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ARTICLE

Differential effects of antidepressant drugs on mTOR signalling in rat hippocampal neurons

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Abstract

Recent studies suggest that ketamine produces antidepressant actions via stimulation of mammalian target of rapamycin (mTOR), leading to increased levels of synaptic proteins in the prefrontal cortex. Thus, mTOR activation may be related to antidepressant action. However, the mTOR signalling underlying antidepressant drug action has not been well investigated. The aim of the present study was to determine whether alterations in mTOR signalling were observed following treatment with antidepressant drugs, using ketamine as a positive control. Using Western blotting, we measured changes in the mTOR-mediated proteins and synaptic proteins in rat hippocampal cultures. Dendritic outgrowth was determined by neurite assay. Our findings demonstrated that escitalopram, paroxetine and tranylcypromine significantly increased levels of phospho-mTOR and its down-stream regulators (phospho-4E-BP-1 and phospho-p70S6K); fluoxetine, sertraline and imipramine had no effect. All drugs tested increased up-stream regulators (phospho-Akt and phospho-ERK) levels. Increased phospho-mTOR induced by escitalopram, paroxetine or tranylcypromine was significantly blocked in the presence of specific PI3K, MEK or mTOR inhibitors, respectively. All drugs tested also increased hippocampal dendritic outgrowth and synaptic proteins levels. The mTOR inhibitor, rapamycin, significantly blocked these effects on escitalopram, paroxetine and tranylcypromine whereas fluoxetine, sertraline and imipramine effects were not affected. The effects of escitalopram, paroxetine and tranylcypromine paralleled those of ketamine. This study presents novel *in vitro* evidence indicating that some antidepressant drugs promote dendritic outgrowth and increase synaptic protein levels through mTOR signalling; however, other antidepressant drugs seem to act via a different pathway. mTOR signalling may be a promising target for the development of new antidepressant drugs.

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Key words: Antidepressant drugs, hippocampal dendritic outgrowth, ketamine, mammalian target of rapamycin, synaptic proteins.

Introduction

Depression is a common, serious, recurrent illness. In American adults, the 12-month prevalence rate of a major depressive episode is 8.3% and the lifetime prevalence rate is 19.2% (Hirschfeld, 2012). Depression can compromise quality of life and daily functioning and can lead to medical disease. The World Health Organization (WHO) ranked depression as the fourth-leading cause of functional disability worldwide (Kessler and Bromet, 2013). Since the 1950s antidepressant drugs have been prescribed to treat depression. Most antidepressant drugs elevate levels of neurotransmitters in the brain, particularly serotonin and norepinephrine (Artigas, 2001). Although antidepressant drugs are generally efficacious for the treatment of depression, many patients are resistant to them (Fava, 2003). Moreover, it takes several weeks to months before antidepressant drugs exert their full effect. Therefore, it is necessary to develop rapid-acting antidepressant drugs (Adell et al., 2005).

In the search for a mechanism for rapid-acting antidepressant drugs, plastic changes in neural connectivity have received a great deal of attention (Castrén, 2005). According to this hypothesis, antidepressant drugs improve neuronal function and mood by regulating synaptic proteins (Castrén, 2005). Glutamate, an excitatory neurotransmitter, may play an important role in synaptic plasticity (Li et al., 2010b). In recent clinical trials, a subanaesthetic dose of ketamine [an *N*-methyl-D-aspartate (NMDA) antagonist] had rapid antidepressant effects

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on patients with treatment-resistant depression (Zarate et al., 2006). Until recently, the mechanism of the rapid antidepressant action of ketamine had not been well understood. However, Li et al. (2010b) reported that a sub-anaesthetic dose of ketamine activates the mammalian target of rapamycin (mTOR) signalling pathway, increases expression of synaptic proteins and synaptogenesis in the prefrontal cortex of mice and causes antidepressant effects as assessed by the forced swim test. Therefore, it is possible that activation of mTOR signalling and synaptogenesis play an important role in the rapid antidepressant effects of ketamine.

mTOR is a serine/threonine protein kinase that regulates the initiation of protein translation and consequently controls the protein synthesis required for synaptogenesis (Tang and Schuman, 2002; Klann et al., 2004; Abe et al., 2010; Hashimoto, 2011). mTOR activity increases with phosphorylation at serine^{2448} by phosphatidylinositol-3 kinase (PI3K)/Akt (Ayuso et al., 2010). In cultured rat neurons, activation of brain-derived neurotrophic factor (BDNF)-induced tyrosine-related kinase B (TrkB) stimulates the mitogen-activated protein (MAP)-extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK and PI3K/Akt signalling pathways (Pizzorusso et al., 2000; Rodgers and Theibert, 2002). These pathways stimulate mTOR signalling, which increases the phosphorylation of p70 ribosomal protein S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP-1). Consequently, p70S6K and 4E-BP-1 promote the initiation of protein translation for synaptic protein synthesis via those critical down-stream targets, small ribosomal protein 6 (S6), eukaryotic translation initiation factor 4B (elF4B) and elF4E (Wang et al., 2003; Parsa and Holland, 2004; Raught et al., 2004).

Dendrites are the sites at which neurons receive information from multiple pre-synaptic partners. Dendritic function is critically dependent on dendritic outgrowth and small protrusions called dendritic spines (Kumar et al., 2005). PI3K/Akt/mTOR signalling has emerged as a key regulator of dendrite size and dendritic complexity as well as of dendritic spine density (Ehrlich et al., 2007). Specifically, the post-synaptic density (PSD) protein PSD-95 is largely localized in these spines and plays an important role in regulating dendritic spine size and shape (Ehrlich et al., 2007; Han and Kim, 2008). A component of the pre-synaptic vesicle membrane, synaptophysin (SYP), is widely used as a marker for synapse activity, which can be used to detect synaptic density and distribution (Valtorta et al., 2004). Therefore, changes in dendritic morphology and synapse-associated proteins might affect synaptic plasticity.

However, few studies have examined whether antidepressant drugs (e.g. selective serotonin transporter inhibitors) stimulate the mTOR signalling pathway. Lin et al., (2010) reported that sertraline inhibits translation initiation by inhibiting mTOR signalling. However, that study focused on the anti-proliferative effects of sertraline and not its antidepressant effects. More recently, it was reported that the NR2B antagonist Ro-35-6891 exerts antidepressant effects by elevating mTOR activity in mouse prefrontal cortex (Workman et al., 2013). Therefore, in this study, we examined whether antidepressant drugs enhance mTOR signalling. In addition, we investigated whether mTOR signalling mediates the influence of antidepressant drugs on dendritic outgrowth and synaptic protein levels. The antidepressant drugs studied included selective serotonin inhibiters (escitalopram, fluoxetine, paroxetine and sertraline), a monoamine oxidase inhibitor (tranylcypromine) and a tricyclic antidepressant (imipramine), and their effects were compared with those of ketamine as a positive control.

Materials and method

Drugs and reagents

Neurobasal medium, fetal bovine serum (FBS), horse serum (HS), B27 supplement, L-glutamine, penicillin–streptomycin and trypsin were purchased from Invitrogen (USA). Escitalopram oxalate was supplied by Lundbeck (Denmark). Fluoxetine hydrochloride, paroxetine maleate, sertraline hydrochloride and tranylcypromine hydrochloride were purchased from Tocris Bioscience (UK). Imipramine was purchased from Sigma (USA). Ketamine was purchased from Huons (Korea). Antibodies used for Western blotting were purchased from the following sources: rabbit anti-goat IgG-horseradish–peroxide conjugates from Santa Cruz Biotechnology (USA); monoclonal anti-α-tubulin and anti-mouse IgG peroxidase conjugates from Sigma (USA); anti-phospho-mTOR (Ser2448, #2971), anti-mTOR (#2972), anti-phospho-Akt (Ser473, #9271), anti-Akt (#9272), anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204, #9101), anti-p44/42 MAPK (ERK1/2), (#4695), anti-phospho-4E-BP-1 (Thr37/46, #2855), anti-4E-BP-1 (#9452), anti-phospho-p70S6K (Thr389, #9205), and anti-p70S6K (#9202) from Cell Signalling Technology (USA); anti-PSD95 (AB9634) from Millipore (USA); and anti-synaptophysin (ab52636) from Abcam (UK). Immunostaining reagents were purchased from the following sources: anti-microtubule-associated protein 2 (MAP-2, AB5622) from Millipore (USA) and AlexaFluor 568 goat anti-mouse IgG and Hoechst 33258 from Invitrogen (USA). Specific kinase inhibitors were purchased from the following sources: PI3K inhibitor LY294002 from Cell Signalling Technology (USA); MEK inhibitor PD98059 and mTOR inhibitor rapamycin from Calbiochem (USA).

