In search of the mechanisms of ketamine’s antidepressant effects: How robust is the evidence behind the mTor activation hypothesis [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract
Extensive evidence on rapid and long-lasting antidepressant effects of intravenous ketamine motivated efforts to identify underlying mechanisms that would enable development of novel drugs with similar efficacy, but improved safety and pharmacokinetic profiles. It has been suggested that the antidepressant-like action of ketamine may be mediated by the activation of mTOR-dependent intracellular cascades. Therefore, without any coordination or pre-existing agreement, research labs at AbbVie, Servier, Pfizer and Alkermes started independent experiments aiming to reproduce and extend published evidence. More than a dozen experiments conducted by these four independent teams failed to detect robust effects of ketamine on markers reported to be affected in the original study by Li et al. (2010). Thus, detection of the effects of ketamine on mTOR seem to require special conditions that are difficult to identify and establish, at least in some labs. Present results emphasize the importance of publishing detailed methods either within the paper or as supplementary material. This information is essential for follow-up studies that any significant research is likely to trigger. Further, our efforts to identify individual labs that tried to establish ketamine’s effects on mTOR highlight the need for a peer-to-peer mechanism of information exchange such as the one being developed by the ECNP Preclinical Data Forum.

Keywords
ketamine, depression, mTOR, data robustness, data sharing
This article is included in the Preclinical Reproducibility and Robustness gateway.

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Competing interests: No other interests beyond employment indicated in this manuscript. MS was a Servier employee at the time when studies were conducted.

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Introduction
Background: Ketamine and search for novel antidepressants
Intravenous ketamine has been shown to induce a rapid and long-lasting antidepressant effect in treatment-resistant patients (Zarate et al., 2006a) and the results have been replicated by several groups (Aan Het Rot et al., 2012). Intravenous route of administration as well as concerns due to psychotomimetic potential of ketamine have triggered a search for alternative medications with improved safety and pharmacokinetic profiles. Ketamine is usually described in the literature as an antagonist acting at N-methyl-d-aspartate (NMDA) subtype of glutamate receptors, and pilot clinical data indicated that its antidepressant effects may be shared at least to some extent by other drugs from this class (e.g. CP 101,606; Preskorn et al., 2008). However, non-competitive NMDA receptor antagonists appear to lack ketamine’s efficacy at least at the doses free from psychotomimetic effects (memantine: Zarate et al., 2006b; AZD-6765: Sanacora et al., 2014). These controversial findings have called for a deeper understanding of specific biological mechanisms of ketamine’s action.

Seminal discovery: Ketamine-induced activation of mTOR pathway
Li et al. (2010) presented a set of data indicating that, in rats, antidepressant-like action of ketamine may be mediated by the activation of mTOR-dependent intracellular cascades. The phosphatidylinositol 3-kinase (PI3K)–Akt–mTOR pathway responds to a variety of growth factors and mitogenic signals and, when activated, mTOR has multiple functions including facilitated translation of proteins involved in synaptic plasticity and memory. In the study by Li et al. (2010), acute injection of ketamine activated the mTOR pathway, leading to increased synaptic signaling proteins and increased number and function of new spine synapses in the prefrontal cortex of rats. Therefore, assuming that something similar can occur in humans, these data may indeed explain why acute infusion of ketamine produces such long-lasting effects in patients with major depression.

Robustness of ketamine effects on mTOR as the triggering factor for follow-up studies
As these results were reproduced by the same group (Liu et al., 2013) as well as by other academic groups (Yang et al., 2013), ketamine-induced mTOR activation seemed to be a robust finding worth further exploration. These effects were observed under a variety of experimental conditions (e.g. using fresh and frozen tissue; Li et al., 2010; Paul et al., 2014) and appeared to be quite robust (note low sample sizes in some of the studies: n=3 in Paul et al., 2014; n=4 in Li et al., 2010).

Therefore, without any coordination or pre-existing agreement, research labs at AbbVie, Servier, Pfizer and Alkermes started independent experiments aiming to reproduce and extend published evidence.

