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Repeated Systemic Treatment with Rapamycin Affects Behavior and Amygdala Protein Expression in Rats

Martin Hadamitzky, Arne Herring, Julia Kirchhof, Ivo Bendix, Matthew J. Haight, Kathy Keyvani, Laura Lückemann, Meike Unteroberdörster, Manfred Schedlowski

Institute of Medical Psychology and Behavioral Immunobiology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany (Dr Hadamitzky, Ms Kirchhof, Dr Lückemann, Dr Schedlowski); Institute of Neuropathology, University Hospital Essen, Essen, Germany (Drs Herring and Keyvani); Department of Pediatrics I/ Experimental perinatal Neuroscience, University Hospital Essen, University of Duisburg-Essen, Essen, Germany (Dr Bendix); Department of Anesthesia, School of Medicine, University of San Francisco, San Francisco CA (Dr Haight); Department of Neurosurgery, University Hospital Essen, University of Duisburg-Essen, Essen, Germany (Dr Unteroberdörster); Department of Clinical Neuroscience, Osher Center for Integrative Medicine, Karolinska Institutet, Stockholm, Sweden (Dr Schedlowski).

Correspondence: Martin Hadamitzky, PhD, Institute of Medical Psychology and Behavioral Immunobiology, University Hospital Essen, University of Duisburg-Essen, Hufelandstrasse 55, 45122 Essen, Germany (martin.hadamitzky@uk-essen.de).

Abstract

Background: Clinical data indicate that therapy with small-molecule immunosuppressive drugs is frequently accompanied by an incidence rate of neuropsychiatric symptoms. In the current approach, we investigated in rats whether repeated administration of rapamycin, reflecting clinical conditions of patients undergoing therapy with this mammalian target of rapamycin inhibitor, precipitates changes in neurobehavioral functioning.
Methods: Male adult Dark Agouti rats were daily treated with i.p. injections of rapamycin (1, 3 mg/kg) or vehicle for 8 days. On days 6 and 7, respectively, behavioral performance in the Elevated Plus-Maze and the Open-Field Test was evaluated. One day later, amygdala tissue and blood samples were taken to analyze protein expression ex vivo.
Results: The results show that animals treated with rapamycin displayed alterations in Elevated Plus-Maze performance with more pronounced effects in the higher dose group. Besides, an increase in glucocorticoid receptor density in the amygdala was seen in both treatment groups even though p-p70 ribosomal S6 kinase alpha, a marker for mammalian target of rapamycin functioning, was not affected. Protein level of the neuronal activity marker c-Fos was again only elevated in the higher dose group. Importantly, effects occurred in the absence of acute peripheral neuroendocrine changes.
Conclusions: Our findings indicate that anxiety-related behavior following rapamycin treatment was not directly attributed to mTOR-dependent mechanisms or stress but rather due to hyperexcitability of the amygdala together with glucocorticoid receptor-regulated mechanism(s) in this brain region. Together, the present results support the contention that subchronic treatment with rapamycin may induce neurobehavioral alterations in healthy, naive subjects. We here provide novel insights in central effects of systemic rapamycin in otherwise healthy subjects but also raise the question whether therapy with this drug may have detrimental effects on patients' neuropsychological functioning during immune therapy.

Keywords: rapamycin, anxiety, elevated plus-maze, amygdala, glucocorticoid receptor

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Significance Statement

Neuropsychological disturbances and mental health problems are frequently associated with long-term immunosuppressive drug treatment and thus impairment of patients' quality of life. However, whether and to what extent neuropsychiatric alterations emerge as a direct result of the patient's medical history or are rather attributed to properties of the drug is difficult to say. However, so far, surprisingly little is known about unwanted central side effects of immunosuppressive and antiproliferative acting compounds. Against this background, the present study investigated the effects of subchronic administration of the small molecule-immunosuppressant rapamycin (RAPA) on brain and behavior. Our data show that treatment with this drug induced neuromolecular alterations in the amygdala and increased anxiety-related behavior. These findings provide important knowledge regarding the central action of RAPA and its relation to neurobehavioral changes, highlighting the controversial nature of this drug's effects.

Introduction

Like its derivates temserolimus (CCI-779) and everolimus (RAD-001), the macrolide and small-molecule drug rapamycin (RAPA, also known as sirolimus) inhibits the serine/threonine protein kinase mammalian target of rapamycin (mTOR) (Sehgal et al., 1975; Sehgal, 2003). This kinase is a member of the phosphatidyl-inositol 3'-kinase (PI3K) family and plays an important role in cell growth and cell proliferation (Schmelzle and Hall, 2000; Sekulic et al., 2000; Aoki et al., 2001; Chong et al., 2010). mTOR inhibitors reached importance in preventing acute graft rejection after organ transplantation (Vezina et al., 1975). Evidence derived from studies in experimental animals and patients further revealed broad antitumor activity of this drug group (Guertin and Sabatini, 2009; Lane and Breuleux, 2009; Dancey, 2010). Even though its definite mechanism of action is not completely understood, RAPA has been shown to form a complex with the FK binding protein 12 that in turn inhibits mTOR-driven T- and B-cell proliferation as well as antibody production (Sehgal, 2003; Guertin and Sabatini, 2009). In the brain, mTOR signaling plays a role in many physiological and pathophysiological processes such as control of protein translation, control of local protein synthesis in dendrites and axons, and autophagy (Russo et al., 2012). The kinase mTOR interacts with several proteins to form mTORC1 (Laplante and Sabatini, 2012), a complex that plays a critical role in neuroplasticity. mTORC1 itself is able to phosphorylate certain downstream target proteins such as the p70 ribosomal S6 kinase alpha (p70s6k), which in turn is involved in the initiation and elongation phases of protein translation in neurons (Schratt et al., 2004; Tavazoie et al., 2005; Jaworski and Sheng, 2006; Parsons et al., 2006; Park et al., 2008).