Primary hippocampal cell cultures

All animal manipulations were performed in accordance with the animal care guidelines of the U.S. National Institutes of Health (NIH publication no. 23–85, revised

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1996). This experiment was approved by the Committee for Animal Experimentation and the Institutional Animal Laboratory Review Board of Inje Medical College (approval no. 2011-036). Primary cultures of hippocampal neurons were prepared from foetal brains (embryonic day 17; E17) obtained from Sprague–Dawley rats (Orient Bio, Korea) in a manner similar to that developed by Kaech and Banker (2006). Briefly, brains were exposed, and the hippocampi were carefully removed and dispersed in neurobasal medium containing 0.03% trypsin for 20 min at 37 °C (5% CO_2). Next, they were suspended in a neurobasal medium containing 1% FBS, 1% HS, 2% serum-free growth medium B27 (components: biotin, α-tocopheryl acetate, α-tocopherol, vitamin A, bovine serum albumin, catalase, insulin, transferrin, superoxide dismutase, corticosterone, galactose, ethanolamine, glutathione, carnitine, linoleic acid, linolenic acid, progesterone, putrescine, selenium, and trido-l-thyronine), 0.25% L-glutamine and 50 U/ml penicillin–streptomycin; this was considered the control condition. Neurons were plated on 6-well dishes coated with poly-L-lysine at a density of 2×10^5 per dish for Western blotting or on 12-well dishes at 2×10^4 per dish for neurite assays. They were grown under the control conditions for seven days (for neurite assays) or ten days (for Western blotting).

The present study utilized a model of toxicity that causes cell death, namely the omission of B27 in the culture medium (Bastianetto et al., 2006). A preliminary experiment found that hippocampal cells were reduced by approximately 33% under B27-deprived conditions (Supplementary Table S1). Following a seven- or ten-day incubation period, cells were treated with drugs in the presence or absence of B27 for four days (Western blotting) and in control conditions for five days (neurite assay) before harvesting for further analysis. Cells were pre-treated for 30 min with LY294001, PD98059 or rapamycin before treatment with antidepressant drugs or ketamine in the presence (neurite assay) or absence (Western blotting) of B27. The culture media, inhibitors and drugs were changed every two days.

Drug treatment

Antidepressant drugs (10 mM) and ketamine (50 mM) were completely dissolved in dimethyl sulfoxide (DMSO) and diluted to various concentrations (final concentration of 1% DMSO) in neurobasal medium. For Western blotting and neurite assays, cells were cultured for four and five days, respectively, with ketamine (50, 100, and 500 μM), escitalopram (10 and 50 μM), fluoxetine (1 and 10 μM), paroxetine (0.1 and 1 μM), sertraline (0.1 and 1 μM), tranylcypromine (10 and 50 μM) and imipramine (0.1 and 1 μM). The concentrations of antidepressant drugs used in this study were based on our previous study, which showed that these concentrations enhance expression of BDNF, PSD-95 and synaptophysin, as well as dendritic outgrowth in rat hippocampal neurons (Seo et al., 2014). Additionally, several studies reported a similar narrow concentration range for antidepressant effects on neurons (Wu et al., 1996; Post et al., 2000; Peng et al., 2008; Horikawa et al., 2010; Dikmen et al., 2011). The concentrations of ketamine were based on the observation that lower concentrations (<50 μM) had no effect on the phosphorylation of mTOR-mediated proteins, and higher concentrations (>500 μM) tended to reduce cell viability (data not shown).

Western blot analysis

Western blotting experiments were performed as previously described (Seo et al., 2014). Immunoblotting were performed with primary antibodies [anti-phospho-mTOR, anti-mTOR, anti-phospho-Akt, anti-Akt, anti-phospho-ERK1/2, anti-ERK1/2, anti-phosho-4E-BP-1, anti-4E-BP-1, anti-phospho-p70S6K, anti-p70S6K, anti-PSD-95, anti-synaptophysin and anti-α-tubulin. All dilution rates were 1:1000 except anti- α-tubulin which was diluted at 1:2000 in TBS-T] at 4 °C overnight. The membranes were washed three times in TBS-T for 10 min. The membranes were subsequently incubated for 1 h in TBS-T containing the horseradish–peroxidase-conjugated secondary antibody [goat-anti- rabbit IgG for anti-phospho-mTOR, anti-mTOR, anti-phospho-Akt, anti-Akt, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-4E-BP-1, anti-4E-BP-1, anti-phospho-p70S6K, anti-p70S6K, anti-PSD-95 and anti-synaptophysin and anti-mouse IgG for anti-α-tubulin. All dilution rates were 1:1000 except anti- α-tubulin which was diluted at 1:10 000] at room temperature.

Neurite assay

Neurites were visualized by immunostaining for MAP-2, a dendritic marker, as previously described (Seo et al., 2014). For neurite analysis, two independent experiments were performed for each condition and five fields were randomly selected from each sample. Dendritic lengths were determined as the distance between the edge of the cell body and the tip of the growth cone. Degrees of branching and soma diameter were measured by tracing dendritic branches and cell bodies using MetaMorph, an automated image-analysis program (Molecular Devices, USA) with images captured by a researcher blinded to the treatment groups. These programs are routinely used for such anlaysis (Lom and Cohen-Cory, 1999; Klimaschewski et al., 2002; Lalli and Hall, 2005). At least 300–400 cells obtained from five randomly picked areas (600–800 in total) were analysed.

Statistical analysis

All statistical analyses were conducted using one-way ANOVAs as values analysed by repeated measures from the same samples. For the *post-hoc* comparison

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Table 1. Effects of antidepressant drugs, ketamine or B27 deprivation on phosphorylation of mTOR, 4E-BP-1, p70S6K, Akt and ERK in primary hippocampal cultures.

Drug	±B27	Dose (μM)	mTOR	4E-BP-1	p70S6K	Akt	ERK
Ketamine	+	0	100.0±3.6	100.0±4.5	100.0±1.5	100.0±4.0	100.0±1.6
		50	119.2±7.0*	–	–	–	–
		100	125.1±6.0**	–	–	–	–
		500	135.5±2.4**	123.0±2.7**	122.7±1.5*	127.4±2.7*	131.4±0.9**
	–	0	38.5±6.4**	46.3±7.0**	50.0±9.6**	46.5±10.8**	36.2±4.1**
Escitalopram	+	0	100.0±4.5	100.0±6.4	100.0±3.2	100.0±4.9	100.0±1.3
		10	100.1±4.4	100.8±7.4	101.6±2.6	101.9±3.2	101.6±2.2
		50	100.7±3.7	101.5±8.0	102.9±3.4	100.9±5.9	101.1±1.8
	–	0	52.4±1.4**	44.9±4.5**	61.5±1.4**	59.1±7.3**	36.2±4.4**
Paroxetine	+	0	100.0±3.4	100.0±2.2	100.0±3.5	100.0±3.0	100.0±3.9
		0.1	99.4±3.0	97.6±1.6	99.5±4.1	98.7±5.6	100.3±3.8
		1	98.5±3.5	96.6±2.6	99.7±4.3	99.3±6.5	99.5±4.2
	–	0	41.8±2.4**	47.0±4.1**	33.2±3.4**	48.1±3.1**	44.1±1.9**
Tranylcypromine	+	0	100.0±3.9	100.0±7.9	100.0±6.2	100.0±1.1	100.0±1.8
		10	102.5±3.6	99.9±7.2	98.0±7.0	99.8±1.6	99.6±2.3
		50	101.5±4.1	103.3±7.3	97.0±4.6	100.8±1.4	101.8±1.2
	–	0	47.0±3.1**	44.5±6.2**	56.1±2.9**	72.4±2.5**	54.6±2.9**
Fluoxetine	+	0	100.0±3.9	100.0±13.2	100.0±3.3	100.0±5.9	100.0±6.8
		1	99.8±5.6	97.0±14.1	100.8±5.0	101.7±5.4	98.9±6.6
		10	102.7±7.1	101.2±10.8	100.5±5.1	102.1±3.6	99.9±6.0
	–	0	34.3±4.6**	34.6±2.6**	44.8±2.6**	52.9±3.1**	33.7±3.7**
Sertraline	+	0	100.0±4.6	100.0±2.0	100.0±8.8	100.0±1.7	100.0±0.9
		0.1	98.5±6.1	98.4±2.3	100.1±8.8	103.4±5.7	99.6±1.8
		1.0	95.4±8.2	102.1±1.0	98.7±5.9	101.7±1.9	103.1±1.7
	–	0	38.3±3.4**	34.5±7.9**	42.7±1.4**	30.0±0.8**	31.6±4.0**
Imipramine	+	0	100.0±2.4	100.0±2.8	100.0±1.7	100.0±4.3	100.0±2.2
		1	102.2±2.3	101.0±1.9	97.3±3.8	99.5±5.6	102.9±5.3
		10	98.3±3.2	101.4±1.9	95.4±2.1	99.6±6.3	104.1±4.6
	–	0	55.8±2.8**	46.1±4.6**	65.5±4.3**	38.8±3.5**	31.6±1.5**

Values represent means±S.E.M expressed as percentages of the values of non-drug-treated, non-B27 deprived cells. *$p<0.05$, **$p<0.01$ *vs.* control cells.

Scheffe's test was used. p-values <0.05 were deemed to indicate statistical significance.

Results

Effects of different classes of antidepressant drugs on mTOR, 4E-BP-1 and p70S6K phosphorylation

Drug-induced changes in the phosphorylation of mTOR and its down-stream targets, 4E-BP-1 and p70S6K, were measured in rat hippocampal cells maintained separately in media with or without B27. Hippocampal cells were incubated with ketamine (50, 100, or 500 μM), escitalopram (10 or 50 μM), fluoxetine (1 or 10 μM), paroxetine (0.1 or 1 μM), sertaline (0.1 or 1 μM), tranylcypromine (10 or 50 μM) or imipramine (0.1 and 1 μM) for four days.

