RESEARCH ARTICLE

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.

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Any reports and responses or comments on the article can be found at the end of the article.

Keywords

ketamine , depression , mTOR , data robustness , data sharing

This article is included in the **Preclinical Reproducibility and Robustness** gateway.

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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, other 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 intravenous 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 8 μl 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 decahydrate, 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 $1000 \times g$ 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 $15,000 \times g$. The resulting pellet was resuspended in $\sim 20 \mu\text{l}$ 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 $33000 \times g$. 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 $33000 \times g$. 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

applied on the E-PAGE™ 48 8% gel (Invitrogen Cat.No.: EP048-08). The electrophoresis was run on an Invitrogen electrophoresis device either a Mother E-Base™ device connected to a power source or a Daughter E-Base™ connected to a Mother E-Base™. Two standard samples (MagicMark™ XP Western Protein Standard [Invitrogen Cat.No.: LC5602] [marker] and SeeBlue® Plus2 Pre-stained Protein Standard [Invitrogen Cat.No.: LC5925] [marker]) were run in parallel to the samples for 24 minutes. After completion of the run the gel was removed and subjected to the Invitrogen semi-dry blotting procedure. Proteins were transferred to a nitrocellulose blotting membrane with a pore size of 0.2 microns (Invitrogen Cat.No.: IB3010-01). The membrane was dried and stored at 4°C for further analysis.

For the following steps the Invitrogen WesternBreeze Chemiluminescent Western Blot Immunodetection Kit for primary antibodies made in mouse (Invitrogen Cat. No.: WB7104) or for primary antibodies made in rabbit (Invitrogen Cat. No.: WB7106) was used. The membrane was allowed to come to room temperature, incubated 30 minutes in a blocking solution from the kit on a shaker, washed

twice with deionized water and incubated with a Primary Antibody Solution for at least 1 hour at room temperature on a shaker. The membrane was washed four times for 5 minutes with a prepared Antibody Wash (included in the kit) and incubated in a Secondary Antibody Solution for 30 minutes. After washing the blots four times with Antibody Wash and rinsing it twice with deionized water the bands were detected using the Chemiluminescent Substrate Solution (included in the kit). The chemiluminescence intensity of the bands was quantified by a CHEMI DOC XRS imaging system (Bio-Rad Laboratories GmbH, Munich, Germany) utilizing an Universal Hood II enclosure.

The following proteins were analyzed for samples taken 1 hour after ketamine injection: phospho-p70S6 Kinase, phospho-Akt (Ser 473), Arc (C-7), phospho-mTor (Ser2448), phospho-S6 Ribosomal Protein (Ser 235/236) and phospho-p44/42 MAP Kinase (Erk1/2) (Thr 202/Tyr 204). The following markers were analyzed for samples taken 3 hours after ketamine application: Arc(C-7), Synapsin I, GluR-1 (E-6), phospho-S6 Ribosomal Protein (Ser 235/236) and PSD-95 (7E3). For details see [Table 1](#) and [Table 2](#).

Table 1. Antibodies used in the studies.

Antibody	Company	Catalog number	Species	Dilution	Detection method	Used by
4EBP1	Cell Signaling	9644	Rb mAb	1:1000	WB	Pfizer
p-4EBP1 (Thr37/46)	Cell Signaling	2855	Rb mAb	1:1000	WB	Pfizer
p-p44/42 MAP Kinase (Erk1/2) (Thr202/Tyr204)	Cell Signaling	9101	Rb pAb	1:500	WB	AbbVie
p-p44/42 MAP Kinase (Erk1/2) (Thr202/Tyr204) (E10)	Cell Signaling	9106	M mAb	1:500	WB	Karolinska
p70S6K	Cell Signaling	2708	Rb mAb	1:1000	WB	Pfizer
p-p70S6K (Thr 389)	Cell Signaling	9205	Rb pAb	1:1000	WB	AbbVie, Pfizer
p-Akt (Ser 473)	Cell Signaling	9271	Rb pAb	1:200 1:250	WB WB	AbbVie Karolinska
Arc (C-7)	Santa Cruz	sc-17839	M mAb	1:400	WB	AbbVie
GAPDH	Sigma	G8795	M mAb	1:1000	WB	Pfizer
GluR1 (E-6)	Santa Cruz	sc-13152	M mAb	1:200	WB	AbbVie
GluR1	Millipore	AB1504	Rb pAb	1:1000	WB	Pfizer
GluR1	Upstate	06-306	Rb pAb	1:1000	WB	Karolinska
p-GluR1 (Ser845)	Thermo Scientific	OPA1-04118	Rb pAb	1:1000	WB	Pfizer
p-GluR1 (Ser845)	Upstate	06-773	Rb pAb	1:1000	WB	Karolinska
mTOR	Cell Signaling	2972	Rb pAb	1:1000	WB	Karolinska, Pfizer
p-mTOR (Ser 2448)	Cell Signaling	2971	Rb pAb	1:1000	WB	Pfizer
p-mTOR (Ser 2481)	Cell Signaling	2974	Rb pAb	1:200 1:500	WB WB	AbbVie Karolinska
p-mTOR (Ser2448) (D9C2)	Cell Signaling	5536	Rb mAb	1:50*	CE	Alkermes
PSD-95 (7E3)	Santa Cruz	sc-32290	M mAb	1:1000	WB	AbbVie
PSD-95	Cell Signaling	2507	Rb pAb	1:1000 1:50*	WB CE	Pfizer Alkermes
S6	Santa Cruz	sc-74459	M mAb	1:1000	WB	Pfizer
p-S6 (Ser240/244) (D68F8)	Cell Signaling	5364	Rb mAb	1:1000	WB	Pfizer
p-S6 (Ser235/236)	Cell Signaling	2211	Rb pAb	1:1000	WB	AbbVie
Synapsin I	Abcam	ab18814	Rb pAb	1:1000	WB	AbbVie
Synapsin I (D12G5)	Cell Signaling	5297	Rb mAb	1:1000	WB	Pfizer

