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Everolimus improves memory and learning while worsening depressive- and anxiety-like behavior in an animal model of depression

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ABSTRACT

Everolimus (EVR) is an orally-administered rapamycin analog that selectively inhibits the mammalian target of rapamycin (mTOR) kinase (mainly mTORC1 and likely mTORC2) and the related signaling pathway. mTOR is a serine/threonine protein kinase regulating multiple important cellular functions; dysfunction of mTOR signaling has also been implicated in the pathophysiology of several neurological, neurodegenerative, developmental and cognitive disorders. EVR is widely used as an anti-neoplastic therapy and more recently in children with tuberous sclerosis complex (TSC). However, no clear correlation exists between EVR use and development of central side effects *e.g.* depression, anxiety or cognitive impairment. We studied the effects of a 3 weeks administration of EVR in mice chronically treated with betamethasone 21-phosphate disodium (BTM) as a model of depression and cognitive decline. EVR treatment had detrimental effects on depressive- and anxiety-like behavior while improving cognitive performance in both control (untreated) and BTM-treated mice. Such effects were accompanied by an increased hippocampal neurogenesis and synaptogenesis. Our results therefore might support the proposed pathological role of mTOR dysregulation in depressive disorders and confirm some previous data on the positive effects of mTOR inhibition in cognitive decline. We also show that EVR, possibly through mTOR inhibition, may be linked to the development of anxiety. The increased hippocampal neurogenesis by EVR might explain its ability to improve cognitive function or protect from cognitive decline. Our findings suggest some caution in the use of EVR, particularly in the developing brain; patients should be carefully monitored for their psychiatric/neurological profiles in any clinical situation where an mTOR inhibitor and in particular EVR is used *e.g.* cancer treatment, TSC or immunosuppression.

1. Introduction

Chronic stress has been implicated as a possible factor in the etiology of psychiatric disorders including anxiety and depression. However, the specific relationship between stress depression and anxiety, remain unclear (Kendler et al., 1999; Mitchell et al., 2003).

Hyperactivation of the hypothalamic-pituitary-adrenal axis is associated with the development of depressive symptomatology; this connection was underlined from evidence that depressed patients often exhibit hypercortisolism (Pariante and Lightman, 2008), while patients undergoing long-term glucocorticoid therapy, often develop psychiatric and cognitive symptoms (Starkman, 2013). Similarly, features of depression/anxiety-like behavior have

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been observed in laboratory animals following chronic glucocorticoid administration. For example, rodents of several strains treated long-term with corticosterone (CORT) show higher levels of depression and anxiety (Aiello et al., 2015; Murray et al., 2008; Sterner and Kalynchuk, 2010) and an impairment in cognitive performance (Aiello et al., 2015; Sousa et al., 2000). Additionally, chronic CORT exposure in rodents causes a significant neuronal remodeling in brain regions associated with depression (*i.e.* hippocampus and amygdala) in agreement with observations of morphological changes in depressed patients' brain (Campbell et al., 2004; Gourley et al., 2013). Furthermore, in rodents, chronic CORT treatment reduces hippocampal neurogenesis (Crupi et al., 2010), although strain differences were also found (Aiello et al., 2015; Hodes et al., 2012). According to the 'neurogenesis hypothesis' of depression, a decrease in the production of newborn hippocampus granule cells is related to the disease pathophysiology (Hanson et al., 2011); however, studies show no direct link between decreased neurogenesis and onset of depression (Petrik et al., 2012; Tang et al., 2012).

Everolimus (EVR) is an orally-administered rapamycin analog that selectively inhibits mTOR kinase activity (mainly mTORC1 and likely mTORC2) (Hasskarl, 2014; Sarbassov et al., 2006). mTOR is a serine/threonine protein kinase regulating multiple important cellular functions; dysfunction of mTOR signaling has also been implicated in the pathophysiology of several neurological disorders (Russo et al., 2012; Wong, 2013). EVR is widely used as an antineoplastic therapy and recently in children with tuberous sclerosis complex (TSC) (Curatolo and Moavero, 2012; Kotulska et al., 2013). In humans, it has been observed that EVR treatment may provide significant improvement in memory and psychiatric symptoms among heart transplant recipients (Lang et al., 2009); conversely, in renal transplant patients receiving rapamycin treatment, cognitive *impairment,* was observed (Martinez-Sanchis et al., 2011). Moreover, no clear correlation was observed between the use of EVR and side effects such as depression, anxiety or cognitive impairment (Curatolo and Moavero, 2012; Hasskarl, 2014). Recently one study has demonstrated that EVR treatment (14 days) had no influence on animal behavior and neurogenesis in mice without any underlying disease (Dubois et al., 2014).

Compared with EVR, the effects of rapamycin on the central nervous system (CNS) have been better characterized; however, opposite results have been reported. Subchronic rapamycin treatment resulted in antidepressant-like activity while long-term treatment *increased* depressive-like behavior (Cleary et al., 2008; Russo et al., 2013c). Moreover, rapamycin significantly decreased depressive and anxiety-like behaviors in mice, possibly by stimulating major monoamine pathways in the brain (Halloran et al., 2012). Rapamycin has shown relevant effects on cognitive performance in rodent models (Brewster et al., 2013; Majumder et al., 2012; Spilman et al., 2010); whereas, systemic inhibition of mTOR prevents hippocampal neurogenesis (Paliouras et al., 2012; Raman et al., 2011, 2013).

The involvement of mTOR signaling in major depressive disorder (MDD) has been suggested by the recent observation that the *rapid* antidepressant effects of ketamine both in animals and in patients, are associated with mTOR activation (Duman et al., 2012) and that depressive symptomatology is correlated with mTOR dysfunction in the prefrontal cortex (Jernigan et al., 2011). Moreover, the inhibitory effect of long-term CORT treatment on mTOR signaling, in mouse cells (Howell et al., 2011), has been indicated as a possible mechanism underlying its pro-depressant activity. Based on this background, we investigated here the effects of EVR treatment on animal behavior and neurogenesis in an animal model of chronic stress induced by chronic administration of betamethasone 21-phosphate disodium (BTM) in DBA/2 mice.