Antidepressant drugs did not affect phosphorylation of mTOR, 4E-BP-1 and p70S6K when B27 was present in the culture medium (control condition, Table 1). On the other hand, ketamine increased mTOR phosphorylation in a concentration-dependent manner ($p<0.05$ or $p<0.001$) and a dose of 500 μM significantly increased phosphorylation of 4E-BP-1 and p70S6K ($p<0.05$ or $p<0.001$, Table 1). B27 deprivation significantly reduced phosphorylation of mTOR, 4E-BP-1 and p70S6K (all $p<0.001$, Table 1).

In B27-deprived cultures, escitalopram, paroxetine and tranylcypromine significantly increased the levels of phospho-Ser^{2448}-mTOR ($F=6.724$, $p<0.05$ for escitalopram; $F=7.578$, $p<0.05$ for paroxetine; $F=34.140$, $p<0.001$ for tranylcypromine; Fig. 1*a*), phospho-Thr$^{37/46}$–4E-BP-1 ($F=64.609$, $p<0.001$ for escitalopram; $F=35.944$, $p<0.001$ for paroxetine; $F=40.961$, $p<0.001$ for tranylcypromine; Fig. 1*b*) and phospho-Thr^{389}-p70S6K ($F=15.517$, $p<0.001$ for escitalopram; $F=60.711$, $p<0.001$ for paroxetine; $F=12.168$, $p<0.001$ for tranylcypromine; Fig. 1*c*). In contrast, fluoxetine, sertraline and imipramine did not affect phosphorylation levels of these proteins under B27 deprivation, suggesting that mTOR

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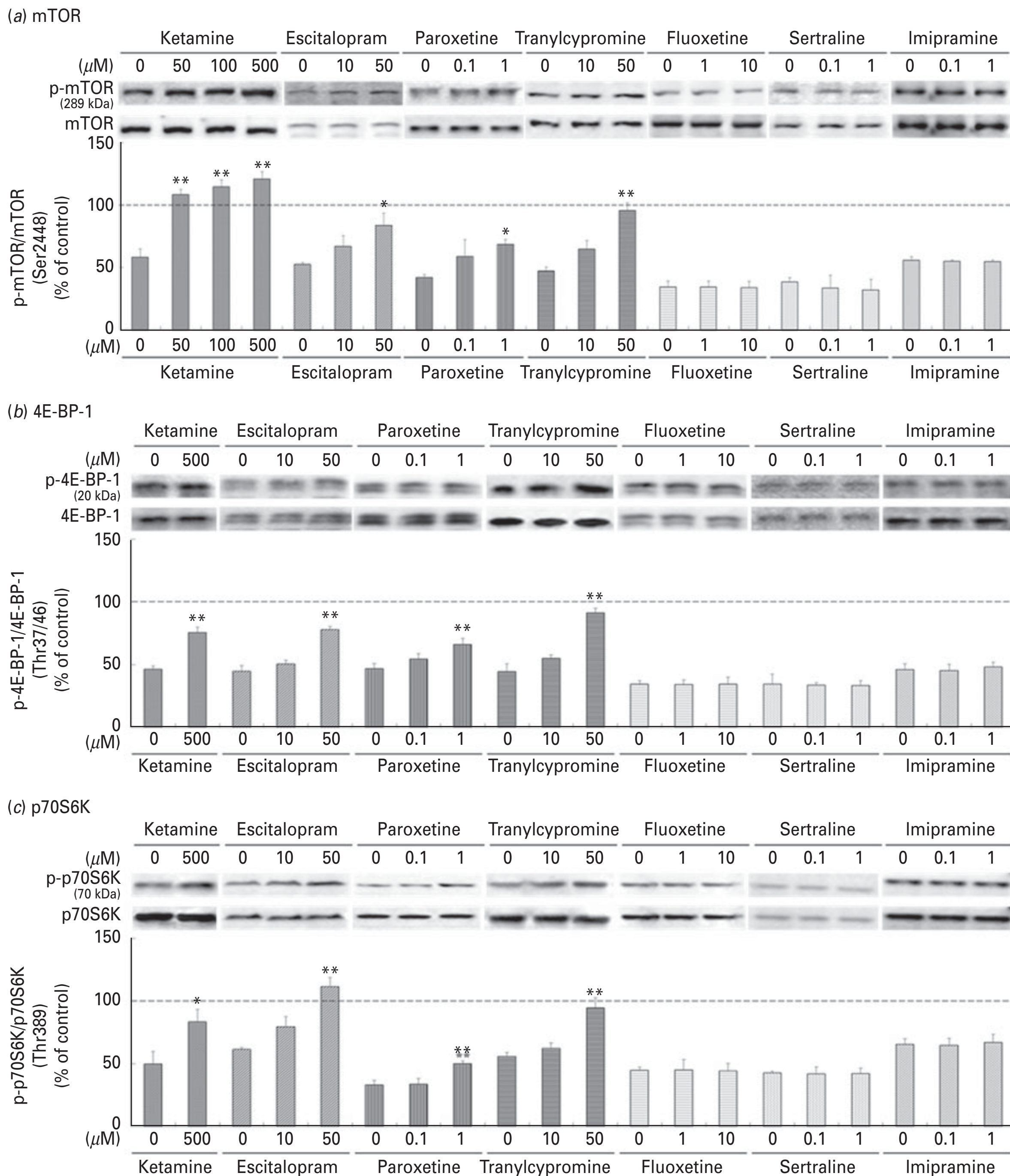

Fig. 1. Antidepressant drug effects on mTOR, 4E-BP-1 and p70S6K phosphorylation in hippocampal neurons. Cells were treated with varying doses of drugs for four days without B27. Three independent experiments were performed. Western blots reveal the levels of phospho-Ser^{2448}-mTOR (*a*), phospho-Thr$^{37/46}$-4E-BP-1 (*b*) and phospho-Thr^{389}-p70S6K (*c*). A representative image and quantitative analysis normalized to the total levels of each protein are shown. Values are means±S.E.M expressed as percentage of values of non-drug-treated, non-B27 deprived cells, considered 100% (dotted lines). *p<0.05, **p<0.01 B27-deprived cells.

activation is not required for the action of some antidepressant drugs (Fig. 1*a*–*c*). As expected, ketamine increased the phosphorylation of mTOR, 4E-BP-1 and p70S6K in this condition (F=26.484, p<0.001 for phospho-Ser^{2448}-mTOR; F=11.184, p<0.001 for phospho-Thr$^{37/46}$–4E-BP-1; F=6.662, p<0.05 for phospho-Thr^{389}-p70S6K; Fig. 1*a*–*c*).

Effects of different antidepressant drug classes on Akt and ERK phosphorylation

Next, we investigated whether different classes of antidepressant drugs differentially regulate phosphorylation of Akt and ERK, potential up-stream activators of mTOR, in hippocampal neurons.

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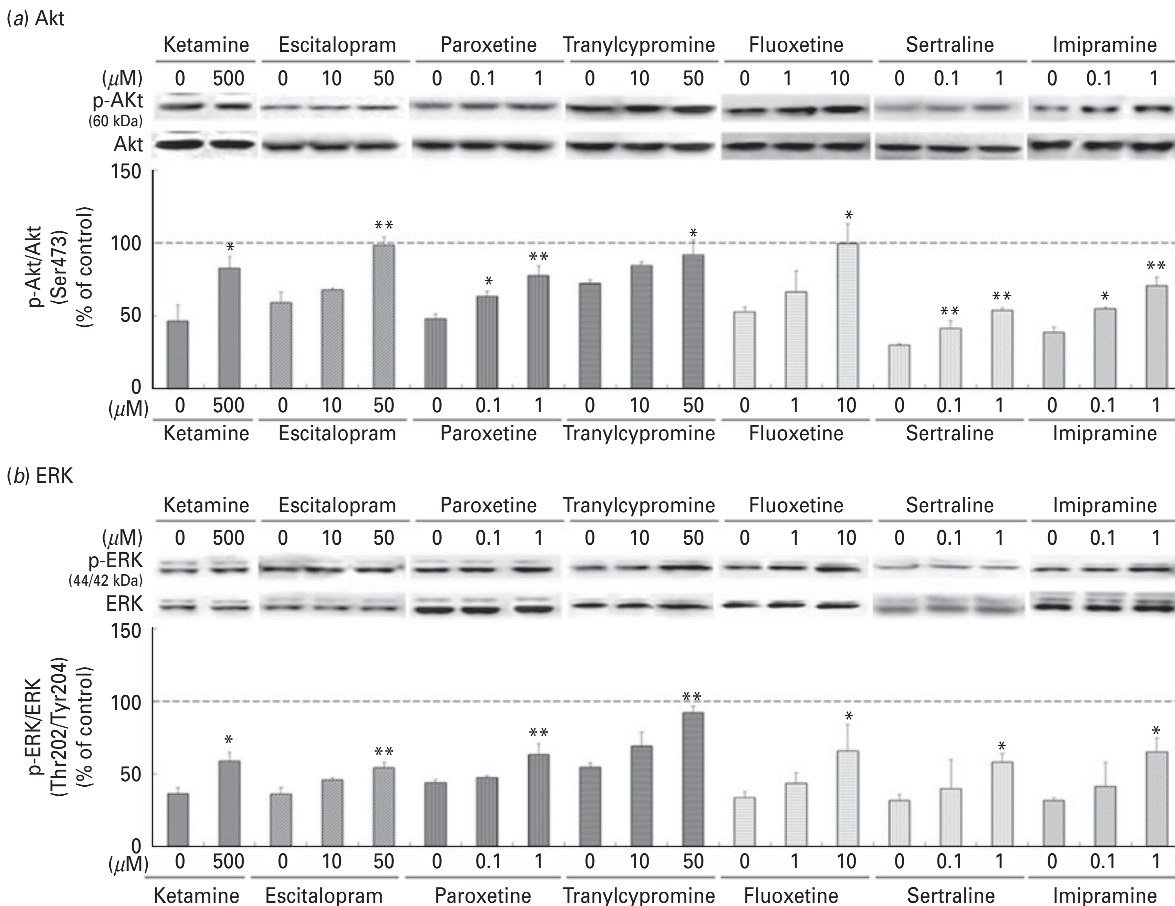

