions about normality entering the inference procedure. Permutation testing is known to provide consistent estimates of means and variances for central and non-central t-tests even in the case of potentially non-normal distributions (Nichols and Holmes, 2002). As such, it is a more stringent approach to assessing statistical significance. In this study, we were interested in identifying brain regions at which the maximal difference between the profile of ketamine and the profile of placebo effects over time was detectible. We thus modeled the difference between ketamine versus placebo, while accounting for the variance across time (4 post-injection time points vs. the first pre-infusion). The mean for each subject effect was modeled. (See Inline Supplementary Fig. S3 for an overview of the design matrix.) An exchangeability block was defined to ensure within-subject permutations. All statistical thresholds were set at threshold-free cluster estimated $p < 0.05$ (Smith and Nichols, 2009).

Inline Supplementary Fig. S3 can be found online at http://dx.doi.org/10.1016/j.neuroimage.2014.12.050.

Drug effects on cortisol, mood and memory factors

Parametric repeated measure statistical analyses were performed using a generalized estimating equation (GEE) within the framework of the GLM, to assess effects of drug, time and drug by time interactions on cortisol and mood factors. GEE constrains typical Generalized Least Squares (GLS) estimation to incorporate the correlations between repeated measurements from each subject, when analyzing the influence of covariates on the outcome variable. This method is recommended for repeated longitudinal measurements of correlated data (Zeger et al., 1988). In all models above, correlation was assumed within subjects. The standard errors of the estimates, were computed using robust Sandwich estimates, assuming an independent working correlation form.

Memory scores were compared using Wilcoxon sign rank test implemented in Prism 6.0, Graphpad, Inc.

Results

Effects of ketamine on salivary cortisol levels

This was a randomized blinded study, however those subjects who received ketamine in their first visit would become unblinded. Since anticipation is considered a significant predictor of stress, we first tested a full GEE model including visit order, drug and time and the interactions to rule out any visit effects on cortisol variations over time. Effects of visit, visit by time, visit by drug, and visit by time by drug were all non-significant (ps > 0.4). We have therefore not included the visit order in the following analyses.

Fig. 2A illustrates the significant drug by time interaction effect on saliva cortisol ($\chi^2_{(df=7)} = 38$; $p < 0.001$), with the main effect being due to drug ($\chi^2_{(df=1)} = 19$; $p < 0.001$). Post-hoc analyses indicated significant differences between cortisol levels in placebo and ketamine sessions after the start of ketamine infusion until the end, with the lowest 95% confidence interval of 9.3 nmol/l (minimum difference between placebo and ketamine at t = 90 min) to 48.7 nmol/l (maximum difference between placebo and ketamine at t = 200 min).

Fig. 2B illustrates the changes in AUC cortisol at the time of scan. Drug by time interaction on AUC cortisol was significant ($\chi^2_{(df=4)} = 31$; $p < 0.001$), with the main effect being due to drug ($\chi^2_{(df=1)} = 29$; $p < 0.001$).

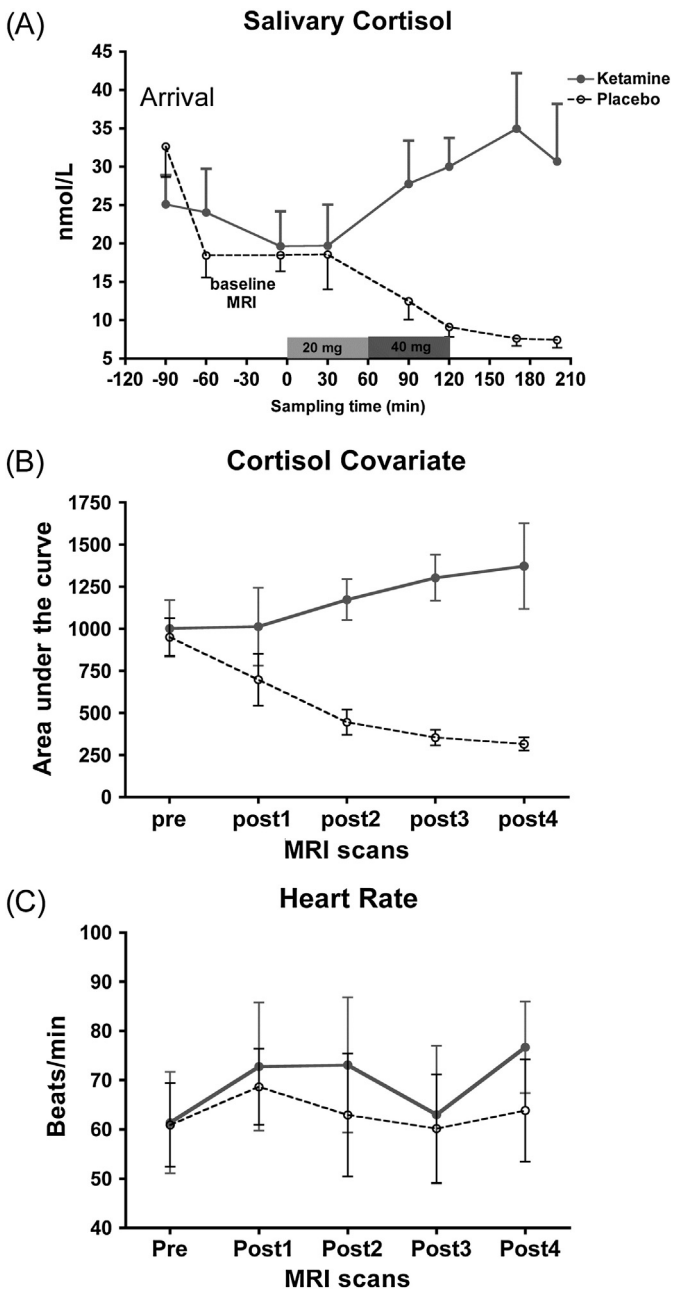

Fig. 2. (A) Ketamine effects on salivary cortisol, shows a gradual reduction of cortisol levels in the placebo, consistent with the diurnal profile of cortisol variation, and a ketamine-induced increase in cortisol until 1 h after the end of ketamine infusion. (B) Area under the curve (AUC) of cortisol; (C) average heart rate.

Ketamine effects on the heart rate were not significant (Fig. 2C).

Effects of ketamine on mood factors

Drug by time interaction effect on alertness was significant ($\chi^2_{(df=7)} = 39.2$; $p < 0.0001$), with significant main effect of drug ($\chi^2_{(df=1)} = 12.5$; $p < 0.0001$) and time ($\chi^2_{(df=5)} = 37.$; $p < 0.0001$). Post-hoc analysis indicated a significant impairment in alertness with ketamine within 30 and 60 min after infusion compared to baseline (95% CI 3.2 to 10.4).