A growing body of clinical observations shows that patients undergoing small-molecule drug immunosuppression (e.g., with the calcineurin inhibitor cyclosporine A or tacrolimus) frequently suffer from mood and anxiety disorders (de Groen et al., 1987; Kahan et al., 1987; Kahan, 1994; Lang et al., 2009; Loftis et al., 2010; Bosche et al., 2015), impairing the quality of life. Whether these neuropsychiatric alterations occur as a direct result of the patient's medical history or are attributed to the action of the immunosuppressive drugs during treatment remains unclear in most cases. In this regard, central effects of mTOR inhibitors have been documented in several clinical trials but also investigated in clinical and experimental settings, revealing opposing effects on brain and behavior (Bosche et al., 2015). For instance, favorable psychiatric outcomes in graft recipients have been described after switching from immunosuppressive drug treatment with the calcineurin inhibitor cyclosporine A to the mTOR inhibitor everolimus (Lang et al., 2009). Moreover, attenuating effects of subchronic and chronic RAPA treatment on depressive-like behavior that occurred comorbidly in subjects with preexisting neurological diseases such as epilepsy, tuberous sclerosis complex, or traumatic brain injury have been observed (Erlich et al., 2007; Chong et al., 2010; Russo et al., 2012; Cambiaghi et al., 2013; Ehninger, 2013). In contrast, clinical therapy with the mTOR inhibitor CCI-779 has been shown to induce striking euphoria followed by melancholy, mimicking bipolar disorder in many breast cancer patients (Raymond et al., 2004). Detrimental effects such as abnormalities in sensorimotor functioning and increased anxiety-related behavior were also seen in the offspring of mice prenatally treated with a single injection of this compound (Tsai et al., 2013).

The FK506 binding protein 51 (FKBP51), a co-chaperone of the glucocorticoid receptor (GR) that also regulates GR sensitivity, has been implicated in the development of anxiety or posttraumatic stress disorder (Binder et al., 2008; Binder, 2009). The protein kallikrein-related peptidase 8 (KLK8) is known to facilitate stress-induced plasticity (Bouvier et al., 2008). Importantly, previous work revealed that in a KLK8-dependent neuronal pathway in the amygdala, KLK8-triggered upregulation of FKBP51 was responsible for stress-induced anxiety-related behavior in mice (Attwood et al., 2011). FKBP51, which belongs to a family of immunophilins, is a target protein for small-molecule immunosuppressive drugs such as RAPA and cyclosporine (Li et al., 2011). This was moreover supported by recent data showing that emergence of anxiety-related behavior was chaperoned by upregulation of FKBP51 and KLK8 following acute RAPA treatment (Hadamitzky et al., 2014).

GR overexpression in the forebrain of genetically modified mice led to an increased anxiety-like phenotype (Wei et al., 2004). GR are known to act as transcription factors, controlling gene expression in the nucleus but also participating in the rapid modulation of neuronal excitability at the membrane (Barik et al., 2013). Interestingly, acute RAPA has been shown to induce neuronal hyperexcitability in the amygdala (Hadamitzky et al., 2014). Together, these findings strongly point out that the mentioned proteins are not only related to each other and are molecular targets for RAPA but are also implicated in the emergence of anxiety-related behavior.

Reliable data of central effects following chronic and/or subchronic treatment with RAPA, better reflecting clinical conditions of patients undergoing therapy with mTOR inhibitors, are still lacking. Against this background, the present study investigated whether repeated treatment with this small-molecule drug affects neurobehavioral functioning in adult rats. Since the emergence of anxiety-related behavior has been particularly linked to alterations of the aforementioned proteins in the amygdala (Kolber et al., 2008; Attwood et al., 2011; Hadamitzky et al., 2014; Arnett et al., 2015), the present study specifically focused on protein expression within this structure.

Methods

Animals and Drugs

Male Dark Agouti rats (DA/HanRj, 220–250 g; Janvier) were housed in groups of 4 with ad libitum access to food and tap water. The vivarium was temperature (20°C) and humidity (55±5%) controlled and maintained on a reversed 12-h-dark/-light cycle (7:00 AM to 7:00 PM). Adult animals were allowed to acclimate to the vivarium and new surroundings for 1 week before initiation of the experiments. All animal facilities and experimental procedures were in accordance with the National Institutes of Health and Association for the Assessment and Accreditation of Laboratory Animal Care guidelines and were approved by the Institutional Animal Care and Use Committee. Permission for the experiments was granted by the local Animal Care and Use Committee (LANUV, NRW, Germany: G1545/16; Az. 84-02.04.2016.A111). Based on previous studies (Pech et al., 2011; Huang et al., 2012; Lu et al., 2015), therapeutically effective doses of RAPA (LC Laboratories) were dissolved freshly every day in a mixture of cremophor (62%), ethanol (33%), and aqua dest (5%). The stock solution was further diluted with sterile saline (0.9% NaCl) to gain the desired dose of 1 and 3 mg/kg at a final injection volume of 0.5 mL administered i.p. Animals were randomly assigned to the treatment groups, receiving only injections of the vehicle solution (n=8), 1 mg/kg (n=9), or 3 mg/kg RAPA (n=8), respectively.

Experimental Design

The experimental design comprised a subchronic drug or vehicle treatment phase followed by neurobehavioral analysis (Figure 1). More precisely, performance on the Elevated Plus Maze (EPM) was assessed at day 6 (i.e., following a total of 6 single injections in 6 days with one daily injection) and locomotor activity in the open field test (OF) was analyzed at day 7 (following a total of 7 single injections in 7 days with one daily injection). At day 8 after a total of 8 single injections in 8 days, animals were decapitated and brains and blood samples were taken for biochemical analyses. Drug treatment was always conducted in the morning between 8:00 and 9:00 AM, behavioral analysis as well as killing the animals started not earlier than 12:00 PM. Based on previous results indicating that the KLK8-pathway and the upregulation of FKBP51 as well as robust hyperactivity of the amygdala were involved in the emergence of anxiety-related behavior following a single injection of RAPA (Hadamitzky et al., 2014), the present study investigated the impact of repeated RAPA treatment, reflecting clinical conditions of patients undergoing therapy with this mTOR inhibitor.

Behavioral Measurements

All behavioral testing was performed during the activity period of the animals (dark phase) under red-light illumination. Prior to testing, rats were transferred to the experimental room and were allowed to habituate for at least 30 min. Mazes were cleaned with 70% ethanol to eliminate possible odor cues of previous animals.

