Xanomeline Modulation of the Blood Oxygenation Level-Dependent Signal in Awake Rats: Development of Pharmacological Magnetic Resonance Imaging as a Translatable Pharmacodynamic Biomarker for Central Activity and Dose Selection

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ABSTRACT

In vivo translational imaging techniques, such as positron emission tomography and single-photon emission-computed tomography, are the only ways to adequately determine that a drug engages its target. Unfortunately, there are far more experimental mechanisms being tested in the clinic than there are radioligands, impeding the use of this risk-mitigating approach in modern drug discovery and development. Pharmacological magnetic resonance imaging (phMRI) offers an approach for developing new biomarkers with the potential to determine central activity and dose selection in animals and humans. Using phMRI, we characterized the effects of xanomeline on ketamine-induced activation on blood oxygen level-dependent (BOLD) signal. In the present studies, xanomeline alone dose-dependently increased the BOLD signal across several regions of interest, including association and motor and sensory cortical regions. It is noteworthy that xanomeline dose-dependently attenuated ketamine-induced brain activation patterns, effects that were antagonized by atropine. In conclusion, the muscarinic 1/4-preferring receptor agonist xanomeline suppressed the effects of the N-methyl-D-aspartate channel blocker ketamine in a number of brain regions, including the association cortex, motor cortex, and primary sensory cortices. The region-specific brain activation observed in this ketamine challenge phMRI study may provide a method of confirming central activity and dose selection for novel antipsychotic drugs in early clinical trials for schizophrenia, if the data obtained in animals can be recapitulated in humans.

Introduction

In light of the 90 to 95% failure rate in neuroscience drug development, there is an urgent need to improve the predictability of preclinical endpoints to clinical outcome (Day et al., 2008, 2009; Feuerstein et al., 2008a,b; De Smaele et al., 2010; Wehling 2011). There are significant unmet medical needs for most neurological and psychiatric disorders, and there is a great demand for improved therapeutics, because many patients still fail to experience significant symptomatic relief when treated for major depression, bipolar disorder, or schizophrenia (Day et al., 2011). Thus, in neuroscience drug discovery and development it is essential to establish translational endpoints that determine whether the experimental therapeutic engages its target receptor and exerts the same pharmacological and physiological effects in animals as it does in humans.

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ABBREVIATIONS: MRI, magnetic resonance imaging; phMRI, pharmacological MRI; BOLD, blood oxygenation level dependent; M1/5, muscarinic 1/5; ROI, regions of interest; NMDA, N-methyl-D-aspartate; mGluR, metabotropic glutamate receptor; CNS, central nervous system; PoC, proof of concept; Amy, amygdala; CA1/3, Cornu Ammonis 1/3 of hippocampus; NAcc, nucleus accumbens; PAG, periaqueductal gray; RSP, retrosplenial cortex; SN, substantia nigra; VTA, ventral tegmental area; LY379268, (1S,2R,5R,6R)-2-amino-4-oxabicyclo[3.1.0]hexane-2,6-dicarboxylic acid; LY354740, (1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid; LY2140023, ((1R,4S,5S,6S)-(1R,4S,5S,6S)-4-amino-2-sulfonylbicyclo[3.1.0]hexane-4,6-dicarboxylic acid.
a drug engages its target, because most neuroscience targets are in the nanomolar to femtomolar range. Unfortunately, most targets do not have a known radiotracer available, and when they are available they have been underused, leading to underdosing and overdosing of drugs in clinical trials (Feuerstein et al., 2008a,b). If such tools do not exist in time for the development of the drug, then it is paramount to find objective and strong evidence that they are centrally active, exerting the predicted biological consequences across animals and humans (Feuerstein, 2007; Feuerstein et al., 2008a,b; Day et al., 2009; Feuerstein and Chavez, 2009; Wehling, 2009).