Drug by time interaction effect on calmness was significant ($\chi^2_{(df=5)} = 26.2$; $p < 0.0001$), with no drug effect, but with significant time effect ($\chi^2_{(df=5)} = 18.1$; $p < 0.003$). Post-hoc analysis indicated reduced calmness at 90 min post-infusion compared to baseline (95% CI 0.4 to 2.8). Treatment effects on contentment were not significant (Fig. 3).

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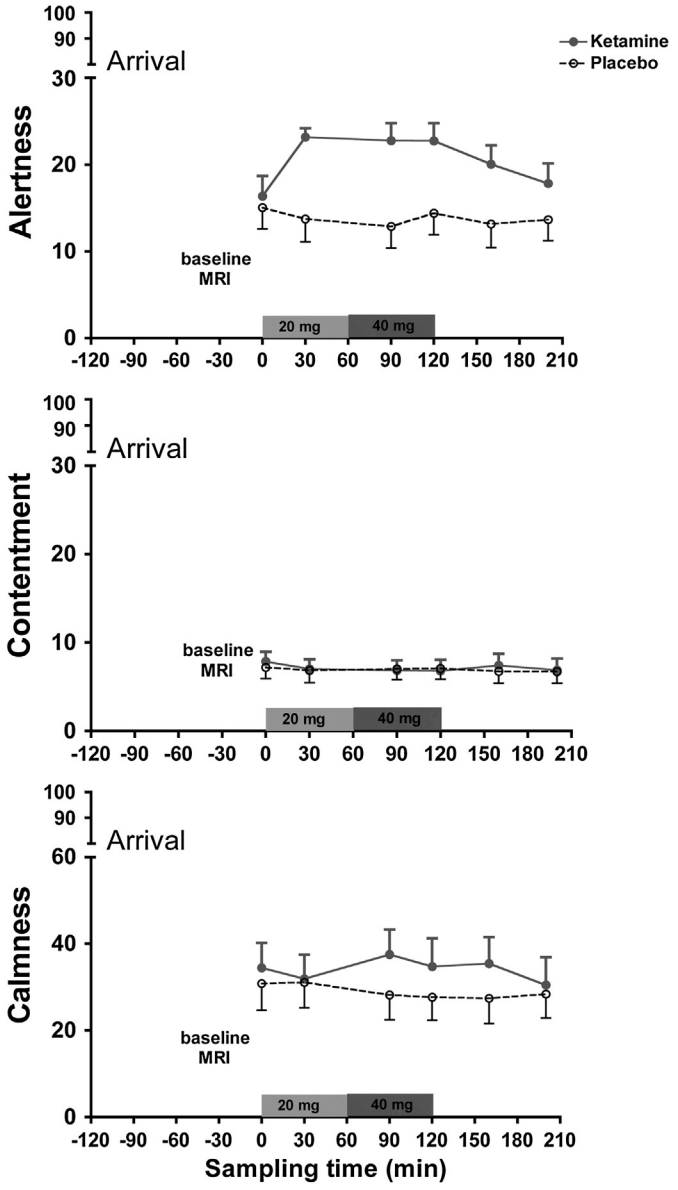

Fig. 3. Ketamine effect on mood scores computed from Bond and Lader visual analog scales. Higher scores correspond to worsening of the mood state.

Effects of ketamine on complex-picture recall

Ketamine impaired performance of both the immediate (Fig. 4A) and the delayed recall (Fig. 4B). A Wilcoxon signed rank test showed that compared to placebo, ketamine increased the number of misses between pre-infusion ($t = -90$) and half an hour after the 20 mg ketamine infusion ($t = 30$), and between pre-infusion and half an hour after 40 mg ketamine infusion had begun ($t = 90$) ($Z's > 2.45$, $p's < 0.02$). Differences in the false alarms were not significant. Only a trend for increased number of misses in delayed recognition ($t = 200$) was observed ($Z = 1.65$, $p = 0.099$). In the final recognition task, we had data only in 10/12 subjects. In 6/10, the performance in recognition declined (due to increased misses).

Effects of ketamine on regional cerebral perfusion

Compared to placebo, ketamine caused a transient increase in global CBF (see Inline Supplementary Fig. S4). The most significant flow effect was observed at min 120 at the end of the 40 mg ketamine infusion (95% CI 3.4 to 21.6 ml/100 g tissue/min). In order to examine the relative changes in CBF, the global CBF was included in the GLM model as a regressor. Anatomical details of the effects are presented in Table 1.

Inline Supplementary Fig. S4 can be found online at http://dx.doi.org/10.1016/j.neuroimage.2014.12.050.

Fig. 5 illustrates the significant reduction of relative CBF compared to placebo in the right hippocampus, bilateral putamen, and bilateral sensorimotor areas, and medial visual cortex, and cerebellum; as well as a significant increase in relative CBF in the medial and dorsolateral prefrontal cortices.

Effects of ketamine on hippocampal connectivity

Figs. 6A and 6B illustrate the brain regions with significant connections to different hippocampal subsections at baseline (averaged predrug conditions). Common connections between different hippocampal regions to the cortex were observed in the posterior parietal regions (namely precuneus and medial occipital cortices) and the cerebellum, but in general the patterns of corticohippocampal connectivity for different subregions of the hippocampus were distinguishable.

At baseline, hippocampal subsections were commonly connected to fusiform, angular, precuneal, opercular, auditory and occipital cortices. Subsection-specific connections were as follows:

1) The hippocampal head was distinctly connected to premotor, primary motor, primary somatosensory, superior frontal gyrus, middle frontal gyrus, orbitofrontal cortices, and left and right hippocampal heads (subicular area), plus subthalamic nuclei, putamen and pons;
2) The hippocampal body was distinctly connected to lingual, temporal pole, medial frontal pole, insula, contralateral HC cornu ammonis, bilateral amygdala, different thalamic nuclei (ventral posteriomedial nucleus, pulvinar and centromedial thalamus) basal ganglia (globus pallidus and caudate) and hypothalamus, as well as to the crus of cerebellum and the midbrain;

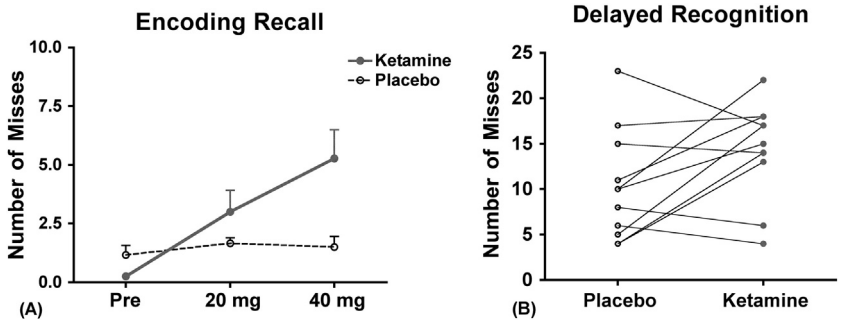

Fig. 4. Ketamine effects on the complex recognition task. (A) Short-term recognition of complex pictures seen during encoding; (B) delayed recognition of complex pictures that were seen during encoding (at minute 200 post-infusion).

