Awake Rat Pharmacological Magnetic Resonance Imaging as a Translational Pharmacodynamic Biomarker: Metabotropic Glutamate 2/3 Agonist Modulation of Ketamine-Induced Blood Oxygenation Level Dependence Signals

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ABSTRACT

Neuroimaging techniques have been exploited to characterize the effect of N-methyl-D-aspartate (NMDA) receptor antagonists on brain activation in humans and animals. However, most preclinical imaging studies were conducted in anesthetized animals and could be confounded by potential drug-anesthetic interactions as well as anesthetic agents’ effect on brain activation, which may affect the translation of these basic research findings to the clinical setting. The main aim of the current study was to examine the brain activation elicited by the infusion of a subanesthetic dose of ketamine using blood oxygenation level dependence (BOLD) pharmacological magnetic resonance imaging (phMRI) in awake rats. However, a secondary aim was to determine whether a behaviorally active metabotropic glutamate 2/3 receptor agonist, (1S,2R,5R,6R)-2-amino-4-oxabicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY379268), could modulate the effects of ketamine-induced brain activation. Our data indicate that ketamine produces positive BOLD signals in several cortical and hippocampal regions, whereas negative BOLD signals were observed in regions, such as periaqueductal gray (PAG) (p < 0.05). Furthermore, pretreatment of LY379268 significantly attenuated ketamine-induced brain activation in a region-specific manner (posterior cingulate, entorhinal, and retrosplenial cortices, hippocampus CA1, and PAG). The region-specific brain activations observed in this ketamine phMRI study may afford a method of confirming central activity and dose selection in early clinical trials for novel experimental therapeutics.

Introduction

In light of high attrition rates in drug development, there is an urgent need to improve the predictability of preclinical behavioral models to clinical outcome. Meeting this demand requires the discovery, validation, and implementation of biomarkers that improve the congruency of preclinical models to clinical reality and the establishment of proof-of-concept for efficacy and safety based on targeted mechanisms of action (Feuerstein et al., 2008; Day et al., 2009). Thus, it is essential to establish translational endpoints that allow the determination of whether the experimental therapeutic has the same pharmacological and physiological effects in a preclinical species as it does in healthy humans and ultimately in a clinical population (Day et al., 2008). In vivo translational imaging, such as positron emission tomography, single photon emission computed tomography, or pharmacological magnetic resonance imaging (phMRI), is one novel pathway to improve the translatability from animal models to clinical outcome (Borsook et al., 2006; Fox et al., 2009; Wong et al., 2009). In particular, phMRI allows the investigation of the effects of an experimental or approved therapeutic on functional endpoints [e.g., changes in blood oxygenation level dependence (BOLD) signals, regional cerebral blood volume (rCBV), and regional cerebral blood flow] and may allow a better understanding of the neurobiological mechanisms (i.e., physiology and pharmacology) underlying the drug effect (Borsook et al., 2006). For example, the glutamatergic hy-
pothesis of schizophrenia is supported by several lines of clinical evidence, including genetic, postmortem, and human psychosis modeling (Krystal et al., 1994; Malhotra et al., 1997). In fact, N-methyl-d-aspartate (NMDA) antagonists, such as phencyclidine (PCP) or ketamine, can induce symptoms that mimic psychosis in healthy volunteers and exacerbate symptoms in schizophrenic patients (Krystal et al., 1994; Lahti et al., 1995). Using in vivo imaging, Littlewood et al. (2006) demonstrated that ketamine increased BOLD signals in the frontal, hippocampal, cortical, and limbic areas with the largest activations observed in the retrosplenial cortex and hippocampus. In addition, it was found that pretreatment of glutamatergic agents seemed to globally modulate rCBV changes in rats challenged with PCP (Gozzi et al., 2008). It is intriguing that ketamine, which can induce psychomimetic symptoms or behavioral aberrations (Moghadam et al., 1997; Nishizawa et al., 2000; Becker and Greckosch, 2004), produced significant changes of brain activity in several cortical, hippocampal, and midbrain regions in animals and humans (Längsjo et al., 2003; Holcomb et al., 2005; Honey et al., 2005; Littlewood et al., 2006). A central translational perspective is to conduct the preclinical studies in a way that would allow the study in humans. However, a translational gap is the use of anesthesia in animal imaging studies. Several lines of evidence have shown that anesthesia can reduce functional responses and lead to discrepancy in observed activation patterns (Lahti et al., 1999; Austin et al., 2005; Chin et al., 2008a). Therefore, awake animal imaging is particularly attractive for investigating drug-induced brain activity, given that anesthetic-drug interactions probably confound the data interpretations and translatability of basic research to clinical investigations.