Pharmacological magnetic resonance imaging (phMRI) offers the potential to improve the translatability from animal models to clinical outcome (Borsook et al., 2006; Fox et al., 2009; Sakoglu et al., 2011). Specifically, phMRI allows the determination of the effects of an experimental or approved therapeutic on functional endpoints (e.g., changes in blood oxygenation level-dependent (BOLD) signals, regional cerebral blood volume, and regional cerebral blood flow) to determine the central activity of the target and allow a better understanding of the neurobiological mechanisms (i.e., physiology and pharmacology) underlying the drug effect (Borsook et al., 2006). In some examples, a drug that can recapitulate disease “symptoms” in a healthy human can be used as a challenge model in phMRI (Chin et al., 2011).

Genetic, postmortem, and human psychosis modeling evidence demonstrate the importance of glutamate in the etiology of schizophrenia (Krystal et al., 1994, 2003; Malhotra et al., 1997). For example, N-methyl-D-aspartate (NMDA) antagonists such as phencyclidine or ketamine in healthy volunteers can produce many of the psychotic and dissociative symptoms observed in schizophrenia, and they can also exacerbate symptoms in schizophrenic patients (Lahti et al., 2001; Krystal et al., 2003). Several studies have also characterized the effects of ketamine on central activity in the CNS (Littlewood et al., 2006a,b; Deakin et al., 2008). In addition, it has been reported that the pretreatment of compounds that activate NMDA receptor via the glycine coagonist site such as d-serine or a glycine transporter inhibitor agent induced inhibition of the global regional cerebral blood volume changes/activation observed in rats challenged with phencyclidine (Gozzi et al., 2008).

A central translational principle is to conduct the preclinical studies in a way that would allow a congruent study in humans. 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; Liang et al., 2012). Therefore, we previously established awake-animal imaging as a feasible approach for developing a pharmacodynamic biomarker to determine central and meaningful biological activity in the brain (Chin et al., 2011). In those studies, ketamine produced positive BOLD signals in several cortical and hippocampal regions. Pretreatment of a mGluR2/3 agonist, (1S,2R,5R,6R)-2-amino-4-oxabicyclo[3.1.0]hexane-2,6-dicarboxylic acid (LY379268), significantly attenuated ketamine-induced brain activation in a region-specific manner. The region-specific brain activations, like those observed with the mGluR2/3 agonist, may provide a method of confirming central activity and lead to a more accurate dose selection in early clinical trials for novel experimental therapeutics.

The main aim of the current study was to use phMRI to develop a pharmacodynamic biomarker that would accelerate compound selection and drug development of muscarinic targets (e.g., M1/M4). In the current studies we used a ketamine challenge. The 30-mg/kg dose was selected based on previously published data characterizing subanesthetic doses of ketamine on locomotor activity, a dose that also produced BOLD signal increases in the frontal, hippocampal, cortical, and limbic areas (Littlewood et al., 2006a,b). In experiment 1, we replicated our previous results on the brain activation elicited by the infusion of a subanesthetic dose of ketamine as assessed using BOLD phMRI in awake rats. To better understand the role of xanomeline on the BOLD signal, experiment 2 determined the effect of behaviorally inactive and active doses of xanomeline (1, 5, and 10 mg/kg i.p.) (Shannon et al., 2000) across several regions of interest (ROI). In experiment 3 we tested whether the ketamine-induced changes in the BOLD signal could be affected by the pretreatment of xanomeline. This study aimed to characterize the influence of muscarinic modulation on the impaired NMDA receptor function by the administration of ketamine. In experiment 4, we used the competitive muscarinic antagonist atropine to determine whether the effects of xanomeline alone on the BOLD signal were driven by the activation of M1/M4 receptors. Finally, in experiment 5, we used atropine to determine whether the xanomeline effects on ketamine-induced changes in the BOLD signal were driven by muscarinic receptor agonism properties.