Fig. 2. Antidepressant drug effects on Akt and ERK phosphorylation in hippocampal neurons. Cells were treated with varying doses of drugs for four days without B27. Three independent experiments were performed. Western blots reveal levels of phospho-Ser^{473}-Akt (*a*) and phospho-Thr^{202}/Tyr^{204}-ERK (*b*). A representative image and quantitative analysis normalized to the total Akt (*a*) and total ERK (*b*) band are shown. Values are means±S.E.M expressed as percentage of values of non-drug-treated, non-B27-deprived cells, considered 100% (dotted lines). *$p<0.05$, **$p<0.01$ *vs.* B27-deprived cells.

Phosphorylation of Akt and ERK were not affected by antidepressant drugs under control conditions (Table 1). On the other hand, ketamine at a 500 μM dose significantly increased phosphorylation of these proteins ($p<0.05$ or $p<0.001$, Table 1). However, B27 deprivation significantly reduced phosphorylation of Akt and ERK (all $p<0.001$, Table 1).

In B27-deprived cultures, Western blotting revealed significant concentration-dependent increases in the levels of phospho-Ser^{473}-Akt and phospho-Thr^{202}/Tyr^{204}-ERK after treatment with all antidepressant drugs tested (Akt levels: $F=32.660$, $p<0.001$ for escitalopram; $F=20.259$, $p<0.001$ for paroxetine; $F=9.382$, $p<0.05$ for tranylcypromine; $F=6.618$, $p<0.05$ for fluoxetine; $F=81.115$, $p<0.001$ for sertraline; $F=34.576$, $p<0.001$ for imipramine; Fig. 2*a*; ERK levels: $F=45.260$, $p<0.001$ for escitalopram; $F=18.802$, $p<0.001$ for paroxetine; $F=8.644$, $p<0.05$ for tranylcypromine; $F=6.189$, $p<0.05$ for fluoxetine; $F=7.348$, $p<0.05$ for sertraline; $F=10.191$, $p<0.05$ for imipramine; Fig. 2*b*). We also found that ketamine increased phosphorylation of Akt and ERK ($F=6.169$, $p<0.05$ for phospho-Ser^{473}-Akt; Fig. 2*a*; $F=7.993$, $p<0.05$ for phospho-Thr^{202}/Tyr^{204}-ERK; Fig. 2*b*) under B27 deprivation. These findings suggest that escitalopram, paroxetine and tranylcypromine induction of mTOR signalling is mediated by PI3K/Akt and MEK/ERK signalling and that other antidepressant drugs may be mediated by mTOR-independent activation of Akt and ERK.

Effects of PI3K, MEK or mTOR inhibitors on antidepressant drug-induced mTOR phosphorylation

To examine the possibility that activation of mTOR signalling induced by ketamine, escitalopram, paroxetine and tranylcypromine in hippocampal cultures is mediated by Akt and ERK signalling, we administered three signalling inhibitors (the PI3K inhibitor, LY294002; the MEK inhibitor, PD98059; and the mTOR inhibitor, rapamycin) in the presence or absence of drugs under B27 deprivation. Our preliminary studies showed that LY294002 and rapamycin concentrations ranging from 0.01 to 1 μM and PD98059 concentrations ranging from 0.5 to 50 μM

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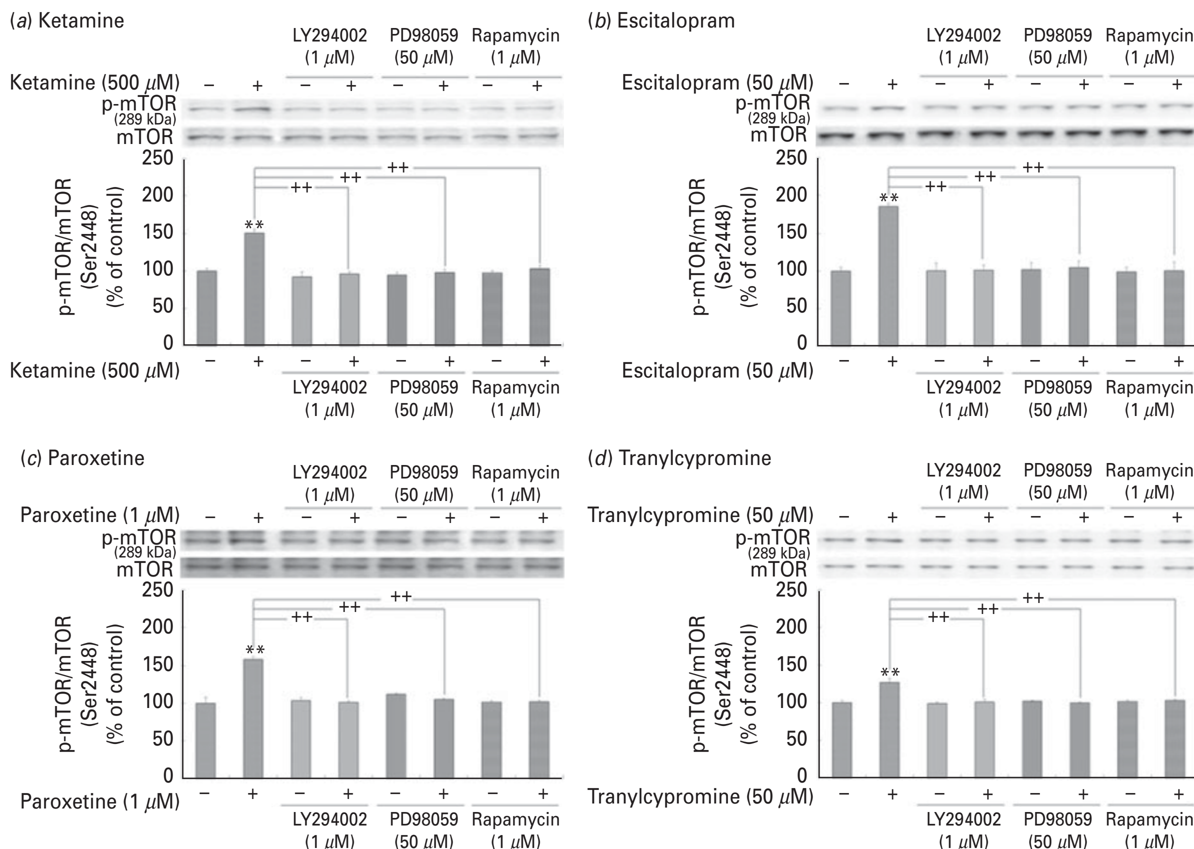

Fig. 3. Effects of PI3K, MEK and mTOR inhibitors on the enhancement of mTOR phosphorylation induced by antidepressant drugs in hippocampal neurons. B27-deprived cells were pre-treated with LY294002 (PI3K inhibitor, 1 μm), PD98059 (MEK inhibitor, 50 μm) and rapamycin (mTOR inhibitor, 1 μm) for 30 min before treatment with ketamine (500 μm, *a*), escitalopram (50 μm, *b*), paroxetine (1 μm, *c*), tranylcypromine (50 μm, *d*), or DMSO (control) for four days. Three independent experiments were performed. A representative Western blot for phospho-Ser^{2448}-mTOR and quantitative analysis normalized to the total mTOR band are shown. Values represent means±s.e.m expressed as percentages of the values of non-drug-treated, B27-deprived cells. ** $p<0.01$ *vs.* control cells, $^{++}p<0.01$ *vs.* drug-treated only cells.

had no significant effects on mTOR phosphorylation or cellular viability; higher concentrations reduced cellular viability (data not shown). Thus, the highest concentrations (1 μm for LY294002 and rapamycin; and 50 μm for PD98059) that did not affect both the levels of mTOR phosphorylation and cell viability were selected. Drug concentrations were selected at concentrations that produced maximum effects on mTOR phosphorylation (i.e. 500 μm for ketamine, 50 μm for escitalopram and tranylcypromine, and 1 μm for paroxetine).

These drug-induced increases in mTOR phosphorylation were completely blocked by LY294002, PD98059 and rapamycin (ketamine: $F=29.458$, $p<0.001$ for LY294002; $F=32.906$, $p<0.001$ for PD98059; $F=32.024$, $p<0.001$ for rapamycin; Fig. 3*a*; escitalopram: $F=27.846$, $p<0.001$ for LY294002; $F=27.846$, $p<0.001$ for PD98059; $F=29.528$, $p<0.001$ for rapamycin; Fig. 3*b*; paroxetine: $F=30.779$, $p<0.001$ for LY294002; $F=31.618$, $p<0.001$ for PD98059; $F=35.644$, $p<0.001$ for rapamycin; Fig. 3*c*; tranylcypromine: $F=11.440$, $p<0.001$ for LY294002; $F=13.727$, $p<0.001$ for PD98059; $F=12.695$, $p<0.001$ for rapamycin; Fig. 3*d*). Taken together, these data indicate that escitalopram, paroxetine and tranylcypromine activate mTOR signalling via the same mechanism as ketamine, through both PI3K/Akt and MEK/ERK signalling in hippocampal neurons.