The main aim of the current study was to examine the brain activation elicited by the infusion of a subanesthetic dose of ketamine using BOLD phMRI in awake rats. Study 1 examined the brain activation elicited by the infusion of a subanesthetic dose of ketamine using BOLD phMRI in awake rats. Considering that positive results have been reported in a phase II schizophrenia trial with the mGluR2/3 agonist prodrug LY2140023 ([1R,4S,5S,6S]-2-thiabicyclo[3.1.0]hexane-4,6-dicarbonylic acid, 4-((2S)-2-amino-4-(methylthio)-1-oxobutyl)laminno, 2,2-dioxide monohydrate) (Patil et al., 2007), we added a secondary aim, to determine whether a behaviorally active metabotropic glutamate 2/3 receptor (mGluR2/3) agonist, (1S,2R,5R,6R)-2-amino-4-oxabicyclo[3.1.0]hexane-2,6-dicarbonylic acid (LY379268), could modulate the effects of ketamine-induced brain activation (Schoepf and Marek, 2002; Imre, 2007). Study 2 aimed to test whether the ketamine-induced changes in the BOLD signal could be affected by the administration of a commercially available mGluR2/3 agonist. To this end, we characterized the effect of LY379268 on ketamine-induced brain activation and hypothesized that LY379268 would modulate BOLD signal changes in specific regions of interest.

**Materials and Methods**

**Animals.** All studies were conducted in accordance with Institutional Animal Care and Use Committee guidelines at Abbott Laboratories and the National Institutes of Health Guide for Care and Use of Laboratory Animals guidelines. Adult male Sprague-Dawley rats (Charles River Breeding Laboratories, Portage, MI) were used for imaging experiments (n = 20; weight: ~300–350 g). Rats were group-housed in temperature-controlled (22°-24°C) rooms and maintained on a 12:12 light/dark cycle with lights on at 6:00 AM. Rat chow and water were provided ad libitum. Facilities at Abbott Laboratories are accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care.

**Drug Preparation and Administration.** LY379268 was purchased from Tocris Bioscience (Ellisville, MO) and dissolved in 5% hydroxypropyl methyl cellulose (Acros Organics, Fairlawn, NJ) in saline. Ketamine hydrochloride solution was purchased from Sigma-Aldrich (St. Louis, MO). Rats were divided into four different treatment groups (n = 5 per group): 1) intraperitoneal pretreatment of vehicle (5% hydroxypropyl methyl cellulose in saline) followed a ketamine challenge (30 mg/kg i.p.); 2) pretreatment of LY379268 (10 mg/kg i.p.) followed by a ketamine challenge (30 mg/kg i.p.); 3) pretreatment of LY379268 (10 mg/kg i.p.) followed by a saline challenge, and 4) challenge with LY379268 (10 mg/kg i.p.) without any pretreatment. The injection volume of LY379268 or vehicle was 2 ml/kg, whereas ketamine was given at 1 ml/kg. It is noteworthy that the choice of a subanesthetic dose of ketamine in the current study was based on previously published work (Ellison, 1995; Becker and Greckosch, 2004) and in-house exploratory findings. In a pilot study, we examined brain activation induced by different doses/routes of administration of ketamine, in which we clearly observed global negative BOLD signals in rats treated with a higher, or presumably anesthetic, dose of ketamine (not shown), whereas a dose of 30 mg/kg i.p. resulted in robust positive BOLD signals.