Materials and Methods

Animals. All studies were conducted in accordance with the Institutional Animal Care and Use Committee guidelines at Abbott Laboratories and the National Institutes of Health’s Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animals. Adult male Sprague-Dawley rats (350–425 g) (Charles River Breeding Laboratories and the National Institutes of Health’s Guide for Care and Use of Laboratory Animals) housed in temperature-controlled (22o-24°C) rooms and maintained under 12-h light/dark cycles with lights on at 6:00 AM. Rats were singly housed in temperature-controlled (22-24°C) rooms and maintained on a 12-h light/dark cycle with lights on at 6:00 AM. Light anesthesia (3% isoflurane) (Baxter, McGaw Park, IL) was administered during a phMRI scan for 10-min, 30-min, and 60-min periods on nonconsecutive days over a 10-day span. The restrained rats were placed in acoustic behavioral chambers with a recording of active MRI acquisition scans playing. Light anesthesia (3% isoflurane) (Baxter, McGaw Park, IL) was administered for 3 to 5 min required to secure animals in the restrainer and maintain the animals at the end of the acclimation exposure.

Drug Preparation and Administration. Xanomeline and atropine were purchased from Sigma-Aldrich (St. Louis, MO) and prepared in a 0.9% saline vehicle to a final volume of 2 ml/kg. Ketamine was purchased from Fort Dodge Laboratories (Fort Dodge, IA) and diluted down from an original concentration of 100 mg/ml to 30 mg/ml by using a 0.9% saline solution. During pretreatment regimen, the compound or vehicle was administered intraperitoneally by using a 1-ml syringe tipped with a 24-g ½-inch needle. During ketamine challenge or vehicle administration, the drug or vehicle was injected first into a length of PE160 tubing that was connected to an intraperitoneal catheter (BD Insyte-W 24 GA 0.75” 0.7 x 19 mm; BD Biosciences, San Jose, CA). The tubing containing the challenge was
in turn connected to a medical infusion pump (Medfusion 2001; Medex Inc., Fort Lauderdale, FL) set at a rate of 0.4 ml/min.

**Awake phMRI.** After the final acclimation exposure animals were given a 48-h recovery period before beginning phMRI acquisition. Animals were secured in the restrainer as described previously. phMRI imaging data were acquired in a 7T Bruker Biospec (Karlsruhe, Germany) MRI magnet. The phMRI data were collected with eight-shot spin-echo echo planar imaging sequence with the following 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 16 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). A period of no less than 96 h was allotted between scans. The dosing and scanning timeline for the study is illustrated in Fig. 1. Animals received either one or two pretreatments depending on the given experiment that used a combination of compound and/or vehicle. In single-pretreatment instances compound was delivered intraperitoneally during the process of loading the animal into the MRI restrainer. The time required to load the animal into the magnet and run through the positioning and set-up procedures before beginning acquisition was 20 min. Total scan time for a single experiment was 42 min. Baseline acquisition was 10 min followed by a drug infusion of 2 min, leaving 30 min for postchallenge data acquisition.

**Data Analysis.** Data analysis was performed by using the Analysis of Functional Neuroimage software package (AFNI) (Cox 1996) and the in-house IDL program (Research Systems, Inc., Boulder, CO). To determine activated pixels, cross-correlation coefficients between the time-course raw data and a step function (off/on phase) were calculated on a pixel-by-pixel basis within the brain parenchyma region for individual animals. Later, z-scores were derived from the calculated cross-correlation coefficients, and the percentage of BOLD signal changes was derived from mean signal intensity calculated from the baseline and post-drug periods. The anatomical images 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. ROI analyses were performed to obtain regional BOLD signal changes, in which specific regions were extracted automatically (InVivoScope multimodality imaging and processing suite; http://www.invivoscope.com/) using a registered brain atlas created in-house by using an anatomical reference guide (Paxinos and Watson, 1998) (Fig. 1B). This allowed for an analysis of 54 cortical and subcortical regions (assuming full brain coverage) for each scan. Subsequently, regional mean BOLD signal changes (mean ± S.E.M.) were calculated for each animal as a percentage change by using an ideal ramp step function corresponding with the time of the phMRI challenge. Whereas areas on the average maps that show (de)activation were thresholded to a significance of p < 0.05, the graphical percentage changes were created from numbers generated by using the same data set after running the InVivoScope analysis. This program uses an automated brain region-masking algorithm to create a linear fit of the phMRI signal for each region, resulting in a percentage change by region without thresholding. The anatomical mask was created in-house and was inspected after each time an analysis was run to make sure the overlay was anatomically correct.