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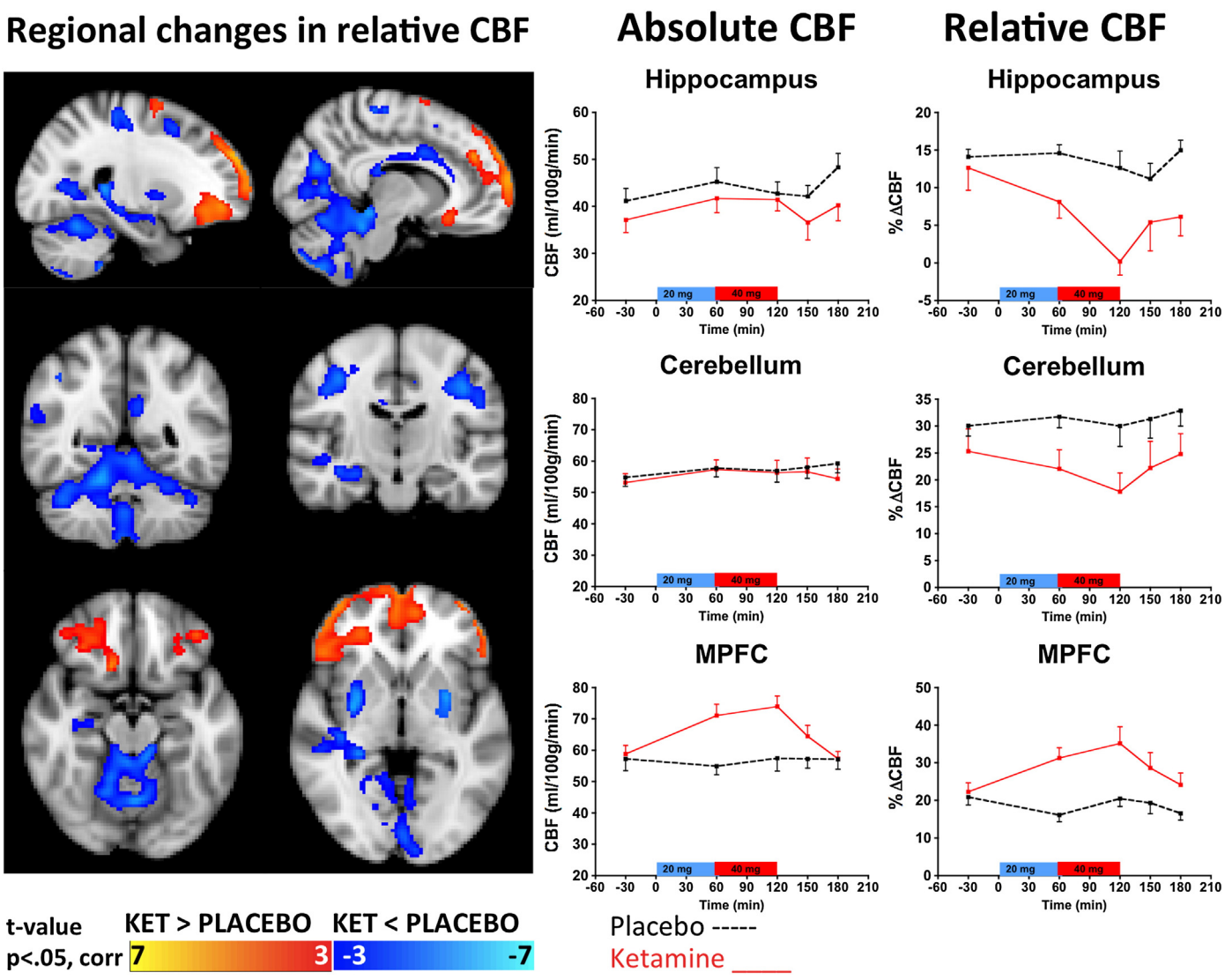

Fig. 5. Regional changes in CBF with respect to average global CBF. The mixed-model estimated the effect of drug by time interaction by modeling the difference between ketamine and placebo and the difference between pre- and the 4 post-infusion times. To illustrate the results, the peak values within the significantly affected ROIs are plotted. $\Delta CBF\% = 100 \times (CBF_{region} - CBF_{global\ average}) / CBF_{global\ average}$. Black dotted line, placebo. Solid red line, ketamine.

3) The hippocampal tail was distinctly connected to primary auditory, parietal lobule, parahippocampal areas, thalamus and lateral occipital cortex, as well as various subregions of the cerebellum (vermis, areas V and VI).

Ketamine induced significant and subsection-specific changes in cortical and subcortical connectivity to the hippocampus as detailed in Table 2 and illustrated in Fig. 6C. The dynamics of change in selected regions of interest are shown in Fig. 6D. The most prominent (in extent) effects were observed in the hippocampal head and body networks. Ketamine effects on the hippocampal head networks (depicted in green in Fig. 6) included an emergence of connectivity to the insular, the medial visual and the posterior parietal cortices. Ketamine effects on the hippocampal body network included bilateral hyperconnectivity to the more superior part of the precuneus as well as to the premotor and the lateral visual cortices. Increased hippocampal connectivity in the posterior parietal and precuneal regions during ketamine infusion returned to baseline after washout (at the end of the session). Other corticohippocampal connections affected by ketamine included the primary visual, sensory-motor, precuneus and the cingulate cortex.