Effects of antidepressant drugs on dendritic outgrowth

Ketamine has been shown to increase synapse density and dendritic spine formation in the prefrontal cortex of rats; the latter effect is blocked by infusion of rapamycin (Li et al., 2010b). To investigate whether mTOR signalling contributes to the regulation of dendritic outgrowth induced by ketamine in hippocampal neurons, we performed a neurite outgrowth assay. In the control conditions, hippocampal cells were incubated for five days with ketamine concentrations ranging from 50 to 500 μm. Ketamine at these concentrations significantly increased dendritic outgrowth of hippocampal neurons ($F=18.294$, $p<0.001$, Fig. 4). The maximum effect of ketamine on dendritic outgrowth was observed at 100 μm.

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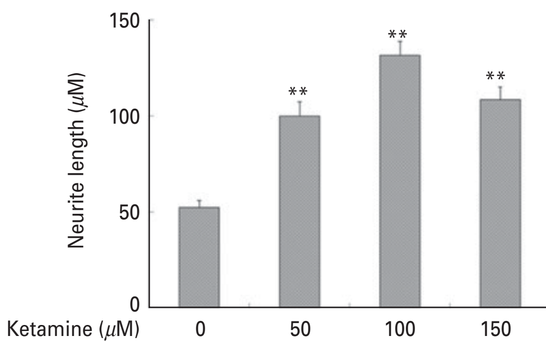

Fig. 4. Effects of ketamine on dendritic outgrowth in hippocampal neurons. In the control conditions, cells were treated with varying doses of ketamine for five days. Two independent experiments were performed. Dendritic outgrowth was scored. In total, 600–800 cells were analysed from each sample. Data are expressed as means±S.E.M. $^{**}p<0.01$ *vs.* control cells.

In a similar fashion, ketamine increased branching (Supplementary Fig. S1A) and did not affect the soma area (Supplementary Fig. S1B). We next examined whether rapamycin blocks the ability of ketamine to enhance dendritic outgrowth. Rapamycin concentrations ranging from 0.01 to 1 μM had no significant effects on dendritic outgrowth or cell viability, but higher concentrations reduced cell viability (data not shown). Thus, the highest rapamycin concentration (1 μM) that did not affect both dendritic outgrowth and cell viability was selected. Hippocampal cells were photographed (Fig. 5*a*) and scored to quantify dendritic outgrowth, branching and soma area. The potentiating effects of ketamine on dendritic outgrowth ($F=77.964$, $p<0.001$, Fig. 5*b*) and branching ($F=46.947$, $p<0.001$, Fig. 5*c*) was significantly decreased by pre-treatment with rapamycin. Neither rapamycin nor a combination of rapamycin and ketamine affected soma area (Fig. 5*d*).

In our previous study, different classes of antidepressant drugs increased dendritic outgrowth in hippocampal neurons (Seo et al., 2014). To determine whether mTOR signalling mediates the enhancement of outgrowth by different classes of antidepressant drugs, we examined the effects of rapamycin on antidepressant drug-induced dendritic outgrowth. In the control conditions, hippocampal cells were incubated for five days with antidepressant drugs at concentrations that produced maximal effects on dendritic outgrowth in the previous study (Seo et al., 2014). Representative images in Fig. 6*a* show the effects of rapamycin on the enhancement of dendritic outgrowth induced by antidepressant drugs. All antidepressant drugs tested significantly enhanced dendritic outgrowth in hippocampal neurons (control= 54.8 μm, escitalopram=86.9 μm, paroxetine=81.0 μm, tranylcypromine=90.9 μm, fluoxetine=69.1 μm, sertraline= 75.8 μm, and imipramine=79.0 μm, $p<0.05$ or $p<0.001$; Fig. 6*b*). However, pre-treatment with 1 μM rapamycin blocked the effects of escitalopram, paroxetine and tranylcypromine ($F=21.170$, $p<0.001$ for escitalopram; $F=24.802$, $p<0.001$ for paroxetine; $F=11.826$, $p<0.001$ for tranylcypromine; Fig. 6*b*). These data suggest that mTOR signalling is involved in the enhancement of dendritic outgrowth induced by these drugs. On the other hand, rapamycin did not block the effects of fluoxetine, sertraline or imipramine. Similar effects were also observed on branching ($F=15.287$, $p<0.001$ for escitalopram; $F=20.859$, $p<0.001$ for paroxetine; $F=13.135$, $p<0.001$ for tranylcypromine; Fig. 6*c*). None of the treatments affected the soma area (Fig. 6*d*).

Effects of antidepressant drugs on synaptic protein expression

Because mTOR signalling plays an important role in new synapse formation, we further examined whether mTOR signalling contributed to the regulation of synaptic protein expression induced by ketamine and antidepressant drugs in B27-deprived hippocampal cultures.

Western blotting analysis revealed that ketamine caused significant increases in levels of the post-synaptic marker PSD-95 and the pre-synaptic marker synaptophysin ($p<0.001$), and these effects were blocked by pre-treatment with rapamycin ($F=38.94$, $p<0.001$ for PSD-95, Fig. 7*a*; $F=56.15$, $p<0.001$ for synaptophysin, Fig. 7*b*).

Our previous study also revealed that antidepressant drugs significantly increase levels of PSD-95 and synaptophysin under B27 deprivation (Seo et al., 2014). Thus, hippocampal cells were incubated for five days with antidepressant drugs at concentrations that produced maximal effects on these synaptic proteins levels in hippocampal neurons. As shown in Fig. 8, all antidepressant drugs tested significantly increased the levels of PSD-95 and synaptophysin (PSD-95: control, 100%, escitalopram, 171%, paroxetine, 171%, tranylcypromine, 171%, fluoxetine, 173%, sertraline, 172% and imipramine, 175%, $p<0.001$; Fig. 8*a*; SYP: control, 100%, escitalopram, 159%, paroxetine, 173%, tranylcypromine, 184%, fluoxetine, 168%, sertraline, 162% and imipramine, 162%, $p<0.001$; Fig. 8*b*). However, rapamycin pre-treatment completely blocked the effects of escitalopram, paroxetine and tranylcypromine on these synaptic proteins (PSD-95: $F=39.200$, $p<0.001$ for escitalopram; $F=24.461$, $p<0.001$ for paroxetine; $F=9.382$, $p<0.001$ for tranylcypromine; Fig 8*a*; SYP: $F=19.800$, $p<0.001$ for escitalopram; $F=20.852$, $p<0.001$ for paroxetine; $F=19.200$, $p<0.001$ for tranylcypromine; Fig. 8*b*). On the other hand, rapamycin treatment did not affect changes in the levels of synaptic proteins induced by fluoxetine, sertraline, or imipramine. These data indicate that the effects of escitalopram, paroxetine and tranylcypromine on the levels of synaptic proteins are mediated by mTOR signalling in hippocampal neurons.

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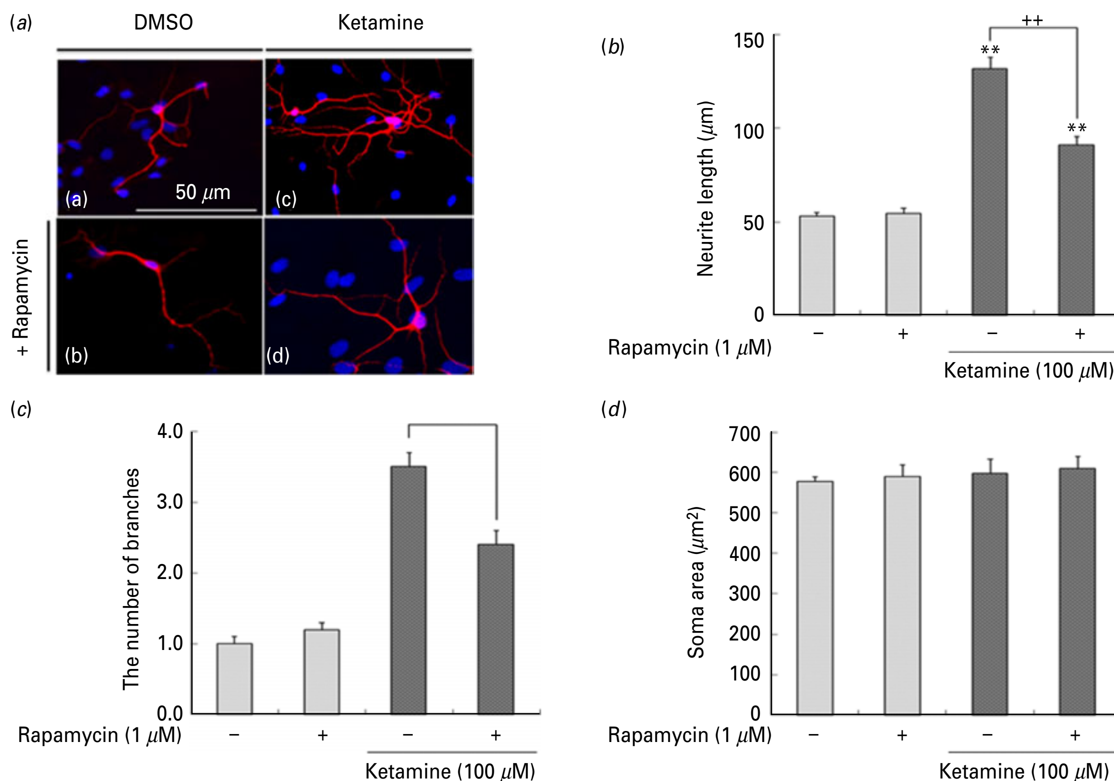

