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Defective regulation of the eIF2-eIF2B translational axis underlies depressive-like behavior in mice and correlates with major depressive disorder in humans

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Major depressive disorder (MDD) is a significant cause of disability in adults worldwide. However, the underlying causes and mechanisms of MDD are not fully understood, and many patients are refractory to available therapeutic options. Impaired control of brain mRNA translation underlies several neurodevelopmental and neurodegenerative conditions, including autism spectrum disorders and Alzheimer's disease (AD). Nonetheless, a potential role for mechanisms associated with impaired translational control in depressive-like behavior remains elusive. A key pathway controlling translation initiation relies on the phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α-P) which, in turn, blocks the guanine exchange factor activity of eIF2B, thereby reducing global translation rates. Here we report that the expression of EIF2B5 (which codes for eIF2Bε, the catalytic subunit of eIF2B) is reduced in *postmortem* MDD prefrontal cortex from two distinct human cohorts and in the frontal cortex of social isolation-induced depressive-like behavior model mice. Further, pharmacological treatment with anisomycin or with salubrinal, an inhibitor of the eIF2α phosphatase GADD34, induces depressive-like behavior in adult C57BL/6J mice. Salubrinal-induced depressive-like behavior is blocked by ISRIB, a compound that directly activates eIF2B regardless of the phosphorylation status of eIF2α, suggesting that increased eIF2α-P promotes depressive-like states. Taken together, our results suggest that impaired eIF2-associated translational control may participate in the pathophysiology of MDD, and underscore eIF2-eIF2B translational axis as a potential target for the development of novel approaches for MDD and related mood disorders.

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INTRODUCTION

Major depressive disorder (MDD) is a neuropsychiatric condition that has remarkably increased in prevalence worldwide [1, 2], currently being the second-leading cause of disability in adults and a significant cause of suicidal behavior and mortality [3]. MDD is characterized by a combination of symptoms that include persistent sadness, anhedonia, sleep disturbances, and often altered appetite and weight. Although knowledge on the pathophysiology of MDD has advanced considerably during the past decades, the specific causes and mechanisms driving depressive behavior are not yet fully understood [2, 3].

The integrated stress response (ISR) comprises a cellular adaptive response to stressful signals mediated by the activity of four known serine/threonine kinases (HRI, PKR, PERK, and GCN2) that phosphorylate the α subunit of the eukaryotic translation initiation factor 2 (eIF2) at serine 51 (eIF2α-P) to attenuate global protein synthesis rates [4]. Increased eIF2α-P inhibits the activity of eukaryotic translation initiation factor 2B (eIF2B), a guanine nucleotide exchange factor that catalyzes GDP-GTP exchange on eIF2 and enables the initiation of mRNA translation [5]. Although ISR-linked translational repression is usually relieved upon resolution of cellular stress, prolonged ISR signaling can trigger programmed cell death [6]. Accordingly, eIF2α-P is considered a major regulator of metabolic, transcriptional, and translational switches during adaptive responses to cellular stress, which may be caused by stimuli such as hypoxia, amino acid and glucose deprivation, viral infection, and endoplasmic reticulum stress [4, 7].

Control of brain mRNA translation has long been implicated in memory and cognition [8–10]. Many studies have established how regulation of the initiation step of neuronal protein synthesis, both in the cell body and at synapses, contributes to memory and behavioral flexibility [11–16]. eIF2α-P is as a central player in protein synthesis-dependent forms of both long-term potentiation and long-term depression [12, 14]. For example, targeted replacement of Ser51 in eIF2α by a non-phosphorylatable alanine (eIF2αS51A) enhances long-term memory [13] and, at the same time, impairs

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hippocampal synaptic plasticity by blocking the endocytosis of synaptic AMPA-type glutamate receptors [17]. Similar findings were reported in PKR constitutive knockout mice [18].

We and others have shown that ISR activation and eIF2α-P-linked translational repression promote memory impairments in Alzheimer's disease (AD) [19–23]. Of note, pharmacological derepression of protein synthesis even in the presence of elevated eIF2α-P restored behavioral impairments in animal models of aging [24], AD [25], and other neurodegenerative disease [26–28]. More recent evidence has connected eIF2-mediated translational control with neuropsychiatric disorders. Translational control by eIF2α-P in midbrain dopaminergic neurons mediates synaptic plasticity and vulnerability to drugs of abuse [29, 30], and stimulation of eIF2-linked translation counteracts phenotypes of anxiety and social interaction in a genetic model of autism-like behavior [31]. However, potential roles for impaired translational control mediated by eIF2α-eIF2B in depressive-like behavior remain elusive. Herein we provide evidence for altered eIF2-eIF2B axis in *postmortem* brain tissue from MDD patients and for roles of eIF2-eIF2B in controlling depressive-like behavior in mice.