By examining the whole-brain BOLD changes with the average maps and a regional approach with InVivoScope, we combined the best features of the whole-brain and regional analyses. Confidence in “real” activation on whole-brain average maps was realized by both averaging the data sets of individual scans per group together and thresholding, both of which reduce the likelihood of random effects on BOLD changes (false positives). Second, confidence in the regional graphical data was increased because of objective computer masking, which can be subjectively verified after an analysis is complete for masking accuracy and individual data per region can be analyzed.

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**Fig. 1.** A, a typical phMRI timeline for the present studies. Pretreatment could either be vehicle or compound depending on the study. For single-pretreatment studies, the compound was injected 20 min before the beginning of the phMRI data acquisition and 30 min before ketamine challenge. For double-pretreatment studies, pretreatments were given 50 and 20 min before data acquisition and 60 and 30 min before the ketamine challenge. B, representative images of InVivoScope-generated masks of a phMRI scan. Individual masks were generated for every slice in a scan series. Each color represents a specific CNS region. The BOLD signal was in turn analyzed by region in accordance with user-specified parameters. Individual sub-cortical regions identified here are the relevant regions of interest in the present study. The real image with BOLD average map at the top is illustrative and was not generated by InVivoScope software.
across a groups population for statistical outliers and remaining data compared as needed between dosing groups.

Statistical significance between groups was determined by using t test (Prism version 5.04; GraphPad Software Inc., San Diego, CA), with differences considered statistically significant at \( p < 0.05 \). Statistical significance of compound alone was determined by linear regression analysis of the BOLD signal for given ROI over time as generated by InVivoScope (Prism version 5.04).

**Results**

**Ketamine-Induced BOLD Signal as a Pharmacodynamic Biomarker.** A previously established in-house pharmacodynamic biomarker model was further confirmed wherein a subanesthetic dose of ketamine (30 mg/kg i.p.) produced region-specific, statistically significant changes in BOLD signaling in awake unanesthetized rats (Chin et al., 2011). This was observed in both the AFNI average maps and region-specific percentage change by means best-fit analysis (Fig. 2). Region-specific linear regression analysis of the entire scan showed significant increases in BOLD signaling in the cortex, anterior cingulate cortex, retrosplenial cortex (RSP), primary motor cortex, primary visual cortex, primary somatosensory cortex, and hippocampus. In contrast, significant decreases in BOLD signaling were observed in the hypothalamus, periaqueductal gray (PAG), substantia nigra (SN), and cerebellum (Fig. 2B). These activated regions were similar to our previous ketamine pharmacodynamic biomarker analysis (Chin et al., 2011). Unlike our previous analysis methodology requiring tedious hand drawing of ROI, the present study automated the process via a new cortical extraction and mapping software tool (see Materials and Methods).

The awake-rat ketamine phMRI model has been previously characterized in-house (Chin et al., 2011). The 30 mg/kg dose of ketamine was chosen because higher doses are behaviorally sedative and potentially neurotoxic and lower doses do not show a significant activation window. Because ketamine intraperitoneally in the rat has a fast onset of action and a half-life of 2 h we are confident that the ketamine pharmacology is consistent with the 30-min postdosing phMRI data acquisition period.