WB: Western blot analysis; CE: Capillary electrophoresis; *Antibody dilution optimized for ProteinSimple WES capillary electrophoresis system

Table 2. Summary of the experimental conditions tried across various studies.

Variables evaluated	Conditions tried at AbbVie	Conditions tried at Karolinska/Servier	Conditions tried at Alkermes	Conditions tried at Pfizer
Rats - supplier - euthanasia	- male Sprague-Dawley, Charles River*, Janvier - under isoflurane anesthesia, guillotine without anesthesia*	- male Sprague-Dawley, Charles River* - under pentobarbital anesthesia, guillotine	- male Sprague-Dawley, Charles River* - CO ₂ asphyxiation, decapitation	- male Sprague-Dawley, Charles River* - guillotine without anesthesia*
Ketamine - source/preparation - route of administration - dose	- powder from Sigma-Aldrich, Ketaset prepared according to the Ketaset® solution, ready solution from WDT, - intraperitoneal*, intravenous - 3, 10*, 20, 30 mg/kg	- powder from LGC Standards - intraperitoneal* - 10 mg/kg*	- Ketaset® solution* - intraperitoneal* - 10 mg/kg*	- Ketaset® solution* - intraperitoneal* - 10 mg/kg*
Tissue sampling	- 1 h* or 3 h after ketamine, 30 min, 2 h or 2.5 h after forced swim test - homogenates, crude synaptosomes*, synaptosomal fraction according to the Percoll method - prefrontal cortex*, hippocampus	- 30 min* after ketamine - frozen tissue samples were sonicated - crude synaptosomes* - prefrontal cortex* (medial vs lateral); hippocampus (dorsal vs ventral)	- 30 min* after ketamine, 24 h* after ketamine - frozen tissue samples were dounce homogenized - crude synaptosomes* - prefrontal cortex*	- 0.5h*, 1h*, 2h*, 6h*, 24h* - frozen tissue samples were sonicated - fresh dounce homogenization* followed by crude synaptosomal prep* - prefrontal cortex*, hippocampus
Markers analyzed	pp70S6K*, pAkt*, Arc*, pmTOR*, pp44/42 MAP Kinase*, pS6, Synapsin I*, GluR1*, PSD95*	pSer831-GluR1, pSer845-GluA1, pThr202-Tyr204-p44/42-ERK*, pSer217-pSer221-MEK, pSer9-GSK3β, pTyr705-TrkB, pTyr816-TrkB, pSer2482-mTOR* total levels of GluR1*, p44/42-ERK*, MEK, GSK3β, TrkB, mTOR*	pmTOR*, PSD95*	pmTOR*, GluR1*, p4EBP1*, pp70S6K*, PSD95*, Synapsin I*, S6

* conditions and markers reported by [Li et al. \(2010\)](#)

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%. Food and water were available ad libitum and rats used for these studies were housed, managed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). All experiments were approved by the Alkermes Institutional Animal Care and Use Committee. Animal studies conducted by Alkermes were reviewed and approved by its IACUC. All animal work conducted by Alkermes is compliant with PHS policies governing the humane care and use of laboratory animals.

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 free-hand 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; wt:vol) 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).