EPM

The EPM was made of grey plastic and consisted of a center platform (15 cm x 15 cm) with 4 branching arms (42.5 x 14 cm), 2 open arms, and 2 opposing closed arms (22.5 cm high). Since the maze was elevated 80 cm above the floor, all edges of the open arms contained a 5-mm lip to prevent animals from falling off the maze. Testing started by gently placing the animal on the center platform always facing an open arm. Using an automatized video tracking system (VideoMot 2, TSE Systems), behavior was assessed for 5 min. The dependent measures in the present study were as follows: number of open arm entries, time spent in open/closed arms, the distance covered on the open/closed arms, and head dips (the frequency of the animal protruding its head over the ledge of an open arm and down towards the floor). An arm entry was defined as the entry of all 4 paws into 1 arm (Pellow et al., 1985; Setem et al., 1999).

OF

An acrylic glass arena consisting of a rectangular acrylic box (75 x 75 cm) with black walls (40 cm height) and a frosted floor with infrared backlighting was used as OF. Testing started by gently placing the animal in the center of the arena, and performance was assessed over a testing period of 10 min using an automatized video tracking system (VideoMot 2, TSE Systems). Parameters analyzed were the horizontal activity (distance) in the whole arena.

Tissue Sample Preparation

Animals were killed on day 8, 1 d after accomplishment of the behavioral analyses. Approximately 3 h after the last drug injection, rats' brains were quickly removed following decapitation, frozen on dry ice, and stored at –80 °C until further processing. Using a freezing microtome (Microm HM560, Thermo Fisher Scientific), coronal brain sections of 200 µm thickness were cut at -5°C and placed on prechilled glass slides. Subsequently, the amygdala was dissected from serial brain sections using a micro punch technique described elsewhere (Cuello and Carson, 1983). Briefly, a prechilled stainless-steel sample puncher (internal diameter of 2 mm; Fine Science Tools) was used to obtain tissue

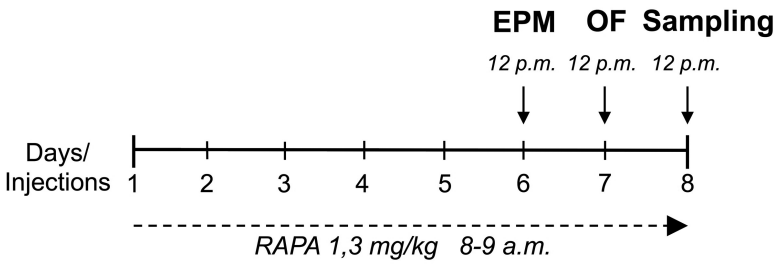

Figure 1. Experimental design. The experiment consisted of a subchronic drug or vehicle treatment phase followed by behavioral testing. Performance on the Elevated Plus-Maze (EPM) was assessed on day 6 after a total of 6 drug injections, locomotor activity was analyzed at day 7 in the Open-Field (OF) after a total of 7 drug injections. At day 8 after a total of 8 drug injections, animals were killed by decapitation, and brain and blood samples were taken for biochemical analyses (sampling).

samples of the left and right amygdala (–1.8 to –2.8 Bregma). Optical tract and hippocampus served as anatomical landmarks to ensure comparable positions of the punched samples across animals (Paxinos and Watson, 1998). Punches of each individual animal were pooled, and proteins from the snap-frozen amygdala tissue were extracted utilizing freshly made radioimmuno-precipitation assay buffer (150 mM NaCl, 20 mM Tris, 0,04 mM EDTA, 1% DOC, 1% Triton X, 0,1% SDS, pH 8). Protein concentrations were calculated via BCA Protein Assay (Pierce Thermo Scientific).

Western Blot

For western-blot analyses of the neuronal activity marker c-Fos and p70s6k, 20 µg protein per sample was diluted with radioimmuno-precipitation assay buffer and loading buffer (Roti Load 1, Carl Roth GmbH + Co. KG). Samples were boiled for 5 min at 95°C, resolved on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with antibodies specific for c-Fos (#2250, 1:1000, Cell Signaling Technology) and phospho (p)-p70s6k (#9208, Ser371; 1:1000, Cell Signaling Technology). For the quantification of kallikrein-related peptidase 8 (KLK8), FKBP51, and the GR, 20 µg protein per sample was diluted with Laemmli lysis buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2% Igpal, 1% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 1% SDS, 1 mM PMSF) and loading buffer (0.5 M Tris/HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue). Samples were boiled for 5 min at 95°C, resolved on 10% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with antibodies specific for KLK8 (ABIN759116, 1:500, antibodies-online.com), FKBP51 (ab2901, 1:500, Abcam), and GR (AB109022, 1:1000, Abcam). Immuno-positive bands were visualized with horseradish peroxidase–conjugated secondary anti-rabbit antibodies (111-035-003, 1:10,000, Jackson ImmunoResearch for KLK8; A2074, 1:10,000, Sigma-Aldrich for FKBP51 and GR; #7074, 1:5000, Cell Signaling for c-Fos and p-p70s6k) and enhanced chemiluminescence (WBKLS0500, Immobilon, Millipore). Chemiluminescence intensities were digitized with a charge-coupled device camera (ChemiDoc XRS, Bio-Rad) and protein levels quantified by densitometry software ImageLab (version 2.0, Bio-Rad). Total protein load via fluorescent gel electrophoresis (TGX stain free gels, 161–0183, Bio-Rad) served for normalization.

Plasma CORT Concentration

Trunk blood was collected in EDTA-treated tubes (Monovette) and stored on ice. Subsequently, plasma was separated by centrifugation (2000*g*, 10 min, 4°C), and stored at –80°C until further analysis. Quantification of plasma corticosterone (CORT) was performed as described previously (Prager et al., 2013). Briefly, CORT levels were determined according to the manufacturer's instructions by using an enzyme-linked immunosorbent assay (RE52211, Corticosterone ELISA, IBL International). Cross-reactivity of the anti-CORT antibody with other relevant steroids was 7.4% (progesterone), 3.4% (deoxycorticosterone), and 1.6% (11-dehydrocorticosterone). The sensitivity of the assay was 0.6 ng/mL.

Statistical Analysis

The descriptive statistics are based on means and variance, indicated by ±SEM. Statistical analyses were calculated using SigmaPlot software (Version 12.3, SPSS). Normality of residuals was examined using the Shapiro-Wilk test, and data were square-root-transformed when necessary. Values outside the 95% CI were defined as outliers and excluded from the analyses. Concerning this matter, one animal of the 1 mg/kg and one rat of the 3 mg/kg treatment group needed to be excluded. Multiple comparisons were performed using 2-way ANOVA followed by Holm-Sidak posthoc corrections. Significance level was set at $P < .05$.