2. Materials and methods

2.1. Animals

Male DBA/2 mice (8 weeks old) were purchased from Charles River Laboratories s.r.l (Calco, Lecco, Italy). Mice were housed five per cage and maintained under stable conditions: humidity (60 ± 5%), temperature (21 ± 2 °C), reversed light/dark (12/12 h) cycle (light on at 19.00) and libitum access to food and water. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. Procedures involving animals and their care were conducted in conformity with the international and national laws and policies (EU Directive, 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept).

2.2. Experimental design

DBA/2 mice were randomized into 4 groups ($n = 20$ animals/group) according to treatment: CTRL group assigned to oral vehicle (water) for 7 weeks; EVR group orally treated with vehicle for the first 4 weeks and, for the subsequent 3 weeks, given oral EVR (5 mg/kg/day; Novartis Pharmaceutical Development, Basel, Switzerland); BTM group was given oral BTM (0.25 mg/kg/day; Sigma Aldrich, Milan, ITALY) for 7 consecutive weeks. Finally, the BTM-EVR group was treated with oral BTM for 7 consecutive weeks and in the last 3 weeks, oral EVR was added to the BTM treatment (Fig. 1A). BTM and EVR were given *ad libitum* in the drinking water in bottles protected from light. Final dilution was calculated on the evidence that mice drink on average 10 ml/100 g/day; this was further confirmed by checking the volume drunk by mice (Aiello et al., 2015). EVR (5 mg/kg/day) and BTM (0.25 mg/kg/day) dosage were chosen according to previous studies (Aiello et al., 2015; O'Reilly et al., 2010). Mice were weighted three times a week for the entire duration of the experiments. Additionally, the mouse coat condition was observed and scored as previously described (Crupi et al., 2011). A different group of mice was only treated with EVR and phospho-p70S6 Kinase (Ser371) was measured by western blotting to ascertain whether drug treatment would inhibit mTOR pathway in the hippocampus (see Supplementary Material S1).

2.3. Behavioral tests

Every experimental group was divided in two subgroups of 10 animals for testing (Test I: Morris water maze group; Test II: All others paradigms used in the following order: Forced swimming test; Open field arena; Elevated plus maze; Novelty suppressed feeding); in Test II series, inter-test interval was at least 1 day (range 1–3 days). All tests were carried out with the support of EthoVision XT8 Software (Noldus, Netherlands) (Aiello et al., 2015).

2.4. Forced swimming test (FST)

The FST has been previously used for measuring the immobility time (IT) and assessing depressive-like behavior in rodents (Nestler and Hyman, 2010; Russo et al., 2013c). Mice were placed individually for 6 min into glass cylinders (height 26.5 cm, diameter 16.5 cm) containing water, maintained at 25 °C, as previously described (Citraro et al., 2015). The IT was scored and analyzed for the last 4 min (Aiello et al., 2015; Russo et al., 2011b).

2.5. Open field test (OFT)

The OFT was a Plexiglas box (50 × 50 cm) divided into 16 squares. The center of the box was defined by 4 squares. The

following variables were measured: time spent in center, number of center entries, total distance moved and mean velocity during a 10 min test (Citraro et al., 2014; Crupi et al., 2011). Reduced exploratory (locomotor) activity in the OFT is usually considered as a measure of increased levels of anxiety/emotionality and vice-versa (Russo et al., 2013b).

2.6. Elevated plus maze test (EPM)

The EPM apparatus consists of two opposing open arms (45 cm × 10 cm) and two opposing closed arms of the same size with walls 10 cm high. The arms were connected by a central platform (10 × 10 cm) as previously described (Russo et al., 2013a). The number of entries into, time spent on each arm and central square were scored for 10 min. The shorter the time spent in open arms and central square, the greater is the number of risk assessments, the higher is anxiety and vice-versa (Crupi et al., 2010; Russo et al., 2013b).

2.7. Novelty-suppressed feeding test (NSF)

The apparatus used for the test was a plastic box (50 × 50 cm) as previously described (Aiello et al., 2015). Twenty-four hours before behavioral testing, food was removed from the home cage. At the time of testing, a single pellet of food (regular chow) was placed in the center of the box as previously reported (Crupi et al., 2011). The latency to begin eating was used as an index of anxiety-like behavior (Crupi et al., 2011).

2.8. Morris water maze test (MWM)

Learning and memory performance was assessed using MWM test (Russo et al., 2013a; Vorhees and Williams, 2006). The apparatus consisted of a circular basin (diameter = 93 cm, height = 45 cm) filled with water (approximately 25 °C) to a depth of 24 cm, with a hidden escape platform (diameter = 8 cm) placed 1 cm below the water surface. Mice ($n = 10$/group) were trained for 4 consecutive days, with 4 trials on each day. The single trial duration was 60 s(s). MWM was performed as previously described (Aiello et al., 2015; Russo et al., 2013a).

2.9. Immunohistochemistry

To label newly-born cells, we used incorporation of the thymidine analogue BrdU into DNA during the S-phase of the cell cycle, as previously described (Aiello et al., 2015; Crupi et al., 2011). BrdU was administered intraperitoneally at 150 mg/kg in saline. Mice were sacrificed 2 h after injection. After anesthesia with sodium pentobarbital (30 mg/kg), mice were transcardially perfused (cold saline, followed by 4% cold paraformaldehyde in phosphate buffered saline (PBS)). All brains were post-fixed overnight in 4% paraformaldehyde at 4 °C, then cryoprotected in 30% sucrose, and stored at 4 °C. Immunohistochemistry was performed, as previously described (Aiello et al., 2015; Crupi et al., 2011).