As can be seen in Fig. 6, the profile of change in different regions appears different. In a post-hoc analysis, we formally tested the relation between the two most prominently affected hippocampal subnetworks as listed in Table 2, i.e. the cluster related to drug-induced precuneal hyper connectivity in the hippocampal body network (Precuneus-HC_{body}, depicted in red in Fig. 6) and drug-induced cingulate hyperconnectivity in the hippocampal head network (Cingulate-HC_{head}, depicted in green in Fig. 6). GEE models were tested to examine the relation between Precuneus-HC_{body} and Cingulate-HC_{head} (Y = Precuneus-HC_{body}; x1 = time, x2 = Cingulate-HC_{head}, x3 = time × Cingulate-HC_{head}); and vice versa. To avoid bias, the relations were investigated separately for placebo and ketamine. This model did not reveal any significant relation between Precuneus-HC_{body} and Cingulate-HC_{head} in either condition.

Post-hoc exploration of the relation between different outcome measures

A visual inspection of the main results (CBF, connectivity, cortisol and mood) indicated regional specificity of the pharmacodynamic effects. We performed these post-hoc analyses to explore whether ketamine effects on different hippocampal subnetworks, or on regional

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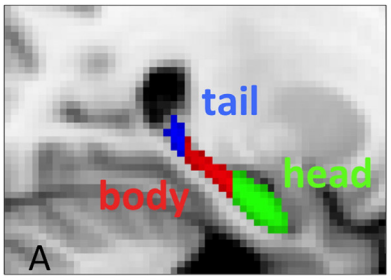

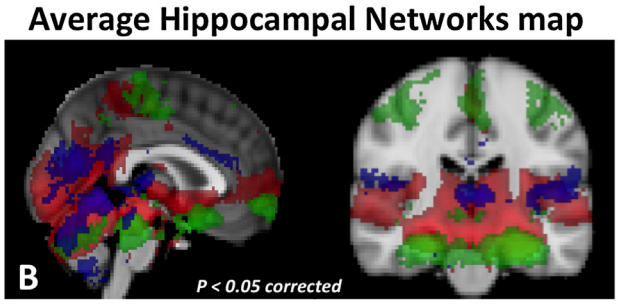

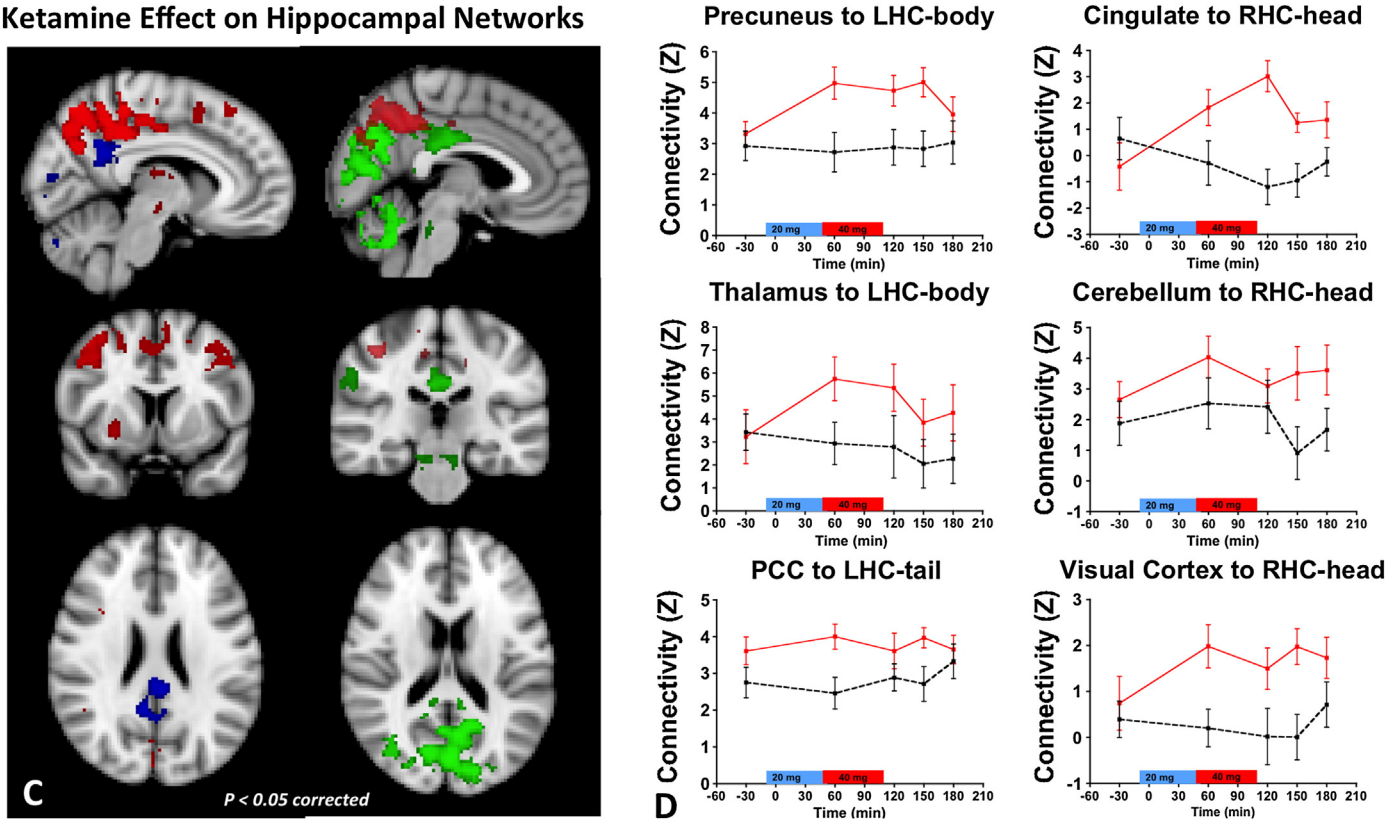

Fig. 6. (A) Sagittal view of different hippocampal subsections; (B) topographic organization of the cortico-hippocampal network obtained by averaging the strength of connectivity (z-scores) of baseline scans and satisfying the TFCE-corrected significance level of $p < 0.05$. (C) Ketamine-induced effects in hippocampal networks (Ketamine > Placebo) revealed from a mixed-model estimation of the effect of drug over time, by modeling the difference between ketamine and placebo and the difference between pre- and the 4 post-infusion times. (D) Profile of change in hippocampal connectivity over time. The average corticohippocampal connectivity (in terms of z-scores) in regions shown in (C) is plotted over time for ketamine (red) and placebo (black) sessions.

Table 2
Ketamine effects on hippocampal connectivity ($p < 0.05$, corrected).