Results

Behavioral Effects

Repeated systemic administration of RAPA (i.e., 6 single injections in 6 days with one daily injection) induced anxiety-related behavior in the EPM test (Figure 2). ANOVA showed a main effect for treatment ($F_{2,22=}4.793$; $P = .019$), and posthoc comparison revealed that RAPA-treated animals entered the open arms significantly less frequently than vehicle-injected controls (1 mg/kg, $P = .02$; 3 mg/kg, $P = .018$; Figure 2a). Closed arm entries did not differ between groups ($F_{2,22=}0.077$; $P = .927$; data not shown). Correspondingly, ANOVA detected main effects for both treatment ($F_{2,22=}3.574$; $P = .045$) and duration on the arms of the EPM ($F_{1,22=}156.637$; $P < .001$). Posthoc analysis showed that time spent in the closed arms (Figure 2b) was markedly increased following treatment with 3 mg/kg RAPA ($P = .042$). Similarly, ANOVA showed a treatment effect for distance ($F_{2,22=}6.671$; $P = .005$), and posthoc comparisons indicate that animals of the 3-mg/kg group covered significantly less distance on the open arms (Figure 2c) compared with the vehicle-injected controls ($P = .003$; Figure 2c). A representative example of the behavioral performance after RAPA treatment is illustrated in Figure 2d as reconstruction of EPM locomotion profiles. The possibility that RAPA impaired general spontaneous locomotor activity or induced sickness-like behavior was ruled out by evaluating the distance on the closed arms ($F_{2,22=}1.140$; $P = .338$; Figure 3a), the total distance covered (horizontal activity) in the OF test ($F_{2,22=}0.466$; $P = .643$; Figure 3b), and the time spent in the center of the OF ($F_{2,22=}0.119$; $P = .889$; Figure 3c).

Molecular Effects

Robust amygdala hyperactivity is considered a high-risk factor for the development of anxiety (Wolfensberger et al., 2008). Following analysis of immunoblotting in amygdala tissue samples (Figure 4a–b), ANOVA revealed a treatment effect on c-Fos protein expression ($F_{2,22=}3.587$; $P = .045$), and posthoc testing indicated a significantly increased protein level in animals treated with 3 mg/kg RAPA ($P = .031$). However, ANOVA showed no treatment effect for the expression of the proteins FKBP51 ($F_{2,22=}0.349$; $P = .709$) and p70s6k ($F_{2,22=}0.618$; $P = .548$), whereas a slight trend towards significance for KLK8 was observed ($F_{2,22=}2.982$; $P = .071$; Figure 4a–b). Interestingly, ANOVA indicated a treatment effect on GR expression ($F_{2,22=}18.091$; $P < .001$), and posthoc analysis revealed that in amygdala tissue samples of animals treated with both doses of RAPA (1, 3 mg/kg), GR protein levels were markedly increased compared with controls (1, 3 mg/kg, $P < .001$; Figure 4c–d). There was no interaction in amygdala GR and c-Fos expressions between groups (2-tailed *t* test, $P = .225$; Figure 5a). Interestingly, Pearson's correlation indicated a positive interaction between the expressions of these 2 proteins in the 3-mg/kg group (2-tailed *t* test, $P = .0068$; Figure 5c) but not in the 1 mg/kg group (2-tailed *t* test, $P = .539$; Figure 5b).

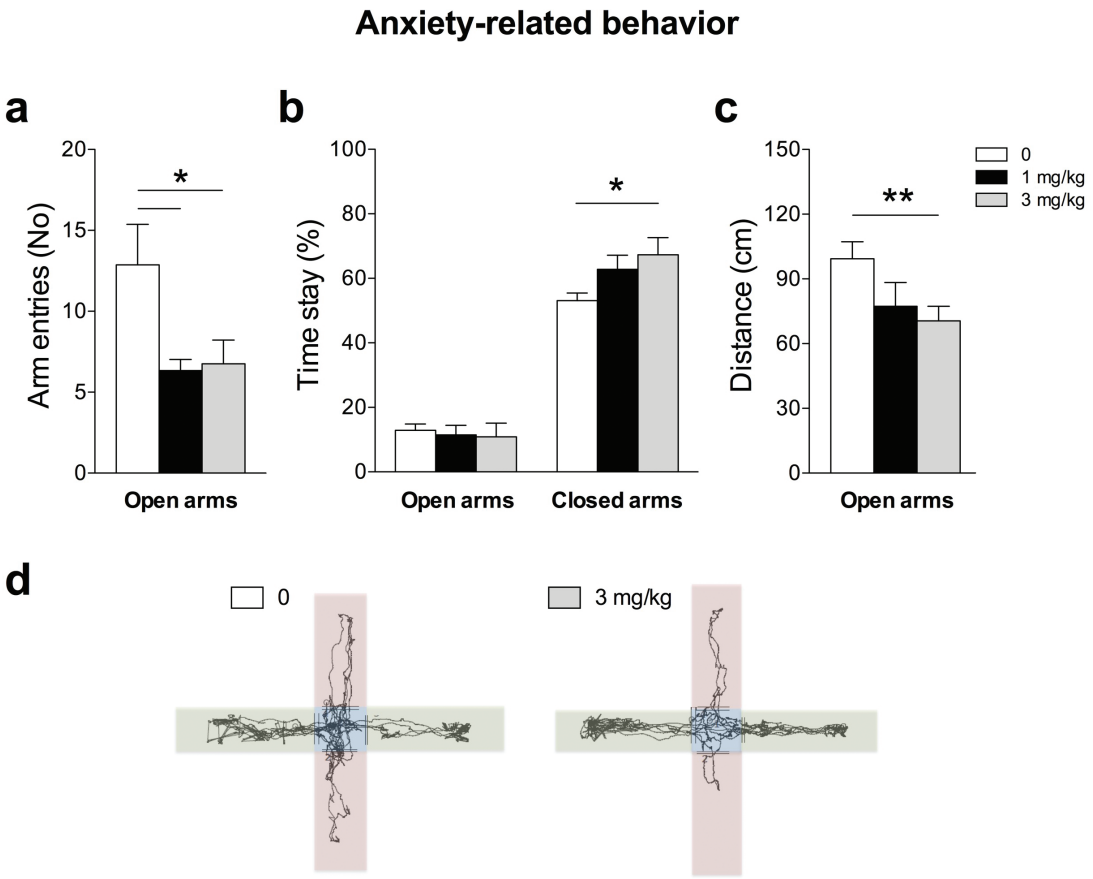

Figure 2. Anxiety-related behavior on the Elevated Plus-Maze (EPM) assessed after 6 consecutive days of drug treatment. (a) Number of open arm entries, (b) percent time spent in open/closed arms, (c) distance covered on open arms, (d) representative reconstruction of EPM locomotion profile after vehicle or rapamycin (RAPA) treatment. Data are expressed as means +SEM (n = 8–9 per group; ANOVA with Holm-Sidak posthoc comparisons; *P < .05 compared with vehicle-treated animals; grey areas = closed arms, pink areas = open arms, blue areas = center of the maze).