**Pharmacological MRI.** A method of reducing and controlling head motion artifacts when imaging awake rats has been characterized previously (Chin et al., 2008a) using a dedicated animal holder (Ekam Imaging Inc., Shrewsbury, MA), which includes a head repositioning system to minimize motion without restricting respiration. To alleviate the stress, each animal was acclimated to this dedicated animal holder for at least 5 min prior to imaging. Functional data. It is noteworthy that Lockhart et al. (1991) reported that the half-life of isoflurane in rabbit brain was approximately 8 min and the value could depend on the duration of anesthesia. For example, a half-life of 16 min was found in rabbits that have been exposed to isoflurane for 90 min (Strum et al., 1986), in contrast to the 30-min time period experienced in experiments by Lockhart et al. (1991). In our study, rats were under isoflurane during the habituation period. On the day of imaging, rats were again briefly anesthetized with isoflurane (5% induction, 3% maintenance in air) and placed into the imaging holder, and an intraperitoneal catheter (PE-160 tubing) was placed for the drug challenge. Rats were allowed to recover from the effects of isoflurane within the imaging holder in the magnet for approximately 20 to 25 min before collecting functional data. It is noteworthy that Lockhart et al. (1991) reported that the half-life of isoflurane in rabbit brain was approximately 8 min and the value could depend on the duration of anesthesia. For example, a half-life of 16 min was found in rabbits that have been exposed to isoflurane for 90 min (Strum et al., 1986), in contrast to the 30-min time period experienced in experiments by Lockhart et al. (1991). In our study, rats were under isoflurane during the preparation work (e.g., intraperitoneal line placement; positioning into the animal holder), which typically takes 15 min or less. Thus, based on the published isoflurane half-life data (Strum et al., 1986; Lockhart et al., 1991) and the current imaging protocol (Fig. 1), we assume that the effect of isoflurane during imaging is negligible. All imaging experiments were carried out on a 7T Bruker Biospec MRI scanner (Karlsruhe, Germany). The phMRI data were collected.
with eight-shot spin-echo EPI sequence with imaging parameters: repetition time/echo time = 3200/50 ms, in-plane resolution = 250 × 250 μm², slice thickness = 1.0 mm, and interslice spacing = 0.25 mm. There were 10 slices prescribed in the coronal plane, centered to cover the whole brain. The imaging protocol included 1) 10-min baseline acquisition, 2) intraperitoneal infusion of either vehicle or drug over 2 min (infusion rate = 0.4 ml/min), and 3) 30-min period of postdrug imaging acquisition (see Fig. 1). It is noteworthy that the pretreatment was typically given approximately 30 min before ketamine or saline challenge.

**Pharmacokinetic Study of LY379268.** To evaluate the exposure levels at the time when the maximum drug-induced BOLD signal changes were observed, a pharmacokinetic study was performed using a satellite group of animals (n = 5). Experiments were carried out to mimic the phMRI study by following the imaging protocol, in which rats were treated with LY379268 at 10 mg/kg i.p. Then, approximately 30 min after drug infusion, blood samples were obtained via cardiac puncture, and brains were then immediately collected and snap-frozen for pharmacokinetic analysis.