2.10. Golgi impregnation

Golgi impregnation was performed according to the directions supplied by FD NeuroTechnologies (FD NeuroTechnologies, Ellicott City, MD, USA). Blocks of brain tissue were placed directly into solutions A and B, without rinsing, and remained there for 2 weeks in the dark at room temperature. Forty-eight hours after placing the blocks in solution C (4 °C), the blocks were frozen on dry ice and stored at 70 °C until sectioning. Cryostat sections (100 μm) were cut and mounted onto gelatinized slides. The tissue had to be stained dark with uniform Golgi impregnation that was uniform throughout the section, to be selected for the analysis. Neurons chosen for tracing met the criteria previously described (Aiello et al., 2015). Cells chosen for analysis had to be well impregnated, clearly distinguishable from adjacent cells and have continuous unbroken dendrites. Spines were counted under oil (X100) using light microscopy, and the entire visible dendritic length measured by imaging computer program (Axio-Vision, Zeiss). Spine density was calculated referring to the length of the dendrite (Crupi et al., 2011).

2.11. Statistical analysis

All statistical procedures were performed using SPSS 15.0.0 software (SPSS Inc., Chicago, Illinois, USA). Comparisons were performed using two-way Analysis Of Variance (ANOVA) followed by Tukey's post-hoc test. Statistically significant differences were considered at $P \leq 0.05$.

3. Results

3.1. Coat state and body weight

The measure of the coat state of the animals has been described as a reliable and well-validated index of a depressed-like state (Crupi et al., 2010, 2011). Long-term oral BTM treatment induced both a significant deterioration of the coat-state of mice (data not shown) and a significant reduction in body weight ($F_{(1,36)} = 7.99$; $P < 0.01$), starting from the second week of treatment, as shown in Fig. 1B. Both these parameters were not significantly re-established after EVR oral administration. On the other hand, mice treated with EVR alone did not differ from control vehicle-treated mice.

3.2. Forced swimming test (FST)

In the FST, long-term BTM treatment had no behavioral effects (Fig. 1D) whereas oral administration of EVR significantly increased ($F_{(1,36)} = 13.24$; $P < 0.001$) the IT both in BTM-treated and vehicle-treated mice. No significant differences were measured between the two EVR-treated groups. Furthermore, independently from BTM treatment, a significant reduction ($F_{(1,36)} = 12.09$; $P < 0.01$) in total distance moved and mean velocity in both EVR-treated groups (EVR and BTM-EVR) in comparison to respective control groups (CTRL and BTM groups, respectively), was observed (Table 1).

3.3. Open field test (OFT)

As shown in Fig. 1G and H, in the OFT, the time spent in the center and the number of entries in the center, were not significantly changed by exogenous chronic BTM treatment. EVR administration, for 3 weeks, significantly reduced these parameters ($F_{(1,36)} = 12.41$; $P < 0.05$) both in BTM-treated and vehicle-treated mice. EVR effects were similar in the two groups. In particular, EVR chronic treatment was able to reduce the time spent in the center by ~39% in vehicle-treated mice and ~34% in the BTM-treated group (Fig. 1G); furthermore, in the EVR group in comparison with the CTRL group, a 47% reduction in the number of entries in the center was found, whereas in the BTM-EVR group, versus BTM-group, the percentage decrease was ~55% (Fig. 1H). Chronic BTM treatment significantly reduced, in the OFT, both the total distance moved and the mean velocity; furthermore, despite BTM treatment, a significant reduction ($F_{(1,36)} = 10.19$; $P < 0.01$) was observed in both parameters in the EVR-treated groups (EVR and BTM-EVR) in comparison to control groups (CTRL and BTM groups, respectively) (Table 1).