**Xanomeline Challenge.** The effects of xanomeline challenge (1, 5, and 10 mg/kg i.p.) to vehicle (0.9% saline) pretreatment in BOLD signaling in awake unanesthetized rats are illustrated in Fig. 3. AFNI average maps and region-specific percentage changes, by means best-fit analysis, showed significant increases in BOLD signaling at 5 and 10 mg/kg across a number of cortical slices (Fig. 3A). Region-
specific linear regression analysis of the entire scan showed significant changes in the percentage of BOLD signal change in the cortex, cingulate cortex, RSP, hypothalamus, thalamus, primary motor, visual and somatosensory cortices, Cornu Ammonis 1/3 of hippocampus (CA1/3), amygdale (Amy), striatum, substantia nigra, ventral tegmental area (VTA), and cerebellum (Fig. 3B).

**Xanomeline Pretreatment to Ketamine Challenge.**
The effects of xanomeline (1, 5, and 10 mg/kg i.p.) pretreatment with ketamine (30 mg/kg i.p.) challenge to BOLD signaling in awake unanesthetized rats are illustrated in Fig. 4. Xanomeline pretreatment dose-dependently blocked ketamine-induced changes in BOLD signaling in a number of specific ROI (anterior cingulate cortex, RSP, cortex, primary motor cortex, primary somatosensory cortex, primary visual cortex, and PAG) as illustrated in both the AFNI average maps and BOLD percentage change by means analysis (Fig. 4).

**Atropine Pretreatment to Xanomeline Challenge.**
The effects of atropine (10 mg/kg i.p.; 30 min) pretreatment with xanomeline (10 mg/kg i.p.) challenge to BOLD signaling in awake unanesthetized rats are illustrated in Fig. 5. Atropine pretreatment in a single-dose study blocked xanomeline-induced changes in BOLD signaling in a number of spe-
specific ROI (anterior cingulate cortex, RSP, motor cortex, somatosensory cortex, visual cortex, hippocampal CA1 and CA3 fields, cerebellum, and thalamus) as illustrated in both the AFNI average maps (Fig. 5, A and C) and BOLD percentage change by means analysis (Fig. 5D). Atropine alone caused a decrease in BOLD signaling in a number of ROI, including the primary visual and motor cortices, VTA, and cerebellum, as illustrated in the AFNI average maps as well as the percentage of BOLD change by means analysis (Fig. 5, B and D).

**Atropine and Xanomeline Pretreatment to Ketamine Challenge.** The effects of atropine (10 mg/kg i.p.) pretreatment (50 min) on xanomeline effect (30 min; 10 mg/kg i.p.) after ketamine (30 mg/kg i.p.) challenge to BOLD signaling in awake unanesthetized rats are illustrated in Fig. 6. Atropine (10 mg/kg) pretreatment in a single-dose study blocked xanomeline (10 mg/kg) attenuation of changes in BOLD signaling after ketamine challenge in a number of specific ROI (primary motor and primary somatosensory cortices) as illustrated in both the AFNI average maps (Fig. 6, A-C) and BOLD percentage change by means analysis (Fig. 6D).

**Discussion**

The current article is the first to report that xanomeline, a putative antipsychotic muscarinic receptor agonist, dose-dependently suppressed the effects of ketamine on the BOLD signal. Moreover, those effects were attenuated with the muscarinic receptor antagonist atropine, providing strong evidence that the effects were driven by the activation of muscarinic receptors. It is noteworthy that xanomeline alone dose-dependently increased the BOLD signal across several specific regions of interest.
regions of interest including association, motor, and sensory cortical regions. A plausible explanation for these seemingly contradictory results can be readily supported by the existing literature. For example, the “xanomeline alone” effect on the BOLD signal can be hypothesized as being driven by direct agonism of the M1/M4 receptors. In contrast, presynaptic modulation of glutamate/NMDA neurotransmission might account for the blockade of the ketamine-modulated BOLD signal.