**Data Analysis.** Data analysis was performed using the AFNI software package (Cox, 1996) and in-house IDL (Research Systems, Boulder, CO) programs. We performed motion correction by coregistering time course data to the first baseline imaging dataset. To determine activated pixels, cross-correlation coefficients between the time-course raw data and a step function (off/on ≡ predrug baseline/postdrug period) were calculated on a pixel-by-pixel basis within the brain parenchyma for individual animals. Later, z-scores were derived from the calculated cross-correlation coefficients, whereas percentage BOLD signal changes were derived from mean signal intensity calculated from the baseline and postdrug periods. Specifically, the percentage BOLD signal changes were derived by the change in average signal intensity between the predrug infusion period (10-min time interval) and average signal intensity during the postdrug infusion period (30-min time period). In addition, baseline levels were not normalized across or within treatment groups. To perform group analysis, anatomical images obtained from individual animals were coregistered into a template image set by rigid body translations and rotations; these calculated transformation matrix parameters were then applied to the functional dataset and derived statistical maps. Regions-of-interest (ROI) analyses were performed to obtain regional BOLD signal changes, in which specific regions were outlined manually based on a rat brain atlas (Paxinos and Watson, 1998). Subsequently, regional mean BOLD signal changes (mean ± S.E.M.) were retrieved from various cortical and subcortical structures for each treatment group. Statistical significance between groups was determined using t test (JMP 7.0.1; SAS Institute, Cary, NC), with differences considered statistically significant at p < 0.05. In addition, group comparisons (unpaired t test) were conducted using AFNI software with a threshold p < 0.05, to determine brain regions affected by the ketamine challenge or pretreatment of LY379268. Finally, to illustrate the temporal dynamics of BOLD signal, representative time-course data for each treatment group were also extracted.

**Results**

Figure 2 shows the anatomical images and 16 brain regions used for ROI analyses. The selection of these brain regions was based on possible involvement of schizophrenia-like symptoms induced by ketamine or PCP, as reported in previous animal studies (Duncan et al., 1998; Gozzi et al., 2008). Table 1 lists regional BOLD signal changes (mean ± S.E.M.) calculated from each treatment group. The results indicated that, compared with saline-challenged rats, ketamine produced a significant increase in BOLD signals (p < 0.05) in brain region, such as the medial prefrontal, cingulate, retrosplenial, entorhinal, auditory, and visual cortices.
as well as CA1 region of the hippocampus, whereas negative BOLD signals were observed in the PAG ($p < 0.05$). A significant increase in BOLD signal was also found in the striatum, but not in nucleus accumbens, ventral tegmental area, or substantia nigra. It is noteworthy that these ROI-based data are consistent with group comparisons results in which brain regions showed a significant effect to the ketamine challenge (Fig. 3A). No significant or robust activation was observed in rats pretreated with LY379268 at 10 mg/kg i.p. then later challenged with saline (not shown). This observation is consistent with our previous saline data (Chin et al., 2008a) and indicate that pretreatment of LY379269 does not modulate the saline response. In addition, we found that pretreatment of LY379268 significantly attenuated the ket-

![Fig. 3. A and B, group comparisons (between groups, unpaired $t$ test) with a threshold of $p < 0.05$ and cluster size of 16 voxels for regions showing significant effects of ketamine versus saline on BOLD signal (A) and regions showing significant pretreatment effects of LY379269 versus vehicle on ketamine-induced BOLD signals (B). C, representative time course data extracted from the brain region located in the retrosplenial cortex for each treatment group. VEH, vehicle; Ket, ketamine; LY, LY379268; Sal, saline.](image-url)
amine-induced BOLD signals in a region-specific manner, including posterior cingulate, entorhinal, retrosplenial cortices, hippocampus CA1, and PAG (Fig. 3B and Table 1). Again, good agreement between data generated from ROI analyses (Table 1) and group comparisons was obtained. Finally, time-course data obtained from the retrosplenial cortex are shown in Fig. 3C. The results indicate that an increase in the BOLD signal occurring immediately after ketamine infusion can be abolished by the pretreatment of LY379268.

Our pharmacokinetic data obtained from the satellite animals indicate that the corresponding LY379268 plasma and brain exposure levels (mean ± S.E.M.) are 2.57 ± 0.25 μg/ml and 0.44 ± 0.09 μg/g, respectively. Considering the affinity of LY379268 for mGluR2/3 receptors (EC50 = 3–6 μM) (Imre, 2007), the drug exposure reached in the current study should be comparable with efficacious doses used in behavioral measures (i.e., ketamine-induced hyperactivity) (Lorrain et al., 2003).