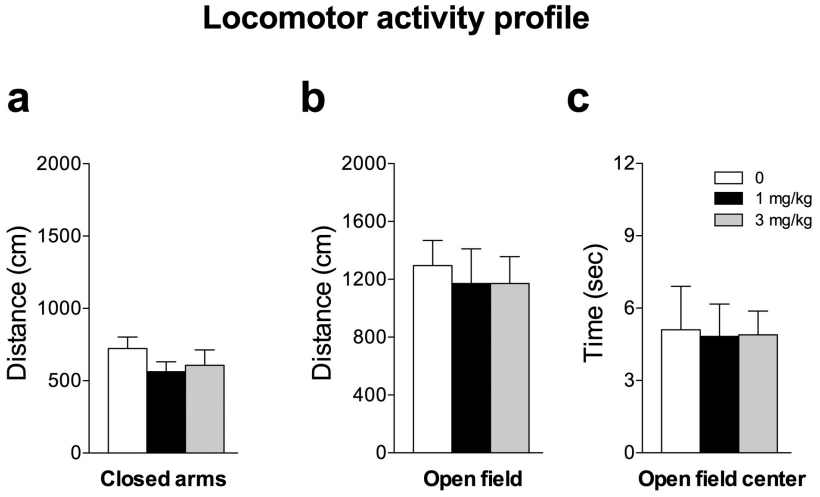

Figure 3. Locomotor activity profile assessed after 6 and 7 consecutive days of drug treatment with rapamycin (RAPA). (a) Distance covered on closed arms on the Elevated Plus-Maze (EPM), (b) distance covered in the Open-field (OF), and (c) time spent in the center of the OF. Data are expressed as means +SEM (n = 8–9 per group; ANOVA).

Physiological Effects

Analysis moreover revealed an effect on total body weight following 8 consecutive RAPA injections ($F_{2,22=}$13.163; $P < .001$). Both RAPA treatment groups displayed significant loss/stagnation in weight compared with vehicle-injected controls (1, 3 mg/kg, $P < .001$; Figure 6a). Blood plasma CORT levels after 8 consecutive RAPA injections did not differ between groups ($F_{2,22=}$0.966; $P = .396$; Figure 6b).

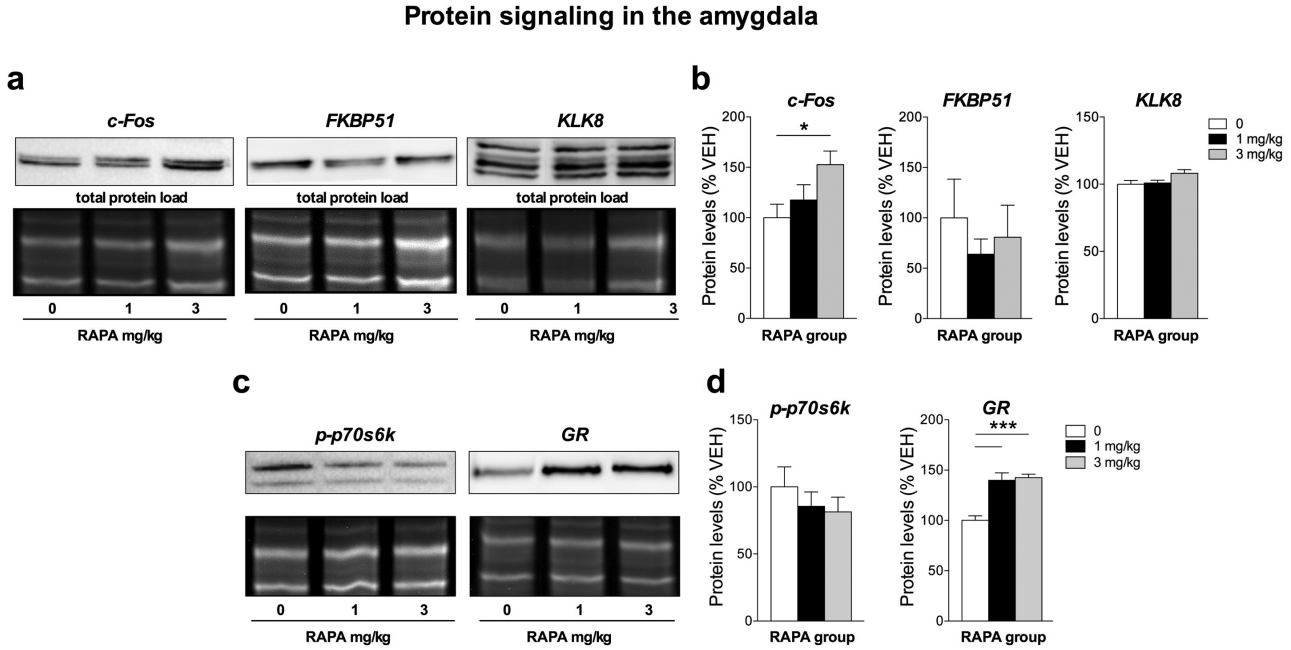

Figure 4. Protein signaling in the amygdala following 8 consecutive days of treatment with rapamycin (RAPA). (a-b) c-Fos, FK506 binding protein 51 (FKBP51) and kallikrein-related peptidase 8 (KLK8) protein levels. (c-d) Phospho p70 ribosomal S6 kinase alpha (p-p70s6k) and glucocorticoid receptor (GR) protein levels. Representative immunoblottings (left hand series) depict the respective proteins in total amygdala homogenates. Data are expressed as means +SEM % vehicle (VEH; n = 8–9 per group; ANOVA with Holm-Sidak posthoc comparisons; *P < .05, ***P < .001 compared with vehicle-treated controls; representative immunoblotting are cropped and merged).