Data analysis

Data are presented as the percentage change from vehicle for each analyte (± SEM). To assess treatment effects of ketamine on p-mTOR, PSD-95 and pp70S6K, pairwise group comparisons were conducted using two-tailed t-test (GraphPad Prism 6.0, San Diego, CA, USA).

Results

Dataset 1. Figure 1 raw data

<http://dx.doi.org/10.5256/f1000research.8236.d117437>

Percentage change from vehicle data and statistical analysis of pm-TOR expression in the synaptosomal fraction of the prefrontal cortex done by the different companies.

Dataset 2. Figure 2 raw data

<http://dx.doi.org/10.5256/f1000research.8236.d117438>

Percentage change from vehicle data and statistical analysis of PSD-95 expression in the synaptosomal fraction of the prefrontal cortex done by the different companies.

Dataset 3. Figure 3 raw data

<http://dx.doi.org/10.5256/f1000research.8236.d117439>

Percentage change from vehicle data and statistical analysis of pp70S6K expression in the synaptosomal fraction of the prefrontal cortex done by the different companies.

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 $\pm$ 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 $\pm$ 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 $\pm$ 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 Pre-clinical 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](https://doi.org/10.5256/f1000research.8236.d117437) (Popp *et al.*, 2016a).

F1000Research: Dataset 2. **Figure 2** raw data, [10.5256/f1000research.8236.d117438](https://doi.org/10.5256/f1000research.8236.d117438) (Popp *et al.*, 2016b).

F1000Research: Dataset 3. **Figure 3** raw data, [10.5256/f1000research.8236.d117439](https://doi.org/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.

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Eero Castren

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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 and 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 confirm that we have read this submission and 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.

Reviewer Report 27 April 2016

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John D. Graef

Genetically-Defined Diseases, Bristol-Myers Squibb Company, Wallingford, CT, USA

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

Comments on this article

Version 1

Author Response 12 Jun 2020

Anton Beshpalov, AbbVie Deutschland GmbH & Co., Ludwigshafen, Germany

Those interested in this subject may want to read a recent report on the results of a clinical trial

on ketamine and rapamycin:

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7162891/>

Competing Interests: I am a co-author of the above paper and an employee / shareholder of PAASP GmbH and EXCIVA GmbH. No other competing conflicts of interest.

Reader Comment 18 Jul 2016

Rene Bernard, Charite - Medical University Berlin, Germany

This paper clearly shows that guidelines for reproducibility are needed. The current paper by Popp et al does not resolve the question about mTOR involvement of as potential mechanisms of action for ketamine. It is example of missed opportunities and/or missing information and communication.

1. Were the corresponding authors of the Li *et al* 2010 and Liu *et al* 2013 paper contacted by the industry consortium prior to the attempt to replicate parts of their study?
2. Were the corresponding authors of the Li *et al* 2010 and Liu *et al* 2013 paper asked to provide their raw data and protocols prior to the replication study?
3. Were the corresponding authors of the Li *et al* 2010 and Liu *et al* 2013 paper provided with the somewhat different protocols of AbbVie, Pfizer, Servier and Alkermes prior to the execution of the replication study?

It would be helpful if the authors would include answers to those questions in an updated version of their article.

It seems clear from the literature that ketamine can induce mTOR Expression, but probably only under very defined conditions. If there is doubt or pre-existing evidence of non-replicability, the authors whose work is being replicated need to be involved during the planning of the replication study. If they decide to opt out, then this should be reported and maybe the editors of journal that published the original study should be informed.

It is clear that a journal method section, most of the time, is an insufficient blueprint for a proper replication study because important details that are often not included or unclear descriptions left for interpretation. For instance, compare the different euthanization methods that are used in the current paper.

A retrospective analysis or trouble shooting with the corresponding authors is much harder to resolve or explain issues of non-reproducibility. In a way, the original authors feel threatened and might be less willingly to cooperate now.

The current paper does not resolve the issue nor does it aim for a solution. Instead of having even more industry laboratories non-replicate the industry protocol, the Popp *et al.* authors should seek for a mediator to work out terms for a common replication scheme. This could include in-house

replication with observer from industry and vice versa industry-replication with members of Duman academic lab present. Only then trust can be restored and scientifically sound answers can be found and then we know when and how ketamine induces mTor Expression and under which experimental conditions it does not.

Competing Interests: I have no competing interests.

Reader Comment 26 May 2016

F1000 Research, UK

The data for this article are also visualized on the Open Science Framework at: <https://osf.io/fng2d/>.

Competing Interests: No competing interests were disclosed.

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