Fig. 5. mTOR inhibitor effects on the enhancement of dendritic outgrowth induced by ketamine in hippocampal neurons. In the control conditions, cells were pre-treated with rapamycin (rapamycin 1 μM) for 30 min, then treated with ketamine (100 μM) or DMSO (control) for five days. Two independent experiments were performed. Cells were photographed (*a*), and dendritic outgrowth (*b*), branching (*c*) and soma area (*d*) was scored. In total, 600–800 cells were analysed from each sample. Data are expressed as means±S.E.M. $^{**}p<0.01$ *vs.* control cells, $^{++}p<0.01$ *vs.* cells treated only with ketamine.

Discussion

The present study is the first to demonstrate that in cultured hippocampal neurons, ketamine, escitalopram, paroxetine and tranylcypromine activate mTOR signalling, and enhance dendritic outgrowth and the levels of synaptic proteins via mTOR signalling. On the other hand, fluoxetine, sertraline and imipramine treatment did not result in similar effects, suggesting that these drug-induced increases in dendritic outgrowth and levels of synaptic proteins may be associated with other signalling pathways involving Akt or ERK.

There is much evidence linking mTOR signalling to synaptic plasticity, memory and neurological disorders (Hoeffer and Klan, 2010). Recent studies reported that phosphorylation of mTOR and its down-stream signalling components (e.g. p70S6K, S6, or elF4B) is significantly decreased in the amygdala of rats exposed to chronic stress and in the prefrontal cortex of individuals diagnosed with major depressive disorder (Jernigan et al., 2011; Chandran et al., 2012). We also observed that immobilization stress significantly decreases BDNF expression and phosphorylation of mTOR and p70S6K in the hippocampus of rats (Fang et al., 2013). Based on these studies, one could hypothesize that mTOR activation followed by increased phosphorylation of down-stream components may underlie antidepressant action.

In fact, ketamine increases phosphorylation of mTOR, 4E-BP-1 and p70S6K in the rat prefrontal cortex (Li et al., 2010b). These effects are consistent with our findings in hippocampal neurons. Recently, it was reported that ketamine stimulates glutamate transmission in the rat prefrontal cortex, resulting in BDNF release and leading to activation of mTOR signalling via Akt and ERK, which consequently stimulates synaptic protein synthesis; these increases are thought to contribute to the rapid antidepressant-like effects of ketamine in behavioural models of depression (Duman and Voleti, 2012; Duman et al., 2012). More recently, it was also reported that acute treatment of rats with ketamine decreased immobility time in the forced swim test and increased expression of BDNF and mTOR in the hippocampus, suggesting an underlying link between antidepressant action and BDNF and mTOR (Yang et al., 2013). On the other hand, antidepressant drugs, such as imipramine or fluoxetine and electroconvulsive treatment do not affect mTOR signalling in the rat prefrontal cortex (Li et al., 2010b). Interestingly, the present

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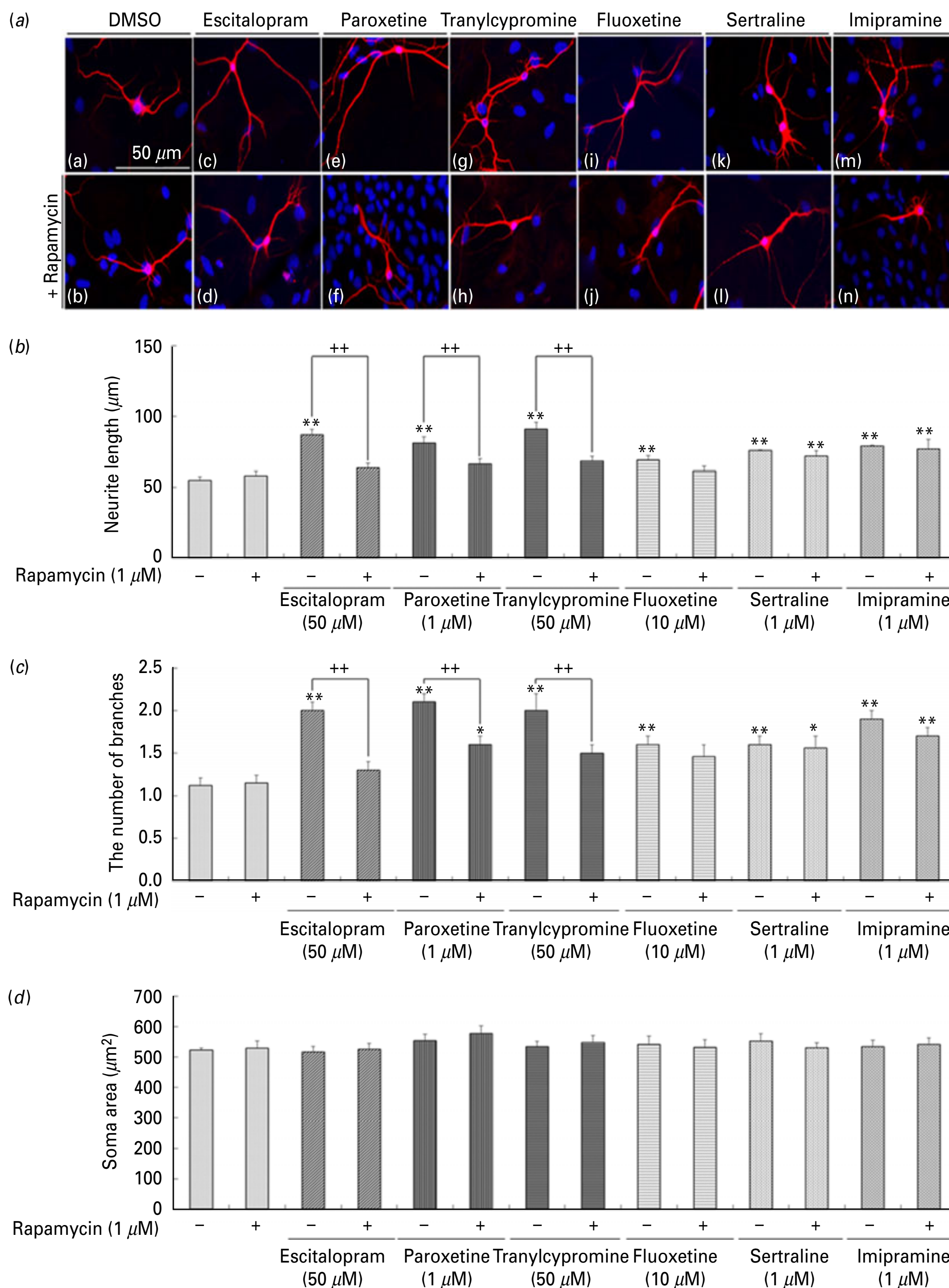

Fig. 6. mTOR inhibitor effects on the enhancement of dendritic outgrowth induced by antidepressant drugs in hippocampal neurons. In the control conditions cells were pre-treated with; rapamycin [1 μM, (b)] for 30 min, then treated with escitalopram [50 μM, (c) and (d)], paroxetine [1 μM, (e) and (f)] tranylcypromine [50 μM, (g) and (h)], fluoxetine [10 μM, (i) and (j)], sertraline [1 μM, (k) and (l)], imipramine [1 μM, (m) and (n)] or DMSO [(a) control] for five days. Two independent experiments were performed. Cells were photographed *(a)*, and dendritic outgrowth *(b)*, branching *(c)*, and soma area *(d)* was scored. In total, 600–800 cells were analysed from each sample. Data are expressed as means±S.E.M. $^{**}p<0.01$ *vs.* control (DMSO-treated) cells, $^{++}p<0.01$ *vs.* cells treated only with antidepressant drugs.

study found that escitalopram, paroxetine and tranylcypromine activated mTOR signalling in hippocampal neurons, whereas fluoxetine, sertraline and imipramine had no effect.

BDNF is a key molecule involved in neural plasticity (McAllister et al., 1999). Many studies suggest that BDNF-induced neural plasticity contributes to the actions of antidepressant drugs (Pittenger and Duman, 2008).