Materials and methods
Methods at AbbVie
Animals. Male Sprague-Dawley rats (150–250 g, Charles River, Germany) were pair-housed, had access to food and water ad libitum and were maintained on a 12-h light/dark cycle in standard cages. Experimental procedures were approved by AbbVie’s Animal Welfare Office (Ludwigshafen, Germany) and were performed in accordance with the European and German national guidelines as well as the recommendations and policies of the U.S. National Institutes of Health “Principles of Laboratory Animal Care”. Animal housing and experiments were conducted in facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Drug administration and harvesting of tissue. Ketamine was purchased either as a 10% solution (WDT, Garbsen, Germany) or as a powder from Sigma-Aldrich (Cat. No.: K2753) and prepared according to the Ketaset® solution (100 mg/mL ketamine and 0.1 mg/mL benzethonium chloride as a preservative in AMPUWA water [ Fresenius Cat. No.: 1080153] at a slightly acid solution [pH=3.5 to 5.5]). The animals were given different ketamine concentrations intraperitoneal (i.p.) either one hour or three hours before being killed or different ketamine concentrations intravenously three hours before being killed. Thirty minutes after ketamine administration some animals underwent a forced swim test. Animals were either killed with an overdose of isoflurane or with a guillotine without anesthesia. The prefrontal cortex, cerebral cortex and/or the hippocampus were dissected from the brain on ice. The brain samples were immediately frozen and stored at -80°C for further analysis.

Preparation of synaptosomal fraction and Western blotting. The brain samples were kept on ice during all stages of the preparation. The tissue was homogenized in 8μl preparation buffer per mg tissue. The preparation buffer contained 10 mM Tris-HCl, 0.32 M sucrose, protease inhibitor complete tablets mini with EDTA (Roche Cat. No.: 04693124001) and phosphatase inhibitor cocktail III (according to the Calbiochem mixture: 10 mM NaF; 0.2 mM Sodium Orthovanadate, 2 mM Sodium Pyrophosphate decahydtrate, 2 mM Glycerophosphate). The brain samples were homogenized with a Teflon-glass tissue grinder (pre-cooled, clearance 0.25 mm) with 10 even strokes (one stroke equals one up and one down action; the first stroke was about 5 s and subsequent strokes around 3–4 s) using a motor-driven pestle at 650 rpm. The homogenate was centrifuged 5 min at 1000 × g and contained a pellet (P1), which was discarded and the supernatant (S1).

For the crude synaptosomes the supernatant (S1) was centrifuged for 30 minutes at 15,000 × g. The resulting pellet was resuspended in ~20μl preparation buffer. The protein concentration was determined by the BCA protein assay according to the manufacturer’s instructions (Thermo Scientific Cat. No.: 23227).

For the synaptosomal fraction of the Percoll method the supernatant (S1) was transferred to a discontinuous Percoll-Gradient containing layers (2%, 6% and 23% Percoll [Sigma-Aldrich Cat.No.: 77237-500ml] in preparation buffer) and centrifuged for 5 min at 33000 × g. The layer between 6% and 23% Percoll (synaptosomal fraction) was collected and diluted with preparation buffer at least 4 times the collected volume and centrifuged for 10 min at 33000 × g. The resulting pellet (P2) contained the synaptosomal fraction and was resuspended in preparation buffer. The protein concentration was determined by the BCA protein assay according to the manufacturer’s instructions (Thermo Scientific Cat.No.: 23227).

For Western blotting, equal amounts of protein (24 μg) for each sample were boiled in an E-PAGE™ loading buffer (Invitrogen Cat.No.: EPBUF-01)/NuPAGE sample reducing agent (Invitrogen Cat.No.: NP0009) for 5 minutes, cooled down and
Table 1. Antibodies used in the studies.

<table>
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<th>Antibody</th>
<th>Company</th>
<th>Catalog number</th>
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<th>Dilution</th>
<th>Detection method</th>
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WB: Western blot analysis; CE: Capillary electrophoresis; *Antibody dilution optimized for ProteinSimple WES capillary electrophoresis system
Methods at Pfizer

Animals. Male Sprague-Dawley rats (150–200 g, Charles River, Wilmington, MA, USA) were pair-housed and allowed to acclimate for three days before handling. Animals had access to food and water ad libitum and were maintained on a 12-h light/dark cycle in standard cages. All procedures related to animal care and treatment were conducted under an Institutional Animal Care and Use Committee-approved protocol, according to the guidelines of the National Research Council Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and the US Department of Agriculture Animal Welfare Act and Animal Welfare Regulations.

Drug administration and tissue collection. Ketamine HCl (Ketaset® 100 mg/mL; Fort Dodge Animal Health, IA, USA) was used to prepare a 10 mg/mL solution in sterile 0.9% saline for injection. Rats received a single acute i.p. dose of either ketamine solution or saline appropriate for their body weight. Animals were sacrificed by live decapitation at 0.5, 1, 2, 6, or 24 hours post dose (n=5). Brains were removed and placed on wet ice for immediate dissection and homogenization, while trunk blood was collected in EDTA tubes to measure drug concentrations.