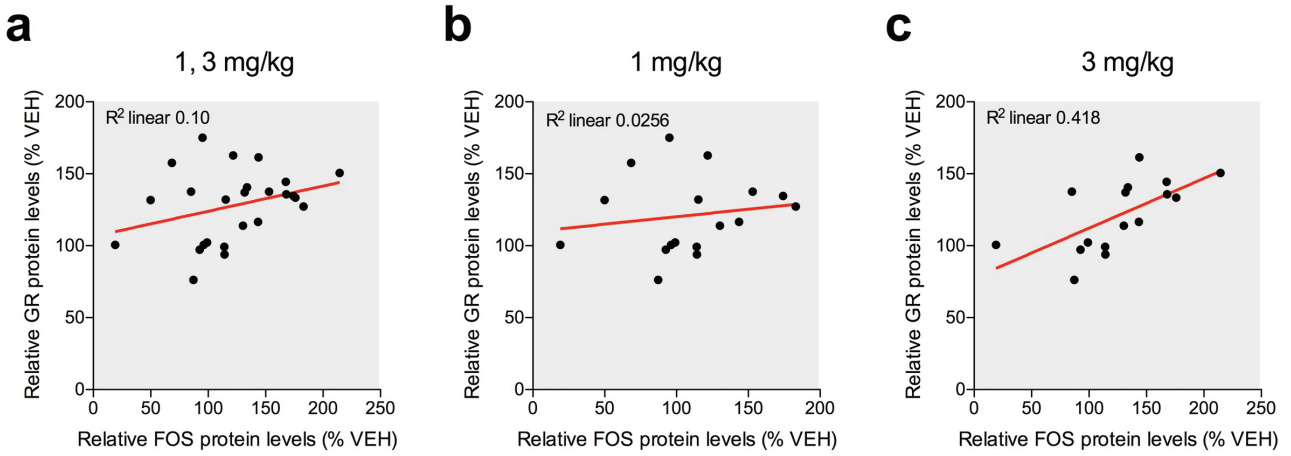

Figure 5. Interaction between amygdala protein expression following 8 consecutive days of treatment with rapamycin (RAPA). (a) Correlation analysis of c-Fos and glucocorticoid receptor (GR) expression of both treatment groups and controls (P = .225), (b) correlation analysis of c-Fos and GR expression in the 1 mg/kg group and controls (P = .539), and (c) correlation analysis of c-Fos and GR expression in the 3 mg/kg group and controls (P = .0068).

Discussion

Therapy with small-molecule immunosuppressive drugs is widely used for treating cancer and autoimmune disease or to prevent graft rejection (Vezina et al., 1975; Murgia et al., 1996; Lane and Breuleux, 2009; Dancey, 2010). Importantly, data of CNS effects in patients as well as experimental settings are inconsistent. The amygdala, a limbic region in the medial temporal lobe, is considered a central element in mood regulation, anxiety in particular (Dantzer et al. 2008). The results of the present study provide novel insights in central "side" effects of the mTOR inhibitor RAPA evolving after repeated systemic administration of drug doses that have been proven therapeutically effective in disease animal models (Pech et al., 2011; Huang et al., 2012; Lu et al., 2015).

Treatment with RAPA for 6 days induced a marked increase in anxiety-related behaviors in the EPM test as indicated by fewer entries into open arms, increased time spent in the closed arms (Gray, 1979; Pellow et al., 1985; Crawley, 1999; Enkel et al., 2013), and reduced activity on the open arms (Lau et al., 2008). In general, rodents' performance on the EPM is a good predictor for anxiety-related behavior. For one, animals avoid being exposed to aversive areas such as open arms and prefer to stay in the more protected zones of the maze (closed arms). For another, open arm entries and exploratory behavior are increasable by anxiolytic agents such as diazepam (Pellow et al., 1985; Lau et al., 2008).

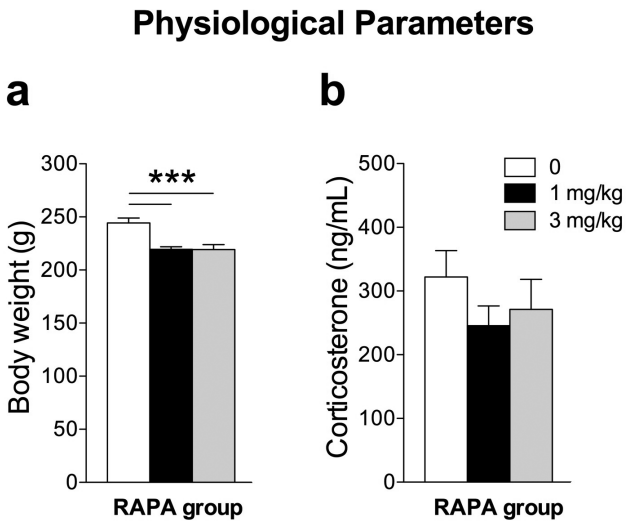

Figure 6. Physiological parameters. (a) Body weight and (b) blood corticosterone after 8 consecutive injections with rapamycin (RAPA; n = 8–9 per group; ANOVA with Holm-Sidak posthoc comparisons; ***P < .001 compared with vehicle-treated controls).

As measures for anxiety, these behavioral patterns have great face validity, given that many anxiety disorders are typified by a pervasive avoidance of feared situations or objects (Cryan and Holmes, 2005). Even though the OF test is also frequently used to pick up anxiety-related events, in the present approach it was specifically conducted to analyze possible impact of RAPA on general locomotor activity. Similar to the EPM, in the OF the avoidance conflict to engage in exploratory activity towards aversive properties (an open, brightly lit arena) is assessed by time/distance in the border/center regions (Bailey and Crawley, 2009). As shown previously, rats repeatedly treated with a high dose of 10 mg/kg RAPA showed no anxiety-related behavior in the OF when tested under red light (Cleary et al., 2008). Contrary, but under white light conditions, rats displayed an increase in those behaviors in the OF following chronic treatment with moderate doses of 1 and 3 mg/kg RAPA (Lu et al., 2015). Since in the present study all behavioral testing was planned and conducted under red light conditions, we neither expected nor discovered any anxiety-related behavior in the OF following RAPA treatment (data not shown).