In our study, xanomeline alone exerted dose-dependent effects on the BOLD signal in brain regions known to have a high density of M1 receptors, implying a direct effect on the receptor activation. Indeed, postmortem schizophrenic patient studies have revealed significant decrease in the density of M1 and M4 receptors in cortical areas and hippocampus (Crook et al., 2000; Dean et al., 2002; Scarr and Dean, 2008). Although it is feasible that both M1 and M4 contribute to the changes in the BOLD activation (experiment 2), the current weight of evidence stemming from RNA and protein expression (Krejci and Tucek, 2002; Volpicelli and Levey, 2004; Tayebati et al., 2006) and electrophysiology experiments (Haj-Dahmane and Andrade, 1996, 1997, 1998) support a dominant role for M1, over M4, in the cortex. Indeed, excitatory cholinergic effects were mostly absent when recording from prefrontal cortical neurons from mice lacking M1 receptors, compared with generally robust cholinergic responses in neurons lacking M3, M5, or both M3/M5 receptor subtypes (Marino et al., 1998; Gulledge et al., 2009). The M4 receptor is localized mainly in dopamine-rich brain areas such as the mesolimbic and nigrostriatal dopaminergic pathways. Studies of M4 knockout mice revealed a hyperactive dopaminergic system with increased levels of dopamine in the nucleus accumbens (NAcc), increased sensitivity to pre-pulse inhibition disruption by pharmacological agents, and increased sensitivity to dopamine release by psychostimulants (Wess et al., 2007). Although these data support the notion that the excitatory effects of xanomeline alone under basal conditions are likely to be dominated, if not exclusive, to M1 activation, they do not prove it. More work is required to dissect the role of M1 and M4 on the BOLD signal (see Potential Study Limitations).

Presynaptic M1 and M4 receptors may mediate the suppressant xanomeline effects on ketamine-induced cortical activation. For example, a converging body of evidence suggests that ketamine increases the activity of cerebral cortical circuits, resulting in enhanced glutamatergic activity throughout the cortical mantle. In contrast, neocortical M1 and M4 receptors are localized on glutamatergic neurons where their

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**Fig. 6.** A and B, average maps of BOLD signal; baseline versus ketamine (A; 30 mg/kg i.p.) and xanomeline (B; 10 mg/kg i.p.) challenges showing statistically significant changes in 6/16 representative slices (unpaired t test, p < 0.05). C, average map of BOLD signal; baseline versus ketamine challenge (30 mg/kg i.p.) with atropine (10 mg/kg i.p.) and xanomeline (10 mg/kg i.p.) pretreatments showing statistically significant changes in 6/16 representative slices (unpaired t test, p < 0.05). D, InVivoScope-automated extraction of select regions of interest showing percentage of change by means using a nonthreshold ideal step-function analysis of the BOLD signal (same data as A-C). Error bars show mean ± S.E.M. (n = 3–5/group). @@, p < 0.01; @, p < 0.05 versus atropine.
activation can decrease glutamate release (Amar et al., 2010), thus providing a potential explanation of how xanomeline may decrease ketamine-induced changes in the BOLD signal. Amar et al.’s experiments, performed using an in vitro slice methodology, confirmed previous electrophysiological studies where cultured neurons grown from the visual cortex where M4 receptor activation suppressed excitatory postsynaptic potentials and M1 receptor activation suppressed inhibitory postsynaptic potentials (Kimura and Baughman, 1997). Thus, activation of both receptors could attenuate external excitatory drive (Rouse and Levey, 1997; Rouse et al., 1999). Furthermore, activation of M1 receptors in pyramidal cells enhances NMDA-mediated responses and long-term potentiation processes (Marino et al., 1998; Fernández and Buño, 2010), suggesting that modulation of excitatory transmission might also account for this effect.

Implications of phMRI for Translational Approaches.