**Discussion**

The main aim of the current study was to examine the brain activation elicited by the infusion of a subanesthetic dose of ketamine using BOLD phMRI in awake rats. Our data are the first to characterize the modulatory effects of ketamine across several brain regions in awake rodents. Strong activations were observed in the medial prefrontal, hippocampal, and cortico-limbic regions. No significant brain activations were observed from saline-challenged rats, which are consistent with our previous findings (Chin et al., 2008a). A secondary aim was to determine whether the mGluR2/3 receptor agonist LY379268 could modulate the effects of ketamine-induced brain activation at doses that are active in behavioral models. To this end, it was demonstrated that the pretreatment of LY379268 selectively modulated changes in the ketamine-induced BOLD signal, including the posterior cingulate, entorhinal, and retrosplenial cortices, CA1, and PAG. Such region-specific activity may indicate the utility of the ketamine model to be used as a translatable pharmacodynamic (PD) biomarker. Previously, Duncan et al. (1998) observed significant changes in uptake of [14C]2-deoxyglucose in specific brain regions in rats, including medial prefrontal cortex, retrosplenial cortex, hippocampus, nucleus accumbens, amygdala, and thalamic nuclei after ketamine challenge. Furthermore, in the same study they found [14C]2-deoxyglucose uptake can be normalized by the pretreatment of clozapine, but not haloperidol. The ketamine-induced BOLD signal changes we report in awake animals are, for several ROIs, in agreement with previous reports using similar imaging methods in anesthetized animals. Littlewood et al. (2006) have shown that ketamine produced positive BOLD signals in the frontal, hippocampal, cortical, and limbic areas with the largest activations observed in the retrosplenial cortex and hippocampus; more importantly, changes in BOLD signal seemed to correlate with the PD profile of the drug. However, in our study, we also found deactivations in the PAG that have not been reported in any previous ketamine phMRI studies in humans and animals. Moreover, the activations of several midbrain ROIs reported by Littlewood et al. were not seen in our study (e.g., activations in nucleus accumbens, amygdala; deactivations in the inferior colliculus). Several other phMRI studies also report a larger number of activated ROIs, albeit using PCP to modulate rCBV (Gozzi et al., 2008) or BOLD signal (Hackler et al., 2010). In these studies, activations of the thalamus, motor cortex, striatum, and nucleus accumbens were reported. To reconcile this discrepancy, in addition to awake imaging, the differences in imaging methods/parameters, doses/routes of administration, or imaging hardware used might collectively contribute to the limited midbrain activation in our results.

For example, in contrast to using rCBV measurements (Gozzi et al., 2008) or a fast low-angle shot MRI method (Hackler et al., 2010), we used an EPI protocol to collect BOLD data, which is the standard imaging method used in human fMRI studies.

One of the key findings in the current study is the region-specific effect of the mGluR2/3 agonist, LY379268, on ketamine-induced brain activations. It is noteworthy that Krystal et al. (2005) have shown that healthy subjects pretreated with an mGluR2/3 agonist show little evidence of memory impairment during ketamine infusion, whereas we observed that pretreatment of LY379268 attenuated ketamine-induced positive BOLD signals in brain regions associated with episodic memory function. Significant modulation of ketamine was seen in specific brain regions (see Table 1), which might afford testing translatability to healthy volunteers, assuming that the same ROIs would be affected. It has been demonstrated that biphenyl indanone-A (BINA), an mGluR2-positive allosteric potentiator, modulated PCP-induced BOLD signals across several ROIs, where hyperglutameric activities were blocked with the pretreatment of BINA in the prefrontal cortex, caudate and putamen, nucleus accumbens, retrosplenial, and motor cortices as well as the mediodorsal thalamus (Hackler et al., 2010). Further, it is known that PCP produces a noncompetitive blockade of NMDA Ca2+ channel, whereas ketamine is a less potent blocker of the same channel. Thus, a comparable effect of PCP and ketamine on brain activation is expected. In fact, it has been shown these drugs induced similar effects on behavioral outcome and neurotransmission (Ellison, 1995; Imre, 2007). Taken together, the use of such mGluR2-selective agents offers the potential to dissect the relative contributions of mGluR subtypes to neuronal modulation as assessed by NMDA antagonist challenge. However, the reconciliation of the studies between the positive allosteric modulator BINA and mGluR2/3 agonists are confounded by the use of anesthesia in those studies. Nonetheless, such data coupled with the positive clinical data (Patil et al., 2007) add to the growing body of evidence indicating the importance and potential utility of glutamatergic agents for treating schizophrenia (Marek, 2004) and the need for translatable biomarkers to accelerate discovery and development of these experimental therapeutics. Therefore, phMRI, using ketamine as a challenge, offers the potential utility to establish a faster clinical path by optimizing dose selection for mGluR2/3 agonism. However, such conclusions are beyond the scope of the current article; to establish such translatability would require the establishment of dose-response relationships in animals and humans to investigate the minimal effective dose, dose range, and specific regions of interest where the BOLD signal is modulated across species.