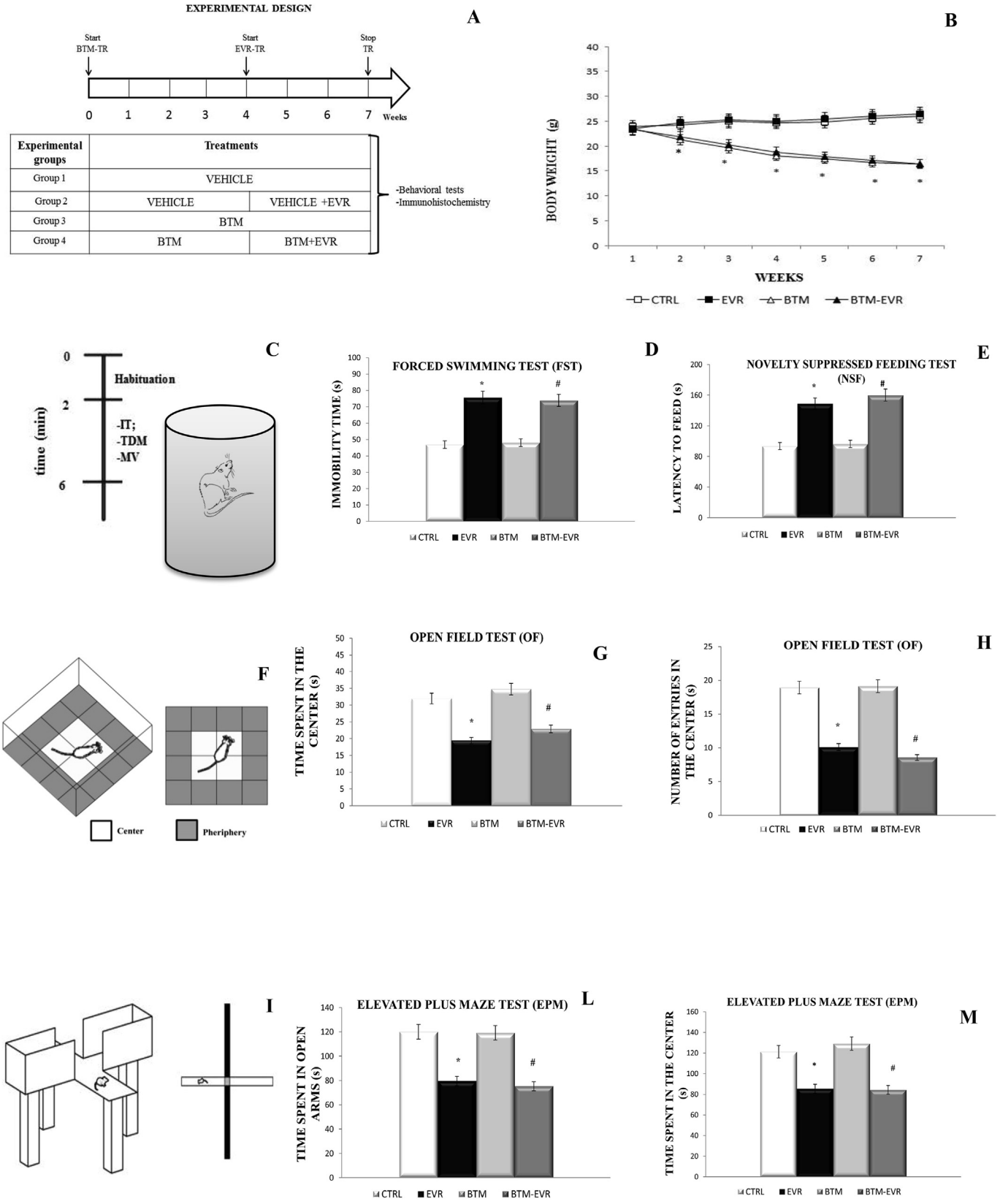

Fig. 1. (A) Schematic representation of the experimental design. DBA/2 mice were orally administered during 7 weeks with vehicle or betamethasone 21-phosphate disodium (BTM; 0.25 mg/kg/day) in the presence or absence of oral everolimus (EVR; 5 mg/kg/day) during the last 3 weeks. (B) Mice body weight (grams) monitored during the 4 weeks of BTM oral administration and the 3 weeks of EVR oral treatment. (D) IT in the FST, expressed in seconds (s). (E) Latency to feed in the Novelty Suppressed Feeding Test (NSF), expressed in

Table 1
Everolimus (EVR) effects on total distance moved (TDM) and mean velocity (MV) in the forced swimming test, open field test and elevated plus maze test.

Groups	TDM (cm)	MV (cm/s)
Forced swimming test (FST)		
CTRL	1815.36 ± 113.96	8.06 ± 0.79
EVR	1275.86 ± 77.96*	4.21 ± 0.22*
BTM	1843.63 ± 69.17	8.45 ± 0.45
BTM-EVR	1397.78 ± 42.30*#	4.45 ± 0.14*#
Open field test (OFT)		
CTRL	5223.61 ± 192.97	8.83 ± 0.33
EVR	3720.53 ± 142.67*	6.17 ± 0.15*
BTM	3815.53 ± 86.63*	4.94 ± 0.15*
BTM-EVR	2902.54 ± 99.89*#	2.76 ± 0.23*#
Elevated plus maze test (EPM)		
CTRL	2591.41 ± 165.40 cm	4.42 ± 0.29 cm/s
EVR	1478.19 ± 103.54 cm*	3.12 ± 0.24 cm/s*
BTM	1637.19 ± 77.08 cm*	3.02 ± 0.21 cm/s*
BTM-EVR	1043.89 ± 28.98 cm*#	1.76 ± 0.21 cm/s*#

Mean velocity (MV; cm/s; Mean ± SEM) and total distance moved (TD; cm; Mean ± SEM) in the FST, OF and EPM. Data marked with "*" are significantly different ($p < 0.01$) from CTRL-group. Data marked with "**#**" are significantly different ($p < 0.01$) from BTM-group. **CTRL**: group assigned to oral vehicle (water) for 7 weeks; **EVR**: group orally treated with vehicle for the first 4 weeks and, for the subsequent 3 weeks, given oral Everolimus; **BTM**: group treated with oral BTM for 7 consecutive weeks; **BTM-EVR**: group treated with oral Betamethasone 21-phosphate disodium (BTM) only for the first 4 weeks and, for the subsequent 3 weeks, oral EVR was added to BTM treatment.

3.4. Elevated plus maze test (EPM)

As shown in Fig. 1L and M, both the time spent in central square (CS) and the time spent in open arms (OA) were not significantly modified by exogenous chronic BTM (0.25 mg/kg/day) treatment. Similarly to the results obtained in the OFT, also in EPM test, EVR administration, significantly reduced these parameters ($F_{(1,36)} = 14.78$; $P < 0.05$) in both BTM-treated and vehicle-treated mice. Specifically, in the EVR group, in comparison to CTRL-group, a reduction of ~30% in the time spent in the central square was found, whereas in the BTM-EVR group, versus BTM-group, the percentage decrease of this parameter was ~35% (Fig. 1L). Furthermore, EVR treatment decreased the time spent in the open arms by ~34% in vehicle-treated mice, and ~37% in BTM-treated mice (Fig. 1M). Similarly to the OFT, also in the EPM test, chronic BTM exposure significantly reduced ($F_{(1,36)} = 10.80$; $P < 0.01$) both the total distance moved and the mean velocity; furthermore, despite BTM treatment, both EVR treated groups (EVR and BTM-EVR), in comparison to their controls (CTRL and BTM groups, respectively) showed significantly ($P < 0.01$) lower scores (Table 1).

3.5. Novelty suppressed feeding test (NSF)

In the NSF test, a long-term BTM treatment had no effects, whereas, as shown in Fig. 1E, EVR oral administration significantly increased ($F_{(1,36)} = 13.52$; $P < 0.01$) the latency to feed in both BTM-treated and vehicle-treated mice. In particular, in EVR and BTM-EVR groups, versus their controls, an increase of ~60% and ~66% in the latency to feed was observed respectively.