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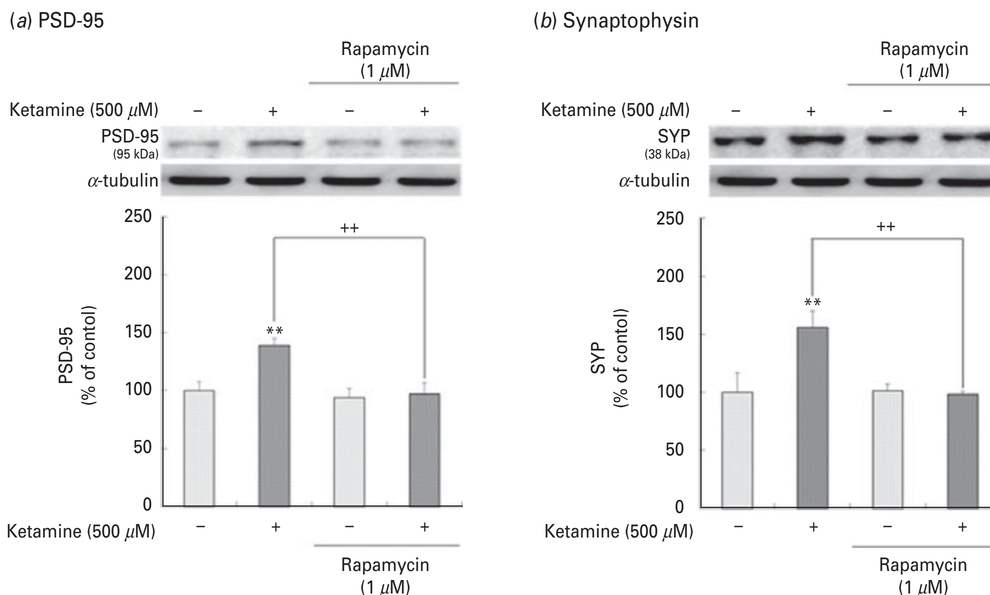

Fig. 7. Effects of ketamine or the combination of ketamine and an mTOR inhibitor on expression of synaptic proteins in hippocampal neurons. B27-deprived cells were pre-treated with rapamycin (1 μM) for 30 min and then treated with ketamine (500 μM) or DMSO (control) for four days. Three independent experiments were performed. Representative Western blots for PSD-95 (*a*) and synaptophysin (*b*) and quantitative analysis normalized to α-tubulin are shown. Values represent means±S.E.M expressed as percentages of the values of non-ketamine-treated, B27-deprived cells. $^{**}p<0.01$ *vs.* control cells, $^{++}p<0.01$ *vs.* cells treated only with ketamine.

Different classes of antidepressant drugs, including SSRIs, TCAs, SNRIs and MOAIs, increase BDNF expression in the hippocampus (Pittenger and Duman, 2008). These increases can also be accompanied by activation of the cyclic-adenosine monophosphate (cAMP) signalling pathway. Phosphorylation of cAMP response element-binding protein (CREB), a key target in the action of antidepressant drugs, occurs via activation of the cAMP/protein kinase A (PKA) pathway in response to different classes of antidepressant drugs (Pittenger and Duman, 2008). Consequently, CREB activation leads to increased expression and secretion of BDNF, which acts on TrkB receptors (Pittenger and Duman, 2008). BDNF-mediated TrkB down-stream signalling includes activation of PI3K/Akt and MEK/ERK (Pizzorusso et al., 2000; Rodgers and Theibert, 2002). Alternatively, G-protein-coupled receptors (GPCRs) can directly transactivate TrkB receptors through Src tyrosine kinase activity (Lee et al., 2002). Thus, antidepressant drugs can induce activation of TrkB receptors via Src kinase down-stream of GPCR in a BDNF-independent manner.

It is generally accepted that mTOR acts as a node of convergence down-stream of PI3K/Akt and MEK/ERK signalling (Hay and Sonenberg, 2004; Mendoza et al., 2011). On the other hand, activation of PI3K/Akt signalling causes inhibition of glycogen synthase kinase-3β (GSK-3β) activity by phosphorylation of serine (Li et al., 2007), which produces antidepressant-like effects in the forced swim test in rodents (Gould et al., 2004; Kaidanovich-Beilin et al., 2004; Rosa et al., 2008). GSK-3β is also phosphorylated and inhibited following SSRI treatment (Li and Jope, 2010). Activation of MEK/ERK signalling increases CREB phosphorylation. In the present study, all antidepressant drugs tested significantly increased phosphorylation of Akt and ERK. Specifically, among different classes of antidepressant drugs, escitalopram, paroxetine and tranylcypromine significantly increased phosphorylation of mTOR, and these effects were also blocked by specific inhibitors of PI3K, ERK, or mTOR, implying that these drugs can also stimulate mTOR signalling via activation of both PI3K/Akt and MEK/ERK. On the other hand, fluoxetine, sertraline and imipramine can stimulate both PI3K/Akt/GSK-3β and MEK/ERK/CREB signaling.

Dendritic morphology plays a critical role in synaptic integration and information processing (Jan and Jan, 2003). Recently, much attention has been directed to mTOR signalling, which is important for dendrite formation and regulation of spine growth (Li et al., 2010a; Okada et al., 2011). Indeed, ketamine-activated mTOR signalling rapidly increases dendritic spine formation in the rat prefrontal cortex and also increases the number of mushroom spines, an indication of increased spine maturation and synaptic strengthening. These effects of ketamine are directly inhibited by the mTOR inhibitor rapamycin (Li et al., 2010b). Similar to these results, ketamine increased hippocampal dendritic outgrowth in the present study, and this effect was prevented by rapamycin.

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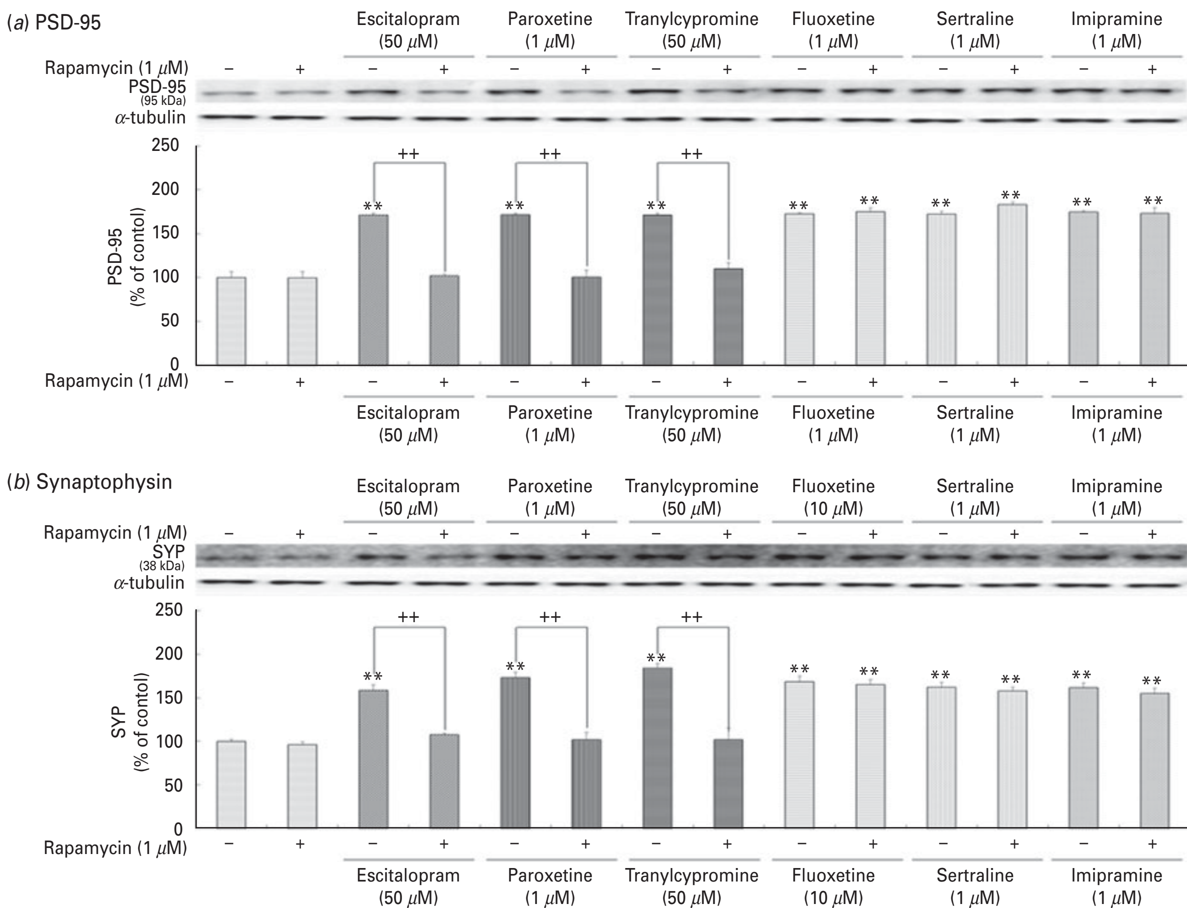

Fig. 8. mTOR inhibitor effects on the enhancement of synaptic proteins induced by antidepressant drugs in hippocampal neurons. B27-deprived cells were pre-treated with rapamycin (1 μM) for 30 min before treatment with escitalopram (50 μM), paroxetine (1 μM), tranylcypromine (50 μM), fluoxetine (1 μM for PSD-95 level, 10 μM for synaptophysin level), sertraline (1 μM), imipramine (1 μM) or DMSO (control). Three independent experiments were performed. A representative Western blot for PSD-95 (*a*) and synaptophysin (*b*) and quantitative analysis normalized to α-tubulin are shown. Values represent means±S.E.M expressed as percentages of the values of non-rapamycin-treated, B27-deprived cells. $^{**}p<0.01$ *vs.* control cells, $^{++}p<0.01$ *vs.* cells treated only with antidepressant drugs.