Seed	# voxels	p-Value	x	y	z	Brain regions connected to seed
HC body	13,796	<0.005	40	31	62	Precuneus, bilateral angular gyrus, superior frontoparietal networks, including pericentral regions
	667	<0.04	24	86	36	Right frontal pole
	666	<0.02	43	57	43	Thalamic region with 80% probability of connection to frontal lobe, right hippocampus cornu ammonis, subthalamic nuclei
	427	<0.03	43	76	40	Caudate
	343	<0.03	31	66	33	Putamen
	333	<0.03	22	29	24	Right cerebellar crus
LHC tail	837	<0.02	46	45	49	Precuneus, posterior cingulate cortex
	415	<0.03	34	21	20	Right cerebellar crus
	385	<0.02	58	23	24	Left cerebellar crus
	326	<0.03	44	19	41	Occipital lobe (intracalcarine cortex)
	70	<0.05	60	18	40	Lateral occipital
	41	<0.05	33	22	27	Occipital fusiform
	40	<0.05	44	59	50	Dorsocaudal ACC
RHC head	14,999	<0.002	49	32	51	Cluster includes cerebellum bilateral vermis & crus; bilateral occipital fusiform; medial visual cortex (including calcarine regions and posterior precuneal region) and caudal CC and parietal lobule
	45	<0.05	35	41	26	Midbrain

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Table 3
Ketamine and placebo effects in post-hoc variable of interest.

Pharmacodynamic endpoint	Biomarker	Placebo Mean (SD)	Ketamine Mean (SD)	Ketamine–Placebo 95% confidence interval
Cingulate-HC_{head}	Connectivity (z score)	1.87 (2.73)	3.38 (2.43)	0.78 to 2.2
Precuneus-HC_{body}		2.88 (2.01)	4.39 (1.78)	0.64 to 2.4
$MPFC_{rCBF}$	Relative CBF % total CBF	18.65 (7.61)	28.27 (12.31)	5.9 to 13.3
HC_{rCBF}		13.48 (5.61)	6.49 (10.0)	−9.7 to −4.3
$MPFC_{CBF}$	Absolute CBF ml/100 mg/min	35.49 (7.21)	40.69 (7.71)	3.1 to 13.3
HC_{CBF}		27.43 (5.86)	24.62 (6.59)	−7.7 to −1.2

CBF response, were sensitive to different pharmacodynamic factors in this study. Dimension-reduced neuroimaging factors included Precuneus-HC_{body} and Cingulate-HC_{head} (where the most significant ketamine effect on connectivity were observed), as well as the averaged CBF values within the medial prefrontal cluster ($MPFC_{CBF}$) and in the right hippocampus (rHC_{CBF}) (where significant ketamine related increase and decrease blood flow were detected). Table 3 presents the average values of each variable during placebo or ketamine condition, as well as the 95% confidence interval of ketamine effect versus placebo, while accounting for baseline (pre-infusion).

To investigate which model better explains the variable of interest, we used GEE modeling, and adopted a step-forward procedure using GEE's corrected quasi-likelihood under independence model criterion, which provides a measure of the relative quality of a statistical model for small samples (Burnham and Anderson, 2002). GEE models are better suited (compared to GLMs) to longitudinal studies such as this, as they adapt the standard errors for within subject dependency, by computing the within subject correlations and adapting the parameter estimates based on such covariance matrices. Note that these post-hoc tests are strictly exploratory and are just used to illustrate the complexity of model selection and data reduction in such pharmacological studies.

First, we explored which other factors would best describe variations in the Precuneus-HC_{body} and Cingulate-HC_{head} connectivity. Consistent with profiles of variation over time observed in Figs. 2, 3 and 6, we found that variations in the Cingulate-HC_{head} were best explained by $AUC_{cortisol}$ ($B = -3.27$, $p < .026$) during the ketamine session; i.e. the higher the cortisol levels, the lower the Cingulate-HC_{head} connectivity. On the other hand, variations in the Precuneus-HC_{body} were best explained by alertness ($B = .08$, $\chi^2_{(df=1)} = 4.27$; $p < 0.04$) and $MPFC_{CBF}$ ($B = .09$, $\chi^2_{(df=1)} = 4.29$; $p < 0.04$), but only if data from both conditions were considered. In other words, the higher the alertness VAS (keep in mind that higher VAS scores indicate loss of alertness), and prefrontal perfusion were, the higher was the Precuneus-HC_{body} connectivity.

Next, we investigated which neuroimaging markers would best fit the variations in cortisol. Since the profile of cortisol response over time is visibly different in placebo and ketamine conditions, the analyses were done separately for each, and time interaction effects were also included in the model. For both ketamine and placebo sessions, model factors rHC_{CBF} and Cingulate-HC_{head} were significant predictors of $AUC_{cortisol}$. A model including both rHC_{CBF} ($B = -.005$, $\chi^2_{(df=1)} = 6.0$; $p < 0.02$) and Cingulate-HC_{head} ($B = -.03$, $\chi^2_{(df=1)} = 5.8$; $p < 0.02$) was the best predictor of variations in $AUC_{cortisol}$. Associations between $AUC_{cortisol}$ and Precuneus-HC_{body}, or $MPFC_{CBF}$ were not significant. These models show that in the ketamine session, the lower the hippocampal perfusion and the functional connectivity between hippocampal head and cingulate cortex were, the higher were the cortisol levels.

Discussion

This study is motivated by a growing interest in ketamine as a probe or a therapeutic drug in various mood or chronic pain disorders (Anticevic et al., 2012; Becerra et al., 2009; Carlson et al., 2013; De Simoni et al., 2013; Deakin et al., 2008; Doyle et al., 2013; Driesen et al., 2013; Musso et al., 2011; Nagels et al., 2012; Niesters et al., 2012; Scheidegger et al., 2012). The critical role of stress hormones (glucocorticoids) in NMDA-neurotransmission is well studied in animals, but not investigated in humans yet. To the best of our knowledge, this is the first neuroimaging study of the relationship between noncompetitive NMDA receptor inhibition, HPA axis activity and the hippocampal network under resting-state conditions in humans.

An important feature of this study is that ketamine infusion was based on a pharmacokinetic model to minimize between-subject variations in drug dose, while functional connectivity and cerebral blood flow were measured repeatedly in the state of 'rest'. Resting-state studies minimize the challenges of controlling for between-subject variations in cognitive performance or behavioral adaptation, affording a more direct measure of the baseline neurophysiological states, thus facilitating translational experimentation. This approach enabled us to examine the stability of each imaging metric over time, and allowed us to cross-examine the dynamics of change in brain function in a more quantitative manner.

We discuss our findings in terms of stressor-like effects of ketamine, and the dynamics of the adaptive changes in the brain function, that partially confirm the negative feedback inhibition theory.

Stressor-like effects of ketamine on cortisol and memory

To investigate whether subanesthetic ketamine infusion would mimic a stress response, we measured salivary cortisol, and a stress-sensitive memory function.