3.6. Morris water maze test (MWM)

As shown in Fig. 2B, BTM chronic treatment caused a significant impairment in learning and spatial memory ($F_{(1,36)} = 11.14$; $P < 0.01$) with a longer latency to find the platform during daily trials starting from day 2. In particular, BTM-treated mice showed a progressive impairment on experimental days with an increase in latency to platform ranging from 50 to 86%. Furthermore, as shown in Fig. 2D, BTM significantly reduced ($F_{(1,36)} = 9.83$; $P < 0.01$) the time spent in the former platform quadrant on day 5.

EVR administration significantly reduced the latency to find the platform, already starting from day 2, in both BTM-treated and vehicle-treated mice. Specifically, EVR-treated mice showed a progressive improvement on experimental days with a reduction in latency to platform ranging from 28 to 54% and 32–47% for EVR and BTM-EVR groups, respectively (Fig. 2B). Furthermore, EVR significantly increased ($F_{(1,36)} = 10.36$; $P < 0.01$) the time spent in the former platform quadrant on day 5 (Fig. 2D) in both BTM-treated and untreated animals. Chronic BTM treatment significantly reduced, both the total distance moved and the mean velocity during the four experimental days and the probe trial on day 5; EVR treatment did not significantly modify both parameters (Table 2).

3.7. Immunohistochemistry

Chronic BTM and EVR exposure significantly ($F_{(1,36)} = 8.08$; $P < 0.05$) increased, both BrdU and DCX-positive cells, in the dentate gyrus (DG) of the adult mouse hippocampus (see Fig. 3A–D). Tracing apical dendrites in DG granule cells also revealed a significant ($F_{(1,36)} = 9.24$; $P < 0.05$) increase of spine density number after administration of BTM and EVR (see Fig. 3E–F).

4. Discussion

The mTOR pathway has been implicated in the pathophysiology of MDD both in experimental animal models and humans (Cambiaghi et al., 2013; Jernigan et al., 2011; Russo et al., 2013c) and more recently it has been indicated as a suitable pharmacological target for novel antidepressant drug development (Abelaira et al., 2014). Deficits in mTOR signaling in the prefrontal cortex (PFC) of rodents and humans contribute to the development of depressive symptomatology (Abelaira et al., 2014; Jernigan et al., 2011). Accordingly, recent findings suggest that the rapid antidepressant effects of the anesthetic agent ketamine, a selective and potent N-methyl-D-aspartate (NMDA) glutamate receptor/ion channel blocker, are strongly related to the activation of mTOR function, followed by enhanced mTOR-dependent protein synthesis in the PFC of rats (Duman et al., 2012; Jernigan et al., 2011; Li et al., 2010). In particular, after ketamine-induced mTOR activation, the increase in protein synthesis seems to be caused by stimulation of p70S6K and 4E-BP1. In fact, p70S6K promotes both the synthesis of ribosomal unit S6 and the protein translation, which is also augmented by the hyper-phosphorylation of 4E-BP1. Furthermore, in the PFC, it was detected that ketamine, through mTOR activation, increases the synthesis of proteins involved in the process of synaptogenesis such as PSD95, synapsin I and GluR1 (Li et al., 2010; Scheuing et al., 2015). In fact, in the PFC of several animal depression models as

seconds (s). (G) Time spent in the center in the OFT, expressed in seconds (s). (H) Number of entries in the center in the OFT. (L) Time spent in the center of the EPM, expressed in seconds (s). (M) Time spent in open arms of the EPM, expressed in seconds (s). **CTRL**: group assigned to oral vehicle (water) for 7 weeks; **EVR**: group orally treated with vehicle for the first 4 weeks and, for the subsequent 3 weeks, given oral Everolimus; **BTM**: group treated with oral BTM for 7 consecutive weeks; **BTM-EVR**: group treated with oral Betamethasone 21-phosphate disodium (BTM) only for the first 4 weeks and, for the subsequent 3 weeks, oral EVR was added to BTM treatment. TR: treatment; IT: immobility time; TDM: total distance moved; MV: mean velocity. Values plotted are mean ± S.E.M. Data marked with "*" are significantly different ($P < 0.01$) from CTRL group; datum marked with (#) is significantly different ($P < 0.01$) from BTM-treated group.