Preparation of synaptosomal fraction and Western blotting. The brains were removed and prefrontal cortex was hand dissected on wet ice. The tissues were placed directly into tubes containing 1 mL of cold Buffer A (Li et al., 2010) and the tube contents (tissue and buffer) were poured directly into a dounce homogenizer and manually dounced 5 times on ice. The homogenized samples were centrifuged at 614 × g for 10 minutes at 4°C (P1 sample is the pellet). The supernatant was removed and centrifuged at 11,269 × g for 10 minutes at 4°C (P2 is the pellet). The supernatant was removed and fresh RIPA buffer (Li et al., 2010) was added to the pellet (400 µL) just prior to probe sonication. The sonicated
samples were centrifuged for 1 minute at the maximum speed of a table top centrifuge (approximately 14000 rpm) and a protein assay was run on the supernatants to normalize gel loading. The samples (15 µg per well) were run on a 4–20% gradient tris-glycine gel and then wet transferred to nitrocellulose membranes for Western blotting. Blots were scanned on an Odyssey 9120 infrared scanner (Li-Cor, Lincoln, NB, USA). Local background was subtracted from all bands prior to normalizing each phospho-protein of interest to its control. For details on the antibodies being used see Table 1.

Methods at INSERM and Karolinska Institute (Servier)

Animals. Adult male Sprague Dawley rats (300–400g; Charles River, France) were housed in pairs in a temperature controlled room with food and water ad lib and under a 12-h light/dark cycle with lights on from 8 am. All procedures were performed in conformity with the National (JO 887-848) and European (86/609/EEC) legislations on animal experimentation.

Drug administration and harvesting of tissue. Animals were anesthetized with pentobarbital (60 mg/kg ip); ketamine was administered (10 mg/kg i.p.; ketamine hydrochloride, LGC Standards) immediately after. Animals were sacrificed 30 min after ketamine administration under isoflurane anesthesia. Brains were dissected into medial and lateral cortices, dorsal and ventral hippocampi and were snap frozen as previously described (Svenningsson et al., 2000) and stored at -80°C until processed.

Preparation of synaptosomal fraction and Western blotting. The cortical samples were sonicated in 1% sodium dodecyl sulfate (SDS), 10mM NaF, transferred to Eppendorf tubes and boiled for 10 min. The protein concentration in each sample was thereafter determined with a BCA-based kit (Pierce, Rockford, IL, USA). Twenty five micrograms of each sample was re-suspended in sample buffer and separated by SDS-PAGE using a 12% running gel and transferred to an Immobilon P transfer membrane (Millipore). The membranes were incubated for 1 h at room temperature with 5% (w/v) dry milk in TBS-Tween 20. Primary antibodies were diluted in 5% dry milk dissolved in TBS-Tween 20 and immunoblotting was performed overnight. Membranes were washed three times with TBS-Tween 20 and incubated with secondary HRP anti-rabbit antibody for 1 h at room temperature. At the end of the incubation, membranes were washed six times with TBS-Tween 20 and the immunoreactive bands were detected by chemiluminescence using ECL reagents (Perkin Elmer). A series of primary, secondary antibody dilutions and exposure times were used to optimize the experimental conditions for the linear sensitivity range of the autoradiography films (Kodak Biomax MR). Films were scanned and the density of each band was quantified using the NIH ImageJ 1.63 software. The levels of phosphorylated proteins were normalized to total levels.

Methods at Alkermes

Animals. Male Sprague Dawley rats (275–300 g; Charles River, Kingston, NY, USA) were pair-housed and allowed to acclimate to the animal colony and handled for at least 3–4 days prior to experimentation. Rats were maintained on a 12:12-h light-dark cycle (0600:1800 h light; 1800:0600 h dark) with a room temperature of 22±3°C and a relative humidity level of 45±10%.

Data analysis

Drug administration and tissue collection. Ketamine HCl (Ketaset® 100 mg/mL; Fort Dodge Animal Health, IA, USA) was used to prepare a 10 mg/mL solution in sterile 0.9% saline for injection. Rats received a single acute i.p. injection of ketamine and were killed 30 min later for phosphorylated mTOR (p-mTOR) or 24 hr for PSD-95 via CO2 asphyxiation followed by decapitation. Brains were removed, placed on wet ice and the prefrontal cortex was freehand dissected and snap frozen on dry ice. Samples were stored at -80°C until further analysis.