We measured a significant increase in cortisol levels by at least 9 nmol/l (compared to placebo) within 30 min after the start of 40 mg dose (per 70 kg body weight/h) but not the 20 mg dose. The magnitude of the effects was comparable to those observed under psychological stressors (Dickerson and Kemeny, 2004). Interestingly, whereas the placebo cortisol levels were decaying consistent with the diurnal pattern, cortisol levels in the ketamine session continued to rise to as high as 48 nmol/l within the next 90 min.

Effects of ketamine on alertness emerged within 30 min (up to 50% with respect to baseline and placebo), but the effects of time on calmness (although mild) emerged only 90 min after infusion began, suggesting a mild psychological stress when experiencing the effects of ketamine. However, the magnitude of ketamine effect on mood states is too small to explain the significant cortisol response as an outcome of psychological stress.

It is common to assess the effect of psychological stress on memory function, however these effects are highly variable and depend on the context and neural circuitry of the particular memory function (Lupien et al., 2007). For this reason, we chose a complex-picture encoding/recognition paradigm that primarily targets the hippocampus (Stern et al., 1996). We expected to replicate memory effects similar to a previous experiment in which a direct link between stress, cortisol response, and changes in hippocampal activation in response to this task was observed in a demographically similar subgroup of psychologically stressed individuals (Khalili-Mahani et al., 2010). Indeed, at the 40 mg dose, we observed a significant reduction of recognition picture-recall

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performance characterized by an increase in the number of misses, as previously observed (Khalili-Mahani et al., 2010).

It should be noted that in the present study, we are not able to dissociate between the effects of psychological stress, cortisol or NMDA-receptor blockade on this memory task. However, these measures provide enough evidence to suggest that at higher dose, ketamine administration was effective in mimicking the expected stress effects on cortisol and memory.

The negative feedback inhibition model of HPA axis control

Increased cortisol response to ketamine supports the notion that a pharmacological "shut down" of the hippocampal glutamatergic signaling pathway (by NMDA-receptor blockade) would lead to disinhibition of the HPA axis activation. The observation of a dose-like reduction of the relative hippocampal CBF could support the idea of a link between hippocampal 'deactivation' and a stress-like response as suggested by Pruessner et al. (2008). In the post-hoc explanatory analyses, we showed that in the ketamine session, there was an inverse relationship between the hippocampal perfusion and total amount of cortisol, and between cingulate-hippocampal head connectivity and total amount of cortisol. However, as it will be discussed shortly, our repeated PCASL and connectivity measurements under controlled pharmacokinetic conditions reveal a more complex picture of the hippocampal physiology under ketamine than "deactivation".

We draw attention to steady levels of absolute CBF in the hippocampus and the MPFC during the placebo session. After ketamine infusion, we observed a significant increase in global perfusion with ketamine, compared to placebo (95% CI 3.4 to 21.6 ml/100 mg/min). In the prefrontal cortex, both relative and absolute CBF were increased, but returned to the baseline levels after ketamine washout (at t = 200 min). Conversely, the absolute CBF in the hippocampus did not change significantly over time, but a dose-dependent relative CBF reduction was observes after ketamine infusion. These results illustrate that the directionality of neuroimaging observations (e.g. increased or decreased activation) is contingent on how signal contrasts are defined or modeled. Because CBF is a quantitative index of brain hemodynamics, our results provide important information about the regional heterogeneity of cerebral perfusion, as well as differences in baseline hemodynamic states, which need to be accounted for when interpreting statistical inference tests. Theoretically, for performing seed-based functional connectivity analysis, stable CBF in the hippocampus would make this structure a more sensitive and less variable reference than most cortical regions (e.g. the prefrontal regions in which significant fluctuations in absolute CBF values were detected after ketamine infusion).

Spatial heterogeneity of neurovascular coupling, a biomarker of neuromodulation

In the absence of a task in pharmacological resting-state studies, regional changes in the brain physiology are likely linked to localized neuromodulation via drug action on local receptors. Repeated PCASL measurements in this study provide a direct quantitative measure of change in cerebral physiology (blood flow), and the spatiotemporal heterogeneity of the effect in different regions underlines the potential importance of neuromodulation of the cerebrovascular coupling.

The dichotomous anterior–posterior topography of ketamine effects on regional CBF observed here is consistent with previous PET and fMRI findings. Several PET studies have reported a ketamine-induced hyperfrontality both in terms of cerebral flow and glucose metabolism (Breier et al., 1997; Holcomb et al., 2005; Lahti et al., 2005; Vollenweider et al., 1997). Decreased connectivity between the anterior and the posterior parts of the default mode network has been reported in a seed based analysis of a placebo-controlled RS-fMRI study (Scheidegger et al., 2012). Other pharmacological fMRI studies of ketamine, fitting the BOLD signal to a model of ketamine effect over time, have reported an attenuation of the BOLD signal in the orbitofrontal but an increase in the cingulate and precuneal areas within 3–6 min after infusion (De Simoni et al., 2013; Deakin et al., 2008; Doyle et al., 2013). Ketamine disruption of the normal fronto/parietal circuitry has been linked to hallucinogenic or memory-disruptive effects of ketamine (Anticevic et al., 2012). Opposite ketamine effects on the frontal and posterior brain regions have been also observed in BOLD signal change in the frontoparietal and the default mode network in pharmacological fMRI experiments involving working memory, episodic memory (Northoff et al., 2005), or visual oddball (Musso et al., 2011) tasks. Although, others have reported a global hyperconnectivity, particularly stronger in the thalamic, cerebellar and precuneal regions (Driesen et al., 2013) — where we observe relative CBF reduction.