Importantly, experimental work has also demonstrated that chronic rapamycin treatment in mice did not induce depressive- or anxiety-like behavior (Cambiaghi et al., 2013) but rather attenuated this behavior (Halloran et al., 2012). The reason why mTOR inhibitors, and RAPA in particular, apparently affect brain and behavior differentially is not clear. This incident most probably depends on a set of distinct aspects comprising species, the age of the subject, the route of administration, and the drug dosage employed as important factors. For instance, acute moderate RAPA (1 mg/kg) administered prenatally in mice (Tsai et al., 2013) or during adulthood in rats (3 mg/kg) (Hadamitzky et al., 2014) induced anxiety-related behavior. Likewise, young rats at 2 weeks of age displayed cognitive impairment and anxiety-related behavior following 4 weeks of chronic moderate treatment (3 mg/kg) (Lu et al., 2015). Contrary, however, chronic high-dose RAPA treatment in mice (6 mg/kg), starting from postnatal day 8 to 40, did not induce any signs of anxiety during adulthood in mice (Cambiaghi et al., 2013). Similarly, chronic inhibition of mTOR by oral RAPA at a nonspecific daily dosage (approximate average dose: 2.24 mg/kg) was shown to enhance learning and memory in old C57BL/6J mice and exert anxiolytic and antidepressant effects in this mice strain (Halloran et al., 2012). Noteworthy, beneficial effects of RAPA on behavior have only been observed in mice. Even though the behavioral impact of RAPA in these studies is very similar, effects on other measures are rather inconsistent. While Halloran et al. (2012) discovered that during continuous oral RAPA the body weight did not change throughout the experiment, Cambiaghi et al. (2013) reported a substantial "anorectic" effect and diminished weight gain when mice were chronically injected with a dose of RAPA at least twice as high as in the former study.

Beside the factors species, age of subject, route of administration, or drug dosage, possible preexisting neuropsychiatric predispositions or experimentally induced neurological damage may also be of importance regarding RAPA-mediated effects. For instance, deteriorated behavioral performance, commonly occurring comorbid to neurological diseases such as epilepsy, tuberous sclerosis complex, or traumatic brain injury, was attenuated or even abrogated by RAPA treatment (Erlich et al., 2007; Cleary et al., 2008; Chong et al., 2010; Russo et al., 2012; Cambiaghi et al., 2013; Ehninger, 2013). These observations may characterize RAPA as a potential candidate for medicating psychiatric symptoms, but only for comorbidities in neurological diseases where mTOR malfunctioning is manifest (Cleary et al., 2008; Chong et al., 2010).

Infected rodents' sickness behavior is characterized by reduced exploration and motor activity, decreased food and water consumption, weight loss due to loss of appetite, general anhedonia, and depressive-like behavior (Hart, 1988; Dantzer, 2001b, 2001a; Dantzer et al., 2008; Steiner et al., 2011; Maes et al., 2012). The possibility that the observed behavioral changes in EPM performance can be attributed to sickness induced by the drug is rather unlikely. First, in the present EPM performance, the number of entries into the closed arms, an action considered to reflect motor activity rather than anxiety (Walf and Frye, 2007; Deacon, 2013), did not differ between groups. Also, no group differences were found regarding overall motor functioning quantified by the total distance covered in the OF. These findings are in line with data reporting that even high doses of subchronic RAPA (5, 10, 20, 50 mg/kg) did not affect overall locomotor activity in the OF (Cleary et al., 2008). Second, subchronic and chronic treatment with RAPA was shown to have no or rather beneficial effects on behavioral-despair pattern, such as immobility time, assessed in the forced-swim test (Cleary et al., 2008; Cambiaghi et al., 2013). Finally, chronic (Deblon et al., 2012) and acute (Hebert et al., 2014) systemic administration of RAPA was indeed shown to reduce both food intake and body weight gain in free-feeding animals. But these results, most probably mediated via inhibited mTORC1 signaling in the hypothalamus (Cota et al., 2006; Toklu et al., 2016), were observed without apparent signs of malaise (Hebert et al., 2014). Moreover, no avoidance behavior towards RAPA was found in a conditioned taste aversion procedure (Herbert and Cohen, 1993), affirming that the effects on behavior in the present study are not attributed to sickness induced by the drug.

Amongst other psychiatric disorders, anxiety is accompanied by abnormalities in amygdala functioning (Lawrie et al., 2003). Especially, robust hyperactivity of this brain structure is considered a high-risk factor for the development of fear and anxiety disorders (Wolfensberger et al., 2008). In contrast, it has been revealed that anxiolytic effects after acute selective serotonin reuptake inhibitors in adolescents were associated with reduced activation of the amygdala and cortical brain regions (Arrant et al., 2013). Enhanced neuronal activation of

the amygdala, characterized by overexpression of the neuronal activity marker protein c-Fos and increased intracerebral electroencephalography signals were detected following an acute injection of 3 mg/kg RAPA (Hadamitzky et al., 2014). Similarly, in the present study c-Fos expression in the amygdala was upregulated in animals treated with 3 mg/kg RAPA. Notably, this observed indication of amplified neuronal activity (Morgan and Curran, 1991) does not necessarily need to be a direct result of RAPA-induced mTOR inhibition within the amygdala. On one hand, the mTORC1 downstream target protein p-p70s6k, a good marker for mTOR functioning (Chiang and Abraham, 2005), was not altered after treatment. This finding is in line with data showing that even an acute, high-dose injection of RAPA (10 mg/kg) did not change rats' baseline p-p70s6k protein expression in the basolateral complex of the amygdala (Gao et al., 2014). On the other hand, in juvenile rats (2 weeks of age) 1 and 3 mg/kg RAPA potently inhibited p-p70s6k in the hippocampus but not in the amygdala, whereas cognitive impairments and anxiety-related behavior in the OF test were impaired following treatment (Lu et al., 2015). Thus, RAPA-mediated effects observed in the present study are presumably partially attributed to the drugs' action in different brain areas.