Synaptosomal preparation and capillary electrophoresis. Crude synaptosomes were prepared from frozen prefrontal cortex samples. Tissues were weighed and dounce homogenized (10:1; w:v) in ice-cold Syn-PER™ synaptic protein extraction reagent (Thermo Scientific; Rockford, IL, USA) supplemented with Halt™ protease and phosphatase inhibitor cocktail (1X, Thermo Scientific). Homogenates were centrifuged at 1200 × g for 10 min at 4°C. The supernatant was centrifuged at 15,000 × g for 20 min at 4°C. After centrifugation, the supernatant was discarded and pellets were resuspended in 200 µL of Syn-PER reagent with inhibitors and proteases. Protein concentration was determined by BCA protein assay according to the manufacturer’s instructions (Thermo Scientific).

Protein levels were quantified using an automated size resolving capillary electrophoresis system, “WES”, from Protein Simple (San Jose, CA, USA). All procedures were performed according to manufacturer’s instructions. Briefly, 8 µL of 0.1 mg/mL of homogenate was mixed with 2 µL of 5X fluorescent master mix and heated at 95°C for 5 min. The samples, blocking reagent, primary antibody, anti-rabbit secondary antibody, chemiluminescent substrate, and wash buffer were loaded into a microplate provided with a 12-230 kDa WES kit (PSD-95) or a 66–440 kDa WES kit (p-mTOR). Primary antibodies used were PSD-95 (rabbit; Cell Signaling [#2507]; 1:50) and p-mTOR (rabbit; Cell Signaling [#5536]; 1:50) (see Table 1). Separation and immunodetection was performed automatically using default plate settings for each kit in Compass software (version 2.7.1; Protein Simple, San Jose, CA, USA). Signal and quantitation of immunodetected proteins were generated automatically by Compass software and the data were graphed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA).
More than a dozen independent experiments conducted by these four teams failed to detect robust effects of ketamine on markers reported to be affected in the study by Li et al. (2010). Given the number of studies and markers analyzed, vehicle- and ketamine-treated groups occasionally appeared to be different but there were no overall consistent and robust differences. Figure 1, Figure 2 and Figure 3 present results from the studies that assessed effects of ketamine on pmTOR, PSD-95 and pp70S6K. Table 2 summarizes experimental conditions that were systematically manipulated in order to enable detection of ketamine-induced biochemical effects.

Independent correspondence with Ronald Duman (senior author in the Li et al. publication) and S. Popp (AbbVie) or J. Joshi (Pfizer) did not help to identify methodological factor(s) that may account for the failure to reproduce ketamine’s effects.

Discussion
What makes clinical effects of ketamine quite appealing is that they are strong enough to be seen even in small studies conducted by different institutions under varying conditions. In contrast, effects of ketamine on mTOR seem to require special conditions that are difficult to identify and establish at least in some labs. Many of

[Figure 1. Expression of pm-TOR in the synaptosomal fraction of the prefrontal cortex done by the different companies (AbbVie, Pfizer, Karolinska-Servier (K-S) and Alkermes (Alk)). Values represent mean ± SEM; n is indicated in the bars for each independent experiment, *p<0.05; student's t-test. Samples were collected at different time points after drug application as indicated in the figure. Karolinska-Servier distinguished between the medial (m PFC) and lateral (lat PFC) prefrontal cortex.]
Figure 2. Expression of PSD-95 in the synaptosomal fraction of the prefrontal cortex done by the different companies (AbbVie, Pfizer and Alkermes [Alk]). Values represent mean ± SEM, n is indicated in the bars for each independent experiment, *p<0.05; student's t-test. Samples were collected at different time points after drug application as indicated in the figure.

Figure 3. Expression of pp70S6K in the synaptosomal fraction of the prefrontal cortex done by the different companies (AbbVie and Pfizer). Values represent mean ± SEM, n is indicated in the bars for each independent experiment, *p<0.05; student's t-test. Samples were collected at different time points after drug application as indicated in the figure.
these phosphorylation events are very sensitive, and subject to high amounts of variability even when environmental conditions are well-controlled. Thus, these kinds of measurements may not be reliable pharmacodynamic markers of efficacy.

Taken together, these data call into question the robustness of the preclinical ketamine mTOR findings and challenge the mTOR hypothesis of ketamine’s antidepressant action. We would also like to emphasize the importance of publishing detailed methods either within the papers or as supplementary materials. This information is essential for follow-up studies that any significant research is likely to trigger.

Decision to publish current results
Efforts to identify individual lab efforts to establish ketamine’s effects on mTOR have followed the peer-to-peer mechanism of information exchange that is being developed by the ECNP Preclinical Data Forum (https://www.ecnp.eu/projects-initiatives/ECNP-networks/List-ECNP-Networks/Preclinical-Data-Forum.aspx) and is suggested as a general tool to identify unpublished data that, when put together and disclosed, could present a value to the scientific community.