In our study, the observed opposite effects in the frontal and posterior cortical regions, as well as the time course of ketamine effects on the CBF — reduced in the hippocampus (seemingly dose-dependent), visual cortex and the cerebellum, and increased in the dorsolateral and medial prefrontal regions (rising after infusion and returning to baseline at the end) are consistent with the expectation that NMDA-receptor blockade leads to glutamatergic and GABAergic modulation of the prefrontal and posterior parietal regions, respectively (Deakin et al., 2008; Moghaddam et al., 1997). The right-lateralization of ketamine effects has been previously reported in a PET study of antidepressant effects of this drug (Carlson et al., 2013). Lateralization of hippocampal NMDA receptor distribution and function has also been reported in schizophrenia (Harrison et al., 2003), although the exact mechanisms are not fully uncovered. Interestingly, the topography of ketamine effects on regional CBF and hippocampal network does not indicate a ubiquitous one-to-one relationship. The overlap in CBF and hippocampal connectivity effects was present in the basal ganglia and cerebellum, which, like the hippocampus, have a high concentration of NMDA receptors (Benarroch, 2011; Rigby et al., 1996). These observations indicate that the neural mechanisms that give rise to changes in cerebral blood flow and resting-state spontaneous fluctuations are spatially heterogeneous and possibly predicted by localized drug-receptor modulation of the electrophysiological state, as we have suggested before (Khalili-Mahani et al., 2014). For instance, here we observed that the absolute CBF in the hippocampus (but not the prefrontal cortex which is also a target for glucocorticoid and NMDA-receptor functions) was stable over time and similar in both ketamine and placebo sessions — although relative CBF in this region was decreased by a dose-dependent manner. A hypothetical explanation might be that given the importance of the hippocampal in maintenance of alert states, it receives tonic and stable blood supplies (Rowland and Kentros, 2008) but relies on local glucocorticoid-facilitated catabolic metabolism for adaptive response to stimuli (de Kloet et al., 2008). Ketamine and brain regions affected by it might serve as useful probe and targets in a quantitative fMRI study of the cerebrovascular coupling.

These observations underline the importance of considering the neurochemical topography of the brain networks, and the neuromodulatory mechanisms that influence the neurovascular coupling. Pharmacological probes, together with multimodal quantitative neuroimaging studies, can be explored in biomarker development for identifying the interindividual variations in general adaptation or in designing personalized therapies.

Functional hippocampal connectivity, a biomarker of adaptive stress response

Our choice of hippocampus as a region of interest is motivated by a wealth of animal research on the relation between this structure and the stress hormone, cortisol. Stress is defined as a generalized adaptive response (Selye, 1951), and is marked by activation of the hypothalamic pituitary adrenal (HPA) axis, followed by the release of corticosteroid hormones that target their most potent receptors in the hippocampus (De Kloet et al., 1998; McEwen et al., 1968; Packan and Sapolsky, 1990). According to Grey and McNaughton's Septo-Hippocampal theory

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of anxiety, the hippocampus is central to adaptive control of stress, as it acts as a comparator to assess the ability of the organism to initiate an optimal adaptive response based on previous learnings (McNaughton, 2006). We therefore proposed the functional connectivity of the hippocampus as a potential biomarker of stress (biological and psychological).

In addition to interesting pattern of blood flow, as discussed above, the transient changes in hippocampal connectivity provide evidence for the role of this structure in adaptive regulation (Fig. 6). Using the hippocampus as a reference we observed increased connectivity to the posterior parietal and precuneal regions during ketamine infusion and return to baseline after washout (at the end of the session). Other corticohippocampal connections affected by ketamine included the primary visual, sensory-motor, precuneus and the cingulate cortex which have been linked to pharmacodynamic outcomes of ketamine infusion such as analgesia (Niesters et al., 2012; Rogers et al., 2004) or psychosis (Driesen et al., 2013).

Because the hippocampus is heterogeneous in terms of regional metabolism and cytoarchitecture, each subsection is specialized for function and thus is susceptible to different neurophysiological risk factors (Small et al., 2011). Recent neuroimaging studies have illustrated that the information flow between the hippocampus and other brain areas is organized in anterior-medial and posterior-lateral gradient (Maruszak and Thuret, 2014; Poppenk et al., 2013; Zarei et al., 2013). In keeping with these findings, we observe a topographical organization of the hippocampal head, body and tail networks, with the hippocampal head being more connected to the anterior brain regions, and the hippocampal body and tail connected to more posterior regions, although significant overlaps were observed in the occipital, precuneal, thalamic and cerebellar regions. Interestingly, with administration of ketamine, the topography of the drug effect on hippocampal subnetworks became more distinct. The most prominent (in extent) effects were observed in the hippocampal head and body networks. The hippocampal head includes the most inferior part of the hippocampal formation, which rests on the subiculum and has bidirectional projections to the hypothalamus, the prefrontal cortex, the nucleus accumbens and the amygdala (Herman and Mueller, 2006). As such, it is a critical part of the behavioral regulation system (McNaughton, 2006) and perhaps more vulnerable to stress disorders (Toffanin et al., 2011; Vythilingam et al., 2005). Ketamine effects on the hippocampal head networks (depicted in green in Fig. 6) included an emergence of connectivity to the insular, the medial visual and the posterior parietal cortices. Ketamine effects on the hippocampal body network included bilateral hyperconnectivity to the more superior part of the precuneus as well as to the premotor and the lateral visual cortices.

In interpreting the neurophysiological significance of connectivity effects, it has to be noted that increased functional connectivity represents stronger correlations or increased synchrony in the temporal profile of the spontaneous BOLD signal fluctuations. The BOLD signal is an indirect measure of neurovascular coupling that depends on the rates of oxygen consumption and regional blood flow. Recent magnetoencephalography studies suggest that the electrical fluctuations in neuronal firing drive the BOLD signal fluctuations measured with RS-fMRI (Brookes et al., 2011). As discussed before, the observed increase in hippocampal connectivity to the cerebellar, posterior parietal and visual areas might reflect the neuromodulatory effect of ketamine (or NMDA receptors) on this corticohippocampal network. It has been shown that NMDA antagonists disinhibit GABAergic interneurons and increase excitatory cholinergic, serotonergic and glutamatergic inputs to the retrosplenial and posterior cingulate cortex (where we find the most robust hippocampal connections) (Olney and Farber, 1995). Ketamine also increases the availability of extracellular glutamate in the frontal cortex and thus increase the excitatory input to the posterior parietal, cerebellar and occipital regions (Moghaddam et al., 1997). This increase in excitatory input to the posterior parietal, cerebellar and occipital regions, or neuromodulation due to increased glutamatergic neurotransmission, might account for increased synchrony (thus increased functional connectivity) between the hippocampus and these brain regions.

Regional specificity of ketamine effects on the hippocampal subnetworks is potentially important in biomarker development. Different profiles of change in each hippocampal subnetwork may be linked to dissociable pharmacodynamics. For example, we observed no correlation between connectivity scores in the Cingulate-HC_{head} and Precuneus-HC_{body} regions. Also, exploring different model factors in the post-hoc analysis showed that each of these regional changes was sensitive to a different set of pharmacodynamic endpoints. For example, the Precuneus-HC_{body} was associated with alertness. The higher the alertness VAS (keep in mind that higher VAS scores indicate loss of alertness), the lower the hippocampal body connectivity. This observation is consistent with the expected role of the default mode network and the precuneus in adaptive maintenance of alertness (Liu et al., 2014; Xie et al., 2011). On the other hand, in the ketamine session the lower Cingulate-HC_{head} connectivity was associated with higher $AUC_{cortisol}$. Hypothetically, this effect can be linked to the negative feedback inhibition of the HPA axis.