Also consistent was our observation that all antidepressant drugs enhanced dendritic outgrowth. Our previous study also showed that calcium/calmodulin kinase II (CaMKII), PKA or PI3K signalling was partially involved in the dendritic outgrowth-promoting effects of different classes of antidepressant drugs in hippocampal neurons. Moreover, the present study revealed that the dendritic outgrowth-promoting effects of escitalopram, paroxetine and tranylcypromine were blocked by rapamycin, whereas the effect of other drugs was not. Taken together, these results suggest that escitalopram-, paroxetine- and tranylcypromine-induced increases in dendritic outgrowth may be associated with mTOR signalling via activation of both PI3K/Akt and MEK/ERK, but other antidepressant drugs may be associated with other signalling through activation of Akt and ERK as these drugs activated these kinase.

Mendoza et al. (2011) demonstrated that PI3K- and ERK-mTOR signalling regulate each other and co-regulate down-stream functions via cross-activation and cross-inhibition. Further research is needed to examine whether PI3K-and ERK-mTOR cross talk are involved in antidepressant drug-induced dendritic growth. The present study simply identifies mTOR as the main signal involved in escitalopram-, paroxetine- and tranylcypromine-induced enhancement of hippocampal dendritic outgrowth.

PSD-95 acts as a scaffold protein that regulates the clustering of glutamate receptors in dendritic spines (Han and Kim, 2008). Appropriate levels of PSD-95 are required for synaptic maturation, strengthening and plasticity. Thus, an increase in PSD-95 protein levels may reflect an increase in the number and size of dendritic spines, which in turn results in a greater number of synapses. Synaptophysin, a Ca^{2+}-binding synaptic vesicle membrane protein, is required for vesicle fusion and neurotransmitter release (Valtorta et al., 2004). Therefore, increased levels of synaptophysin could indicate increased pre-synaptic activity. Given the functions of PSD-95 and synaptophysin, the current results suggest

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that different classes of antidepressant drugs may induce new synapse formation in hippocampal neurons (Seo et al., 2014).

Activation of mTOR signalling has been linked to the protein synthesis required for the formation of new spine synapses (Tang and Schuman, 2002; Klann et al., 2004; Abe et al., 2010; Hashimoto, 2011). Ketamine administration increased the levels of the post-synaptic proteins, PSD-95 and GluR1, as well as of the pre-synaptic protein synapsin I. Rapamycin infusion completely blocked the induction of these synaptic proteins (Li et al., 2010b). Consistent with these results, we observed that ketamine enhances expression of PSD-95 and synaptophysin in hippocampal neurons and that rapamycin blocks these effects.

The present study showed that escitalopram, fluoxetine, paroxetine, sertraline, imipramine and tranylcypromine significantly increased the levels of PSD-95 and synaptophysin. This result is in line with our previous data demonstrating an increase in the expression of the synaptic proteins, PSD-95, synaptophysin and BDNF, after treatment with different classes of antidepressant drugs (Seo et al., 2014). In the present study, the effects of escitalopram, paroxetine and tranylcypromine on the expression of PSD-95 and synaptophysin were completely blocked by the mTOR inhibitor rapamycin, whereas the effects of fluoxetine, sertraline and imipramine were not.

Primary cortical neurons treated with BDNF up-regulate protein synthesis by increasing levels of active eukaryotic elongation factor 2 (eEF2), a down-stream target of mTOR (Takei et al., 2009). Additionally, eEF2 levels can be regulated in an mTOR-independent manner, possibly by ERK activation (Wang et al., 2001). Thus, fluoxetine, sertraline and imipramine may increase synthesis of the synaptic proteins PSD-95 and synaptophysin by an mTOR-independent mechanism through ERK activation. However, a limitation of the present study is it did not address whether signalling via ERK or Akt mediated these effects, as it focused only the effects of antidepressant drugs on mTOR signalling. Additional studies are needed to fully examine the signalling pathways by which fluoxetine, sertraline and imipramine promote synthesis of synaptic proteins.

All antidepressant drugs used in the present study increased PSD-95 and synaptophysin expression, but escitalopram, paroxetinem, and tranylcypromine caused greater dendritic outgrowth. This may not mean that escitalopram, paroxetine and tranylcypromine have greater effects on dendritic outgrowth, because the concentrations of antidepressant drugs varied among treatment types. For comparison, therapeutic concentrations must be administered to up-regulate dendritic outgrowth and synaptic protein levels *in vivo*. Moreover, the number of dendritic spine must be considered *in vivo*, due to the possibility that imipramine and fluoxetine increase dendritic spine synapse formation and the number of

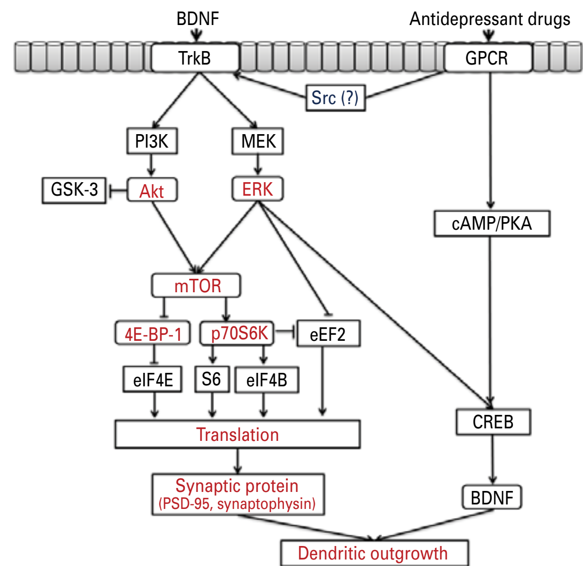

Fig. 9. Signalling pathways regulated by different classes of antidepressant drugs. Antidepressant drugs up-regulate cAMP/PKA/CREB signalling. CREB can also be regulated by MEK/ERK signalling. CREB activation increases BDNF expression. Release of BDNF stimulates TrkB and down-stream signalling pathways (PI3K/Akt and MEK/ERK). These pathways stimulate mTOR signalling, which activates p70S6K and 4E-BP-1 and increases translation for synthesis of synaptic proteins. GPCR: G-protein coupled receptor; TrkB: tyrosine-related kinase B; Src: sarcoma; PI3K: phosphoinositide 3-kinase; MEK: MAP/ERK kinase; ERK: extracellular signal-regulated kinases; GSK-3: glycogen synthase kinase-3; mTOR: mammalian target of rapamycin; 4E-BP-1: 4E-binding protein 1; p70S6K: p70 ribosomal protein S6 kinase; eEF2: eukaryotic elongation factor 2; eIF4E: eukaryotic translation initiation factor 4E; S6: small ribosomal protein 6; eIF4B: eukaryotic translation initiation factor 4B; PSD-95: post-synaptic density 95; cAMP: cyclic-adenosine monophosphate; PKA: protein kinase A; CREB: cAMP response element binding protein; BDNF: Brain-derived neurotrophic factor. The molecular pathways shown in red illustrate author's novel observations, and those in black are generally accepted signalling pathways for antidepressant action.

synapses in the rat hippocampus (Hajszan et al., 2005; Chen et al., 2008, 2010). Further research examining dendritic spine morphology and synaptic density is required.

Because mTOR is expressed in neurons and glial cells, the effects we observe may result from its roles in both cell types. Thus, the present study needs to be confirmed in animals. Further studies are also necessary to characterize the time correlation of the reported effects in animal models, as the rapid antidepressant response to ketamine suggests rapid effects on mTOR activity.

Furthermore, rapamycin has been shown to have positive behavioural effects in animal models of tuberous sclerosis complex (TSC), related to abnormal mTOR signalling (Wong, 2013). While sub-chronic treatment (seven days) with rapamycin had no effect on forced

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swim behaviour in healthy Wistar rats (Russo et al., 2013), this treatment caused antidepressant-like effects in the WAG/Rij rat, an animal model of epilepsy exhibiting depressive-like behaviour. On the other hand, chronic treatment (17 wk) caused depressive-like behaviour in both strains. These results suggest that mTOR might have different roles depending on the underlying pathology.

Although antidepressant drugs show positive effects on dendritic outgrowth and synaptic proteins levels from our *in vitro* data, it is not clear whether these drugs reach concentrations *in vivo* that are sufficient to activate mTOR signalling. The concentrations of antidepressant drugs used here to produce positive effects are generally higher than the concentrations normally observed in plasma. This discrepancy might be attributable to the different conditions between cell culture and brain tissue.

Overall, the results of the present study suggest that different classes of antidepressant drugs might regulate synaptic plasticity by changing dendritic outgrowth and the levels of synaptic proteins in hippocampal neurons; some antidepressant drugs require mTOR signalling to produce these changes (Fig. 9). The clinical effects of these drugs are not identical to the effect of ketamine, which produces rapid antidepressant action in depressed patients. Thus, these findings cannot lead to firm conclusions regarding the relationship between mTOR-mediated changes induced by some antidepressant drugs and the clinical effects of these drugs. However, the results of this study suggest that mTOR signalling may be a promising target for the development of new antidepressant drugs.

Supplementary material

For supplementary material accompanying this paper, visit http://dx.doi.org/10.1017/S1461145714000534.

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Statement of Interest

None.

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