We feel that information about failed attempts to establish ketamine’s effects should be disclosed to allow scientific community to judge on the robustness of these effects.

After the manuscript was prepared for submission, the authors have received information from colleagues at the Lilly Research Labs, Indianapolis, IN USA (H. Wang, J.M. Witkin, and J.W. Ryder, personal communication) that their lab was also unable to establish effects of ketamine on p-mTOR(pS2448), consistent with the data reported in this manuscript.

Data availability
F1000Research: Dataset 1. Figure 1 raw data, 10.5256/f1000research.8236.d117437 (Popp et al., 2016a).
F1000Research: Dataset 2. Figure 2 raw data, 10.5256/f1000research.8236.d117438 (Popp et al., 2016b).
F1000Research: Dataset 3. Figure 3 raw data, 10.5256/f1000research.8236.d117439 (Popp et al., 2016c).

Author contributions
Study design: SP, BB, MS, TL, PS, ES, TMJ, JC, DD, AB; Conducted experiments: SP, BB, DC, JJJ, TMJ, JC; Analysis: SP, MS, ES, BB, DC, PS, DD; Writing: AB, SP, TL, DD.

Competing interests
No other interests beyond employment indicated in this manuscript. MS was a Servier employee at the time when studies were conducted.

Grant information
This research was funded by AbbVie (SP, BB, AB), Pfizer (TL, JJJ), Servier (ES, PS, DC, TMJ), Alkermes (JC, DD), INSERM (TMJ) and IMI-Newmeds (TMJ).

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Popp S, Behl B, Joshi JJ, et al.: Dataset 1 in: In search of the mechanisms of ketamine’s antidepressant effects: How robust is the evidence behind the mTor activation hypothesis. F1000Research. 2016a. Data Source
Popp S, Behl B, Joshi JJ, et al.: Dataset 2 in: In search of the mechanisms of ketamine’s antidepressant effects: How robust is the evidence behind the mTor activation hypothesis. F1000Research. 2016b. Data Source
Popp S, Behl B, Joshi JJ, et al.: Dataset 3 in: In search of the mechanisms of ketamine’s antidepressant effects: How robust is the evidence behind the mTor activation hypothesis. F1000Research. 2016c. Data Source
Open Peer Review

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Version 1

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Eero Castrén, Plinio Casarotto
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It is very important that also negative results get published, especially failures to replicate previously published data, in this regard, this paper is welcome. However, I feel that the results and conclusions are presented in an unnecessarily negative light. Although several sites are involved, many of them report essentially pilot data with a very low n. For pm-TOR, the apparently most thorough study from the Alkermes group robustly confirmed the finding of Li et al. at 30 min after ketamine (12 rats in the ketamine and control groups, P= 0.0109) and the other group studying the same time point (Pfizer) found an almost significant increase (P=0.0509) with only 5 and 3 animals in ketamine and control groups, respectively, begging for a replication with higher n. In the Servier/Karolinska/INSERM lab, the assay does not seem to be working, with almost 4 fold differences between samples in the control group and there are too few rats for any clear conclusions. So the only site where the results were clearly not replicated was AbbViel lab, assayed at 1 h after ketamine. To me these data provide evidence that ketamine increases pmTor at 30 min after ketamine, as reported by Li et al, but apparently not at 1 h or later. As pointed out by the authors, phosphorylation events are sensitive and may take place rapidly which may contribute to the finding that pmTor as the mediator of ketamine effects may not be as robust as the initial studies suggest, but altogether I find that the data is presented and discussed in unnecessarily negative light.

One source of variation between the groups in the consortium and between them an others is what gets included into PFC. I am wondering whether the consortium attempted to standardize their dissection, at least this was not discussed.

The consortium has listed a long list of antibodies used, but data is shown only for 3 targets. Why is that? Please publish the rest of the data as well.

In sum, I do not think that this study represents “failed attempts to establish ketamine’s effects”, rather it provides suggestive, albeit not conclusive evidence that pm-Tor levels are increased by ketamine at 30 min, but perhaps not later. The authors correctly point out that methods should be described better

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.
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The authors have highlighted an important issue concerning the inability to reproduce published data supporting the activation of mTOR-dependent pathways as a potential mechanism for the rapid antidepressant effects of ketamine. The authors have provided in-depth methodological details from all labs involved, allowing for a direct comparison of the experimental procedures carried out by all research groups involved in the study. The authors suggestions of including detailed methods within the manuscript or as supplementary information as well as the disclosure of failed attempts to reproduce published data, are valid and would be a benefit to the scientific community.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.