There is a clear immediate need for translational approaches to decrease the 90% failure rate in CNS clinical trials. For neuroscience targets, phMRI can be a powerful translational tool affording a window into the drug action on discrete regions of interest and disease-relevant circuits in the actual brain. Although no CNS-based biomarkers have been validated to the extent that they can predict efficacy, the use of such biomarkers can increase the probability of success by ensuring that the clinical PoCs are real shots on goal (confirming central and disease-relevant activity) and enrich our understanding of the drug effects in the entire CNS. That is, they can add real value to the company. The uptake of phMRI in research and development has gathered some momentum in recent years as reflected by some elegant, powerful, and thought-provoking work that has been conducted on a variety of targets. Moreover, many of these studies conducted early in the discovery process have afforded enriched translational approaches on disease-relevant central effects of existing and novel experimental therapeutics such as dopamine 3 receptors (D3), 5-hydroxytryptamine 2c, glycine transporter inhibitors, nicotinics, cannabinoid receptor 2/1, and mGluR2/3 (Schwarz et al., 2004, 2007; Chin et al., 2008, 2011; Gozzi et al., 2008; Beaver et al., 2011; McKie et al., 2011).

There are threats, real or otherwise, to the uptake of phMRI in a clinical setting. For example, there is a perception that phMRI is an expensive and complicated way of determining CNS activity, especially when contrasted with scopolamine or ketamine behavioral challenge models. A phase 1 phMRI study, to determine central activity over multiple regions of the brain, only needs to be run at a single site because the study aims are simple: 1) does my drug get into the human brain and recapitulate the regional and biologically meaningful effects that were observed in the animal models; and 2) is the effect dose-dependent? The typical cost for such a study is approximately $400,000 to 600,000, and it can be completed within 6 months of starting the study. Moreover, although a reversal of a behavioral challenge (e.g., scopolamine) in animals and humans clearly adds value to a program, all that is learned is whether the drug reversed the effect or not. Failure to reverse a behavioral challenge does not prove that the drug was not centrally active. A failure in this example, scopolamine challenge, leaves the fate of the program in limbo because little was learned about the drug. In contrast, imaging will afford the clinical team information on the drug effects throughout the brain, perhaps leading to additional alternative indications. As such, for CNS indications, knowing what the drug is doing in the entire CNS seems intuitive. However, our perspective is that biomarkers are most powerful when used in combination. Demonstrating that when a target is engaged it produces a series of biological effects that can be demonstrated with, for example, phMRI, cerebrospinal fluid, and electroencephalogram, provides powerful objective data to empower the clinical team.

In our development of phMRI as a potential biomarker, we used a simple strategy: to characterize mechanisms that have achieved recent positive proof of concept (PoC) in clinical trials. The use of subanesthetic doses of ketamine represents a valuable tool for approaching the model of NMDA hypofunction associated with schizophrenia (Krystal et al., 2002). Ketamine binds noncompetitively to the NMDA receptor and is able to exacerbate psychotic episodes in schizophrenic patients, producing symptoms similar to those observed in schizophrenia in healthy volunteers (Krystal et al., 1994, 2005; Lahti et al., 2001). First, we essentially back-translated the human ketamine-induced brain activation studies with respect to both anatomical and pharmacological validation (Deakin et al., 2008; Chin et al., 2011) in awake animals. We demonstrated that mGlu2/3 receptor agonism (LY379268) blocked the effect of ketamine-induced changes in the BOLD signal of awake rats (Chin et al., 2011). Our phMRI data are intriguing data because we observed effects across several regions of the brain implicated in different forms of information processing. For example, LY379268 decreased the ketamine-induced BOLD response in the posterior cingulate, entorhinal/retrosplenial cortices, and the hippocampus CA1 region. It is seductive to note that in a human study (LY3S79268)-2-aminobicyclo[3.1.0]-hexane-2,6-dicarboxylic acid (LY354740) produced a significant dose-related improvement in working memory during ketamine infusion (Krystal et al., 2005). It is noteworthy that(+)-(1R,4S,5S,6S)-2-amino-2-sulfonyl-bicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY2140023), a mGluR2/3 agonist, yielded positive olanzapine-like efficacy in a phase II PoC (Patil et al., 2007).