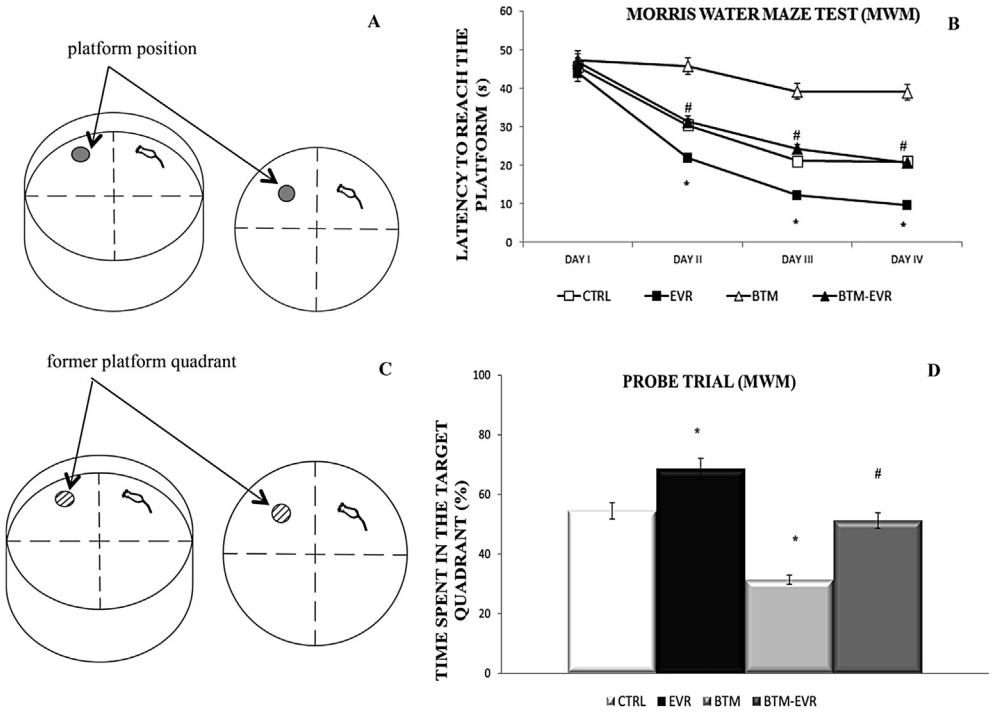

Fig. 2. Everolimus (EVR) effects on cognitive performance in the Morris Water Maze test. (B) Morris water maze results. Latency to platform during daily trials (seconds). BTM chronic treatment caused a significant impairment in learning and spatial memory with a longer latency to find the platform during daily trials starting from day 2. EVR administration for 3 weeks significantly reduced the latency to find the platform, already starting from day 2, in both BTM-treated and vehicle-treated mice. (D) Morris water maze results. Time spent in target quadrant in the probe trial on day 5 (%). Chronic BTM significantly reduced the time spent in the former platform quadrant on day 5 whereas, EVR exposure for 3 weeks significantly increased this parameter in both BTM-treated and untreated animals. **CTRL**: group assigned to oral vehicle (water) for 7 weeks; **EVR**: group orally treated with vehicle for the first 4 weeks and, for the subsequent 3 weeks, given oral Everolimus; **BTM**: group treated with oral BTM for 7 consecutive weeks; **BTM-EVR**: group treated with oral Betamethasone 21-phosphate disodium (BTM) only for the first 4 weeks and, for the subsequent 3 weeks, oral EVR was added to BTM treatment. Values plotted are mean ± S.E.M. Data marked with "*" are significantly different ($P < 0.01$) from CTRL group; datum marked with (#) is significantly different ($P < 0.01$) from BTM-treated group.

well as in subjects with major depressive disorder (MDD), a reduction both in the synaptic spine density proteins (Feyissa et al., 2009) and in the protein levels of mTOR and p70S6K (Jernigan et al., 2011) was observed. Furthermore, Park et al. (2014) have also reported that several widely prescribed antidepressants including escitalopram and paroxetine significantly increased, in primary neuronal cell cultures, the levels of phospho-mTOR and its downstream regulator phospho-p70S6K. According to the authors, the augmented protein synthesis was matched with an increased synaptogenesis (Park et al., 2014).

Moreover, it has been shown that intracerebroventricular (i.c.v) infusion of rapamycin, into the PFC of rats, completely abolishes ketamine's ability to ameliorate depressive symptoms, suggesting that the effect of ketamine is mTOR-dependent (Li et al., 2010). Thus, inhibition of mTOR signaling experimentally would seem to be pro-depressant; however, there are no data demonstrating the potential pro-depressant effects of mTOR inhibitors in patients. Conversely, a clinical study demonstrated that EVR is able to *ameliorate* psychiatric symptoms and *improve* cognitive functions in heart transplant recipients (Lang et al., 2009).

Our results indicate that 3 weeks of treatment with EVR, at a dose corresponding to the one used in clinical practice (O'Reilly et al., 2010), does indeed induce depressive- and anxiety-like behavior in DBA/2 mice independently from an underlying stress. However, the DBA/2 mice used in behavioral tests (Costall et al., 1989; Fish et al., 2010) already show an anxious behavioral profile in comparison to CD1 strain (Aiello et al., 2015) while having shorter IT in the FST; these data are in agreement with previous reports regarding the effects of *chronic* rapamycin treatment on depressive behavior. Specifically, *acute* treatment with rapamycin had no effects on depressive-like behavior in rodents (Cleary et al., 2008; Russo et al., 2014) although the development of an anxiety-like phenotype following systemic administration of a single dose of rapamycin in rats has recently been demonstrated (Hadamitzky et al., 2014). In this light, considering our protocol it could be argued that EVR effects might be due to the last administration of the drug more than to a chronic effect; although this point cannot be excluded, generally mTOR inhibitors seem to

Table 2
Everolimus (EVR) effects on total distance moved (TDM) and mean velocity (MV) in the Morris Water Maze test.

Day	CTRL	EVR	BTM	BTM-EVR
Total distance moved (cm)				
1	410.59 ± 14.08	406.11 ± 8.30	341.75 ± 8.23*	338.27 ± 13.30*
2	352.52 ± 9.42	341.65 ± 23.03	312.01 ± 15.96*	307.51 ± 6.54*
3	335.97 ± 8.18	325.87 ± 7.57	303.78 ± 9.41*	298.40 ± 8.75*
4	303.79 ± 7.21	304.53 ± 16.99	257.39 ± 5.35*	259.93 ± 12.42*
PROBE	294.51 ± 20.64	297.71 ± 1.85	229.41 ± 5.59*	226.89 ± 2.02*
Mean velocity (cm/s)				
1	25.15 ± 1.02	22.98 ± 1.02	18.62 ± 0.85*	17.19 ± 0.55*
2	30.09 ± 0.75	27.81 ± 1.35	19.27 ± 0.59*	17.70 ± 0.85*
3	37.22 ± 1.21	35.11 ± 0.63	22.11 ± 0.78*	20.99 ± 0.73*
4	38.18 ± 0.67	36.06 ± 1.29	24.12 ± 0.65*	23.23 ± 1.19*
PROBE	36.06 ± 0.74	34.00 ± 1.17	25.17 ± 1.11	23.34 ± 0.69*

Mean velocity (MV; cm/s; Mean ± SEM) and total distance moved (TD; cm; Mean ± SEM) in the MWM test during daily trials and probe trial. **CTRL**: group assigned to oral vehicle (water) for 7 weeks; **EVR**: group orally treated with vehicle for the first 4 weeks and, for the subsequent 3 weeks, given oral Everolimus; **BTM**: group treated with oral BTM for 7 consecutive weeks; **BTM-EVR**: group treated with oral Betamethasone 21-phosphate disodium (BTM) only for the first 4 weeks and, for the subsequent 3 weeks, oral EVR was added to BTM treatment.