The neural correlates of cortisol response

Between-subject variations in HPA axis regulation over time, and the negative feedback nature of the cascade, present a challenge in modeling of the relationships between neuroendocrine and neural state measurements. Interestingly, we observed similar patterns of cortisol and brain activity in some subjects (Inline Supplementary Fig. S2), suggesting that it may be possible to estimate normative pharmacokinetic/pharmacodynamic models in a larger dataset. The small sample size of our study precludes controlling for factors that might explain interindividual variations in HPA axis activity, such as personality traits, diet or quality of sleep, just to name a few. In fact, existing reports on the neural correlates of cortisol response are scarce, contradictory and experiment dependent. For instance, in a primate study, higher rostroventral medial prefrontal activation (measured in terms of glucose metabolism) predicts higher plasma cortisol levels, irrespective of stress stimuli (Jahn et al., 2010). In a seed-based RSfMRI study, a negative correlation between hippocampal and medial prefrontal networks and $AUC_{cortisol}$ has been reported (Kiem et al., 2013). Yet in another study, the direction of relation between cortisol and prefrontal connectivity is a function of the magnitude of cortisol response, and the dynamics of change in cortisol over time (Vaisvaser et al., 2013; Veer et al., 2012).

Inline Supplementary Fig. S2 can be found online at http://dx.doi.org/10.1016/j.neuroimage.2014.12.050.

In this study, the effects of ketamine and cortisol are confounded, therefore with the current analysis we cannot separate the cortisol effects on the brain. Furthermore, we have observed a significant between-subject variability in the temporal profile of cortisol in individual subjects (Inline Supplementary Fig. S2). For this reason, we post-hoc explored the relation between cortisol and the brain regions that were significantly affected by ketamine, and asked which one of these regional signals fit the profile of cortisol variations best. We found that a model including rHC_{CBF} and Cingulate-HC_{head} provided the best fit for variations in $AUC_{cortisol}$. These results are consistent with previous neuroimaging studies that have shown that the medial temporal and cingulate areas are functionally and structurally linked to endogenous or pharmacological corticosteroids — in resting-state (Kiem et al., 2013; Veer et al., 2012), under psychological stress (Lord et al., 2012; Pruessner et al., 2008; Thomason et al., 2011; Wang et al., 2005) or under exogenous corticosteroid administration (de Leon et al., 1988; de Quervain et al., 2003; Lovallo et al., 2010; Oei et al., 2007; Symonds et al., 2012).

It is important to note that, because the relation between cortisol and brain states is governed through a closed feedback system, linear models are not the most suitable predictors of how cortisol interacts with the brain. More complex models (e.g. nonlinear mixed effect models or dynamic causal models) must be explored in future analysis

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in larger samples to provide an accurate PK/PD model of the relations between cortisol, ketamine and their interactions with the brain.

Limitations and future work

This study is performed in healthy male individuals with no history of substance abuse, mood disorder or chronic illness; therefore these results are not generalizable across age, sex and clinical populations. For instance, the therapeutic value of subanesthetic ketamine as antidepressant has been demonstrated (Carlson et al., 2013; Scheidegger et al., 2012; Zarate et al., 2013b), which seems contradictory to our report of ketamine as a "stressor". However, it its interesting that in major depression disorders higher baseline cortisol levels (Vreeburg et al., 2009) and blunted cortisol response to normal daily stressors (Burke et al., 2005; Peeters et al., 2003) have been observed. It would be interesting to repeat this prototypical approach in a demographically similar population of clinically depressed patients to compare the therapeutic impact of ketamine in relation to their psychological and HPA axis resiliency.

Another important point is that in our small sample of young healthy men ($n = 12$) between-subject variations in hippocampal morphology were negligible. However, accurate characterization of hippocampal shape is an important consideration in clinical or age- and gender-related studies. Given the structural complexity of the hippocampus, our rough segmentation of the head, body and tail and the low resolution of the EPI sequence used to obtain $T2^*$ images, we cannot make conclusive judgments about the actual anatomical topology of this structure. It is remarkable that despite all these limitations, we can detect dissociable ketamine effects in hippocampal subnetworks. Our findings underline the critical sensitivity of seed-based approaches in functional connectivity analysis. Also, note that our segmentation protocol produces hippocampal subsegments that differ in size (volume $HC_{head} > HC_{body} > > HC_{tail}$), which might influence the signal to noise ratio and thus reduce the detectability of effects linked to HC_{tail}.

Summary and conclusion

Similar to previous ketamine neuroimaging studies in humans, we confirm an anterior–posterior gradient of change in brain function (in our case) with increase in the prefrontal and decrease in the hippocampal, visual and parietal CBF. The stable perfusion in the hippocampus, plus its regional sensitivity to ketamine effects, suggests that the hippocampus may serve as a reliable target for glutamatergic probing. Ketamine induced hyperconnectivity was observed in the hippocampal networks that are vulnerable to mood and cognitive disorders. We also measured a robust cortisol response that was associated with hippocampal perfusion and hippocampal head connectivity, suggesting that these ensemble variables can serve as biomarkers of glutamatergic activity in the brain.

Our findings underline the importance of the cross-talk between exogenous and endogenous neuromodulators (such as ketamine and cortisol) in neuropharmacological research. By showing that NMDA-receptor blockade is associated with an increase in HPA axis activity, we corroborate the negative feedback inhibition theory and provide evidence that ketamine induces stressor-like changes in cortisol and cognitive performance; as well as changes in the physiology of brain regions that are important for behavioral and cognitive adaptation. The spatiotemporally heterogeneous response profile in the brain challenges the simple interpretation of neuroimaging phenomena as "activation or deactivation" and necessitates more quantitative studies of how local receptors can modulate neurovascular coupling.

The scope of our study prohibited extensive psychometric testing, thus we are limited in drawing conclusions about behavioral aspects of the observed effects. Nevertheless, objective and quantifiable indices of regional changes in brain function might help narrow down the choice of the behavioral test batteries in future experiments. A method that captures the dynamics of interactions between NMDA-receptor function, stress hormones and cortico hippocampal function *in vivo* may thus provide a translational biomarker for studying abnormalities of glutamatergic signaling pathways in different clinical populations. We propose CBF, salivary cortisol and the hippocampus as sensitive biomarkers for investigating the link between stress and glutamatergic dysregulation.

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