RESULTS

Expression of EIF2B5 is reduced in the PFC of patients with MDD

eIF2B is composed of five subunits (α/β/γ/δ/ε). The epsilon subunit (eIF2B5 or eIF2Bε) specifically catalyzes the exchange of eIF2-GDP for eIF2-GTP enabling formation of the eIF2-GTP·Met-tRNAi ternary complex for initiation of protein synthesis [32, 33]. Therefore, we quantified eIF2B5 expression in the dorsolateral prefrontal cortex (dlPFC) from MDD or healthy control (HC) subjects (comprising both males and females) from two *post mortem* brain tissue cohorts (Tables 1 and 2). We found that eIF2B5 expression was decreased by about 50% compared to healthy controls ($p = 0.0018$; two-tailed Student's t-test) in the dlPFC of MDD cases from the School of Medicine/University of São Paulo Brain Bank (FMUSP) (Fig. 1a), while only a trend of reduction was detected in the dorsal hippocampus (HP) in the same cohort ($p = 0.08$; two-tailed Student's t-test) (Fig. 1b). In samples from the Stanley Medical Research Institute Brain Bank, we confirmed that eIF2B5 mRNA content is reduced in the dlPFC from MDD compared to healthy subjects ($p = 0.03$; two-tailed Student's t-test) (Fig. 1c). Suicide as cause of death (Supplementary Fig. 1) did not significantly impact the PFC eIF2B5 mRNA reduction in the Stanley cohort. Together, these results indicate that the expression of eIF2B5 is reduced in the PFC of humans diagnosed with MDD.

Social isolation induces depressive-like behavior in mice and reduces the expression of eIF2B5 in the frontal cortex

We next investigated the expression of eIF2B5 in a murine model of depressive-like behavior. Male C57BL/6 mice were housed either individually or in groups for 60 days (Fig. 1d). Social isolation (SI) increased the immobility time of mice in the tail suspension test (TST; 167 ± 11.6 s for controls vs. 215.3 ± 11.3 s for SI, $p = 0.0068$; two-tailed Student's t-test), consistent with depressive-like behavior (Fig. 1e).

We next assessed eIF2B5 mRNA content by qRT-PCR in the frontal cortex (FC) and hippocampus (HP) of mice. SI reduced by

Table 1. Demographic information of the Biobank for Aging Studies from the University of São Paulo Medical School (FMUSP, Brazil).

	HC	MDD	Statistics (test performed)
Sample size	16	20	-
Sex (F/M)	11/5	12/8	$p = 0.58$ (X^2 test)
Age (years)	65.6 ± 14.4	66.0 ± 14.1	$p = 0.94$ (Unpaired t test)
Race	10 W/2Bl/4B	13 W/2Bl/5B	$p = 0.97$ (X^2 test)
PMI (hours)	13.4 ± 2.89	14.4 ± 3.31	$p = 0.34$ (Unpaired t test)
Suicide (%)	0	0	-
Antidepressant use (%)	0	25.0	-
Antipyschotic use (%)	0	18.7	-

Values represent the mean ± standard deviation (SD).
F Female, *M* Male, *W* White, *Bl* Black, *B* Brown, *PMI* post mortem interval (hours), *HC* healthy control, *MDD* major depressive disorder.

Table 2. Demographic information of the Stanley Medical Research Institute Brain Bank cohort (USA).

	HC	MDD	Statistics (test performed)
Sample size	10	19[a]	-
Sex (F/M)	3/7	9/10	$p = 0.36$ (X^2 test)
Age (mean years)	46.40 ± 11.57	42.89 ± 11.90	$p = 0.45$ (Unpaired t test)
Race	9 W / 1H	18 W / 1H	$p = 0.63$ (X^2 test)
Duration of Illness (years)	-	12.50 ± 7.82	-
PMI (mean hours)	23.50 ± 10.77	29.95 ± 12.23	$p = 0.17$ (Unpaired t test)
Suicide (%)	0	73.6[b]	-
Antidepressant use (%)	0	73.6	-
Antipyschotic use (%)	0	31.6	-

Values represent mean ± Standard Deviation.
F Female, *M* Male, *W* White, *H* Hispanic, *PMI* post mortem interval (hours), *HC* healthy control, *MDD* major depressive disorder.
[a]combining individuals diagnosed with MDD with or without psychotic features.
[b]81.8% of the individuals diagnosed as MDD with psychotic features (11 patients) had suicide as cause of death.