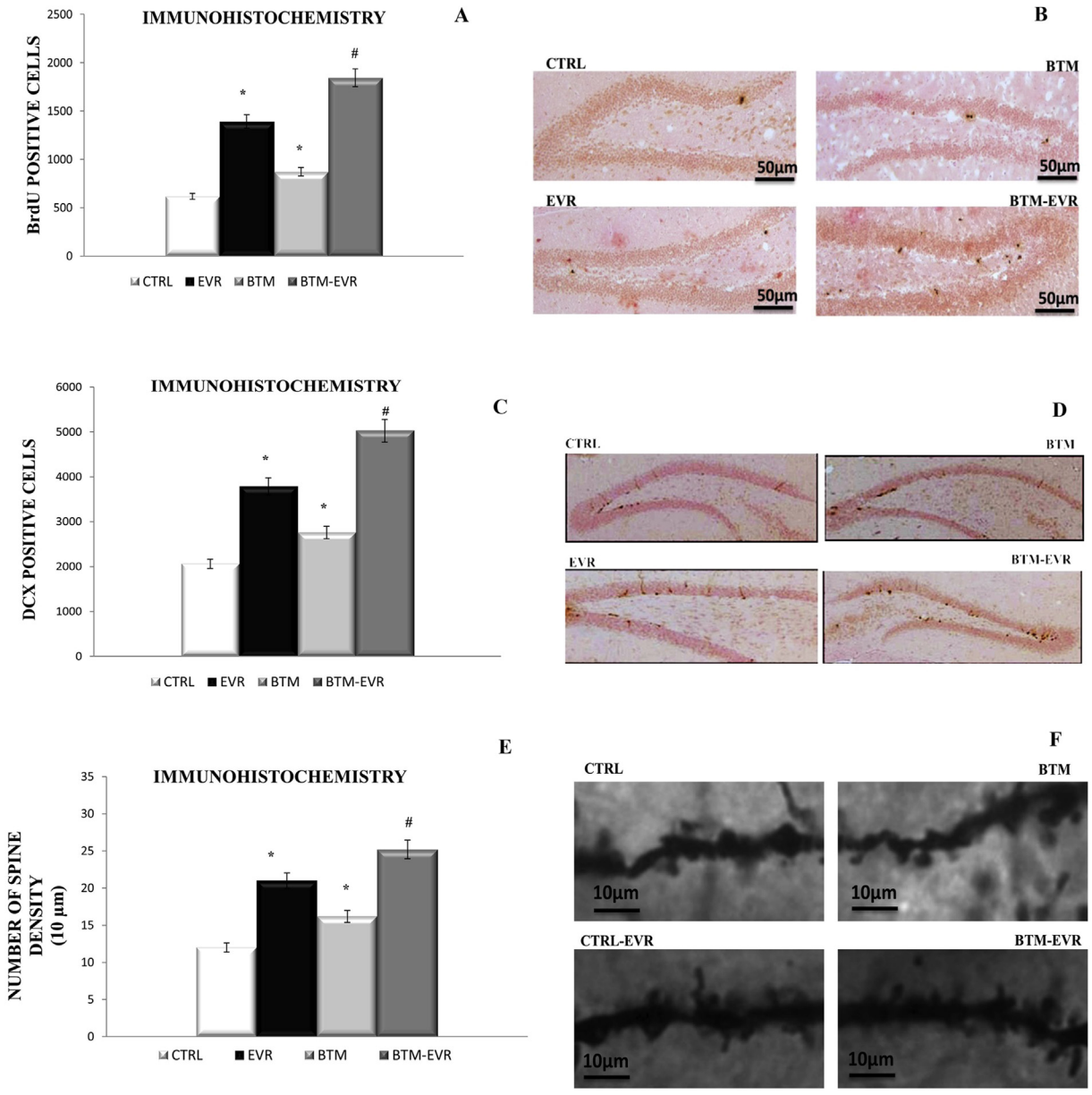

Fig. 3. Everolimus (EVR) effects on neurogenesis and synaptogenesis. (A) Cell proliferation in the dentate gyrus (DG) of mice hippocampus: number of BrdU-positive cells. Chronic BTM and EVR exposure significantly increased BrdU-positive cells in the dentate gyrus (DG) of the adult mouse hippocampus. (B) Representative images of BrdU immunohistochemistry for CTRL, EVR, BTM and BTM-EVR groups, respectively. (C) Cell proliferation in the dentate gyrus of mice hippocampus: number of DCX-positive cells. A significant increase in DCX-positive cells, after chronic BTM and EVR administration, in the adult mouse hippocampus, was found. (D) Representative images of DCX immunohistochemistry for CTRL, EVR, BTM and BTM-EVR groups, respectively. (E) Number of spine density in the dentate gyrus granule cells of mice hippocampus. Long-term BTM and EVR treatment significantly increased the number of spine density in the DG of mice hippocampus. (F) Representative images of spine density number in DG of hippocampus, for CTRL, EVR, BTM and BTM-EVR groups, respectively. **CTRL**: group assigned to oral vehicle (water) for 7 weeks; **EVR**: group orally treated with vehicle for the first 4 weeks and, for the subsequent 3 weeks, given oral Everolimus; **BTM**: group treated with oral BTM for 7 consecutive weeks; **BTM-EVR**: group treated with oral Betamethasone 21-phosphate disodium (BTM) only for the first 4 weeks and, for the subsequent 3 weeks, oral EVR was added to BTM treatment. Values plotted are mean ± S.E.M. Data marked with "*" are significantly different ($P < 0.01$) from CTRL group; datum marked with (#) is significantly different ($P < 0.01$) from BTM-treated group.

have poor effects after acute administration and in any case, the results observed might represent the sum of chronic and acute effects. Finally, our administration protocol leads to stable concentrations during the day in experimental animals (Russo et al., 2011a, 2013c). In agreement, sub-chronic exposure to rapamycin produces *anti*depressant-like properties in both rats and mice (Cleary et al., 2008; Russo et al., 2013c) whereas, long-term rapamycin administration can have either pro-depressant activity (Russo et al., 2013c) or antidepressive-anxiolytic-like effects (Cambiaghi et al., 2013; Halloran et al., 2012) depending on the animal model tested.