FKBP51 is a co-chaperone of the GR suggested to be a key molecule in the stress response due to its action in stress adaptation and recovery (Albu et al., 2013). KLK8, highly expressed in amygdala and hippocampus, is a protein known to facilitate stress-induced plasticity (Bouvier et al., 2008). Previous work discovered a KLK8-dependent neuronal pathway in the amygdala, in which KLK8-triggered upregulation of FKBP51 was responsible for stress-induced anxiety-related behavior in mice (Attwood et al., 2011). Following an acute injection of RAPA, recent work showed increased amygdala expression of these 2 proteins concomitantly with increased anxiety-related behavior. The data indicate that the KLK8 pathway and the upregulation of FKBP51 are not only implicated in the development of stress-related affective disorders (Binder et al., 2004, 2008; Binder, 2009), but also seem to play a role in triggering anxiety-like behavior in general (Hadamitzky et al., 2014). However, after repeated RAPA treatment no changes in FKBP51 expression were found while expression levels of KLK8 were only slightly elevated in the 3-mg/kg treated group. Thus, upregulation of KLK8 and FKBP51 most probably reflect early effects emerging after acute RAPA treatment, which are no longer detectable following subchronic treatment. Nevertheless, both treatment groups showed highly elevated protein levels of GR in amygdala tissue samples. Under "normal" or healthy conditions, GR is a widely expressed ligand-dependent transcription factor that modulates a broad range of neural functions, such as stress responsiveness or cognitive functioning (Sapolsky et al., 1984; McEwen and Sapolsky, 1995; Roozendaal et al., 2003). GR are located throughout the brain and particularly in limbic areas like the amygdala, but the mechanisms of their central regulation are still poorly understood (Meaney et al., 1985; Groeneweg et al., 2011). Kolber et al. (2008) showed that disruption of GR specifically in the central nucleus of the amygdala led to attenuation of freezing in a conditioning fear paradigm during contextual fear, which was associated with decreased expression of c-Fos and corticotropin releasing hormone. Likewise, early-life stress has been shown to reduce GR mRNA expression in brains of mice with a notable reduction in the amygdala. This diminished GR expression was moreover associated with decreased anxiety and fear responsiveness (Arnett et al., 2015). Tronche et al. (1999) have shown that knockout mice with decreased GR activity in the CNS displayed diminished anxiety-related behavior but profound alterations in the neuroendocrine system. Vice versa, we here report increased anxiety-related behavior accompanied by enhanced expression of GR in the amygdala while baseline plasma levels of circulating CORT remained equal between treatment groups and controls. Our observations are also compatible with those in the study of Wei et al. (2004) demonstrating in genetically modified mice that GR overexpression in the forebrain led to an increased anxiety- and depressant-like phenotype with no apparent alterations in plasma CORT levels. Interestingly, ex vivo-analyzed amygdala samples showed a significant interaction of c-Fos and GR protein expression in animals treated with the higher dose. A direct correlation between GR density and c-Fos protein expression and anxiety-like behavior was also revealed in previous work showing a significantly greater concentration of c-Fos / GR co-localized neurons in animals highly responding to conditioned fear compared to low responding rats (high anxiety rats). Thus, co-localized c-Fos and GR may interact within cortical and limbic neurons to provide transcriptional regulation such as repression or stimulation of neurotransmitter and neurotransmitter receptor gene expression (Lehner et al., 2009). Due to the fact that GR acts as transcription factor, controlling gene expression in the nucleus and participating in the rapid modulation of neuronal excitability at the membrane (Barik et al., 2013), in the present study enhanced GR expression may have also been responsible for elevated neuronal activity in the amygdala reflected by increased c-Fos protein expression.

Conclusion And Limitations Of The Study

The present study showed that animals treated with RAPA (1, 3 mg/kg) displayed alterations in EPM performance with more pronounced effects in the higher dose group. Here, the result of "milder" anxiety-related behavior (just one EPM measure was affected) and a trend towards elevated c-Fos protein expression after low-dose treatment with 1 mg/kg slightly point into the direction of a dose-dependent effect. We therefore hypothesize that anxiety-related behavior observed after repeated RAPA treatment was not directly attributed to mTOR-dependent mechanisms or stress but rather due to hyper-excitability and GR overexpression in the amygdala. It is suggested that chronic RAPA administration possibly stimulates major monoamine pathways in the brain as shown in mice whose depressive-like behavior was attenuated due to this intervention (Halloran et al., 2012). Thus, alterations in neurotransmitter levels (e.g., serotonin) may also play a role in modulating this drug's effects on neuromolecular alterations in the amygdala and anxiety-related behavior.

One drawback of the present study is limitation of protein expression to one brain region. Neuromolecular involvement of other structures like the hippocampus, which has already been shown to be susceptible to RAPA (Lu et al., 2015), should be taken into account in future studies. Likewise, due to the complexity of the amygdala structure with its subnuclei, gaining more specific information regarding regional neuronal activity would be advantageous. Another cutback is the sole use of the EPM to measure anxiety-like effects. Rodent behaviors have limitations when compared with the complexity of human behavior. Employing multiple tests to address a broader range of specific domains relevant to anxiety may therefore improve translation from animals to humans (Freudenberg et al., 2017). However, the ideal animal model of anxiety does not exist, and the tests available (EPM, OF, and the Light-dark box) are characterized by their originality. Moreover, it is proposed that short-term, intraindividual variations in emotionality constitute an

important factor for investigating anxiety-related behavior that may differ between tests. Thus, to gain broader understanding about underlying mechanisms and to increase validity of data, multiple behavioral tests should be used in future studies to characterize anxiogenic/anxiolytic properties of mTOR inhibitors (Bourin and Hascoet, 2003; Ramos, 2008).

Together, the present results support the contention that, regardless of the underlying mechanism of action, subchronic treatment with RAPA may induce neurobehavioral alterations in healthy, naive rats. Moreover, our data once more support the hypothesis that RAPA and its impact on the mTOR and associated signaling pathways apparently exerts both beneficial and unfavorable effects on neurobehavioral outcomes. However, these outcomes most likely depend on conditions such as species, age, possible preexisting predispositions, as well as on duration and dosage of drug intake. To better understand the exact beneficial but also detrimental effects of RAPA on brain and behavior, further research implementing anxiolytic treatment options is needed to track down relevant brain structures and proteins within the mTOR and associated signaling pathways.

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Statement of Interest

None.

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