In this article, we provide additional support for the potential role of awake-animal ketamine phMRI as a pharmacodynamic translational imaging biomarker. In this study, we used behaviorally effective doses of xanomeline, based on previously published preclinical studies reporting antipsychotic-like profile for xanomeline (Shannon et al., 2000; Stanhope et al., 2001). Thus, the effect of xanomeline at 10 mg/kg attenuating ketamine-induced BOLD phMRI signal is consistent with efficacy in pharmacological/behavioral studies in rodents. Alone, xanomeline dose-dependently increased in the regions associated with the midbrain and cortical and medial temporal lobe (see Fig. 3), whereas pretreatment of xanomeline seemed to attenuate all of the ketamine-induced BOLD signal changes. It is interesting that xanomeline improved the behavioral outcomes measured by the Clinical Global Impression, the Brief Psychiatric Rating Scale, and Positive and Negative Syndrome Scale and improved cognition, albeit in a schizophrenia PoC pilot study (Shekhar et al., 2008). Furthermore, a larger, 6-month, randomized, double-blind, placebo-controlled clinical study with xanomeline in patients with Alzheimer’s disease revealed a significant treatment effect in the Alzheimer Disease Assessment Scale

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Cognitive scale as well as significant improvement across behavioral symptoms such as delusions, agitation, and hallucinations (Bodick et al., 1997a,b).

**Potential Study Limitations.** Although we were able to demonstrate that atropine was able to block the effects of xanomeline, we cannot rule out the possibility that the effects, seen in our phMRI studies, could be driven by M2, M3, or M5 receptors. The phMRI data in the present experiments may provide strong evidence for xanomeline CNS activity. However, because of the poor side effect profile of xanomeline, several companies are trying to develop selective M1 or M4 agents. It is important, therefore, to establish whether M1 and/or M4 receptors are the major drivers of the CNS activity we have reported. An optimal way to determine the effects of muscarinic receptor subtypes would be to use M1 and M4 receptor knockout mice. Future experiments should use this technology to dissect the relative contribution of the M1 and M4 receptors on xanomeline-induced modulation of the BOLD signal. In our study, we used atropine to determine that xanomeline effects were driven by muscarinic receptor activation. However, because our focus was to characterize the effect of xanomeline by using an established methodology of dosing and imaging parameters, atropine was used only as a way of gaining an extra degree of confidence that our effects were caused by the muscarinic effects. After characterizing a single dose of atropine alone (10 mg/kg; see Fig. 5B) we demonstrated that atropine blocked the effects of xanomeline. However, we cannot rule out that atropine may inhibit nicotinic acetylcholine receptors. Another potential caveat to our study is the repeated exposure to isoflurane. In our studies, animals were imaged while they were conscious. However, getting the animal into a restrainer before imaging can induce stress on the animal. In our studies, we lightly anesthetized the animals before placing them into the head restraints. The effect of isoflurane anesthesia during animal preparation on later phMRI imaging was evaluated and ruled out as a confounding factor in our previous awake-animal phMRI study (Chin et al., 2011). Previously, Lockhart et al. (1991) reported that the half-life of isoflurane in rabbit brain was approximately 8 min; however, it was also concluded that this 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 used in Lockhart et al.'s experiments. In our study, rats were under isoflurane during the preparation work (e.g., intraperitoneal line placement; positioning into the animal holder), which typically took 15 min. Thus, in light of previous work (Strum et al., 1986; Lockhart et al., 1991) and our current imaging protocols (where rats were off isoflurane at least 20–25 min before functional MRI data collection), the effect of isoflurane during imaging should be negligible.

In conclusion, the M1/M4 receptor-prefering agonist xanomeline suppressed the effects of a NMDA channel blocker, ketamine, in a number of brain regions, including the association cortex, motor cortex, and primary sensory cortices. Those data suggest that phMRI could be a powerful pharmacodynamic biomarker for the determination of central biological activity and dose selection, if the data can be recapitulated in humans.

**Authorship Contributions**

Participated in research design: Baker, Chin, Fox, Marek, and Day.

Conducted experiments: Baker and Chin.

Performed data analysis: Baker.

Wrote or contributed to the writing of the manuscript: Baker, Basso, Fox, Marek, and Day.

**References**


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