To date, studies endorse a possible role of autophagy in depression. However, the signaling pathways of autophagy are very complex and still not fully understood, in fact, there are several crucial points where the pathways could be modulated. Therefore, by virtue of this it is difficult to address the link among mTOR, autophagy and depression (Jia and Le, 2015). However, it was reported how several antidepressants are able to promote this

process in neuronal cells (Gassen et al., 2014; Zschocke et al., 2011). According to Cleary et al. (2008) the antidepressant-like effect, observed after sub-chronic treatment with the mTOR inhibitor rapamycin, could be linked to an induction of autophagy, which is also regulated by mTOR pathway (Egan et al., 2011).

Dubois et al. (2014) recently showed that EVR orally administered for 14 consecutive days did not affect emotional reactivity, spontaneous activity, learning and memory, behavioral flexibility and object recognition memory when mice were tested starting from 7 days after EVR withdrawal. These data are in contrast with our results both for mouse behavior and memory/learning results. Considering that we have used the same dose, these differences might be attributed both to the shorter duration of the treatment (14 *vs.* 21 days) and the protocol used, since in our experiments tests were carried out while mice were still under drug treatment. Furthermore, it is not possible to exclude that EVR might differentially affect animal behavior also in a genetic background-dependent manner, similarly to the differences observed for BTM (Aiello et al., 2015).

Interestingly, EVR treatment had positive effects on cognitive performance both in BTM-treated and control (vehicle-treated) mice. This result, together with the above reported data for depressive and anxiety-like behavior, indicate that EVR effects are independent of the current background pathology and the drug is very likely to directly affect CNS function in areas involved in psychological and cognitive functions. Indeed, differences in the EVR response were observed between BTM and vehicle-treated mouse groups, however, the experimental outcome was not in contrast and, even if effectiveness measured as the extent of the modification observed in comparison to the respective control group might be different, EVR had consistent effects, worsening animal behavioral and emotional responses while improving cognitive performance.

EVR effects are in agreement with some previous results observed using rapamycin, which ameliorated age-dependent learning and memory deficits (Majumder et al., 2012) and decreased cognitive decline associated with neurological disorders such as *status epilepticus* (Brewster et al., 2013) and Alzheimer's disease (Spilman et al., 2010). In this scenario, it is not surprising that EVR effects on neurogenesis and synaptogenesis in our study are controversial. Rapamycin's ability to *reduce* neurogenesis in rodents has been extensively demonstrated (Paliouras et al., 2012; Raman et al., 2011, 2013; Russo et al., 2012). In the study by Dubois et al. (2014), EVR had no effects on neural precursor cell proliferation or vascular component distribution in the hippocampus after 14 days of treatment. We found a BTM-dependent *increase* in neurogenesis and synaptogenesis in the hippocampus, in agreement with our previous findings (Aiello et al., 2015). EVR itself, both in BTM and vehicle-treated animals, had similar effects, increasing neurogenesis and synaptogenesis; however, these effects were accompanied by a worsening in animal behavior and an improvement in cognitive performance. These effects are in contrast both to: 1) our own data, where BTM increased neurogenesis and impaired cognitive function without affecting mouse behavior (Aiello et al., 2015), and 2) previous data supporting a negative correlation between depression and neurogenesis (Eisch and Petrik, 2012). Indeed, the correlation between neurogenesis and neurological/psychiatric diseases/functions is still not well understood (Eisch and Petrik, 2012; Hanson et al., 2011; Petrik et al., 2012; Tang et al., 2012). In this light, our results show once again the difficulty in predicting drug effects only from immunohistochemical results. Overall, EVR-dependent altered neurogenesis appears in line with the improvement in cognitive function and in contrast with increased depressive- and anxiety-like behavior.

4.1. Conclusions

In conclusion, we report for the first time the potential detrimental effects of chronic EVR administration on animal behavior correlated with depression and anxiety. Furthermore, we confirm the possible positive effects of this drug on cognitive performance; this point seems to be linked to an increased neurogenesis and synaptogenesis in the hippocampus. However, this latter effect either does not influence animal behavior or is contributing to its aggravation. Our results, demonstrating the pro-depressant properties of EVR both in control and BTM-treated animals, supports and might extend the knowledge that deficits in the mTOR signaling are involved in the pathophysiology of depression, further extending its potential role in anxiety. For the first time, we show that EVR and possibly other mTOR inhibitors might improve cognitive function or protect from cognitive decline by increasing neurogenesis. Overall, EVR effects on neurogenesis underline the need for a critical re-evaluation of this phenomenon in all brain diseases indicating that alterations in neurogenesis might not be directly correlated with measurable outcomes such as depression or cognitive decline. Finally, our findings suggest caution in the clinical use of EVR, above all in the developing brain; patients should be carefully monitored for their psychiatric/neurological profiles in any clinical situation where an mTOR inhibitor will be used *e.g.* cancer treatment, TSC or immunosuppression.

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Contributors

Russo and Cuzzocrea designed the study and wrote the protocol. Constanti, De Sarro and Citraro managed the literature searches and analyses. Aiello, Lippiello and Leo undertook the statistical analysis, and Russo wrote the first draft of the manuscript. Crupi, Aiello, Chimirri, Citraro and Leo managed animal experiments. Spiga performed western blotting experiments. All authors contributed to and have approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpsychires.2016.03.008.

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