The current study essentially back-translates the ketamine-induced brain activation that has previously been
done in humans. In the human positron emission tomography study, ketamine elevated regional cerebral blood flow, with the largest effects in the anterior cingulate cortex (Längsjö et al., 2003; Holcomb et al., 2005). Likewise, positive BOLD signals in the frontal, midposterior cingulate, temporal, motor cortex, and hippocampus were observed in healthy subjects infused with ketamine (Deakin et al., 2008). In a human BOLD fMRI study using an episodic memory task, it was found that ketamine infusion potentiated frontal activation during the encoding phase, but attenuated hippocampal activation during the retrieval phase (Honey et al., 2005); therefore the effect of ketamine on BOLD signal changes seems to be region-specific, albeit these observations could be task-dependent (Abel et al., 2003). In our study, we also found cortical and hippocampal activation in awake rats, which agrees with regional specificity reported in the human study (Deakin et al., 2008). In addition, ketamine seemed to increase glutamate metabolism in the anterior cingulate cortex, as revealed via $^1$H-magnetic resonance spectroscopy study in healthy subjects (Rowland et al., 2005), and these findings are consistent with the proposition that NMDA receptor hypofunction may increase glutamate release and result in hypermetabolism in cortico-limbic regions. Thus, in view that both the anterior cingulate cortex and retrosplenial cortex are involved in various aspects of information processing; the results observed from these studies imply that brain activation in such regions may be reconciled with the underlying psychosis or memory deficits (Sharp et al., 1991). In fact, it has also been consistently demonstrated that systemic administration of subanesthetic doses of ketamine can induce psychotomimetic symptoms or behavioral aberrations in animals (Moghaddam et al., 1997; Nishizawa et al., 2000; Becker and Grecksch, 2004), which supports the validity and translatability of the ketamine model for schizophrenia. Nevertheless, the molecular insights on this hypoglutamatergic hypothesis remain elusive. For example, it was found that in the medial prefrontal cortex local infusion of ketamine does not elevate the extracellular glutamate concentration, whereas systemic administration results in increased extracellular glutamate, implying that the interplay between excitatory and inhibitory neurons is critical to understanding the response to systemically applied NMDA antagonists in vivo (Lorrain et al., 2003). Thus, the observation of increased extracellular glutamate levels after ketamine administration has been ascribed to its actions on GABAergic interneurons as well as glial cells (Olney et al., 1999).

In summary, we have demonstrated ketamine infusion can produce significant changes in BOLD signal in awake rats, and that the observed brain activation pattern is consistent with previous work (Littlewood et al., 2006; Deakin et al., 2008). Furthermore, we also found that pretreatment of LY379268 attenuated ketamine-induced BOLD signals in brain regions that are associated with episodic memory function. The regional specificity observed in the current study suggests that this ketamine awake rat model could be used as a translational biomarker for confirming central activity and dose selection in early clinical trials for novel experimental therapeutics, although the utility and translatability remain to be seen across species. This translational imaging approach may ultimately be translatable to human pMRI in early clinical studies of novel therapeutic compounds.

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Performed data analysis: Chin, Upadhyay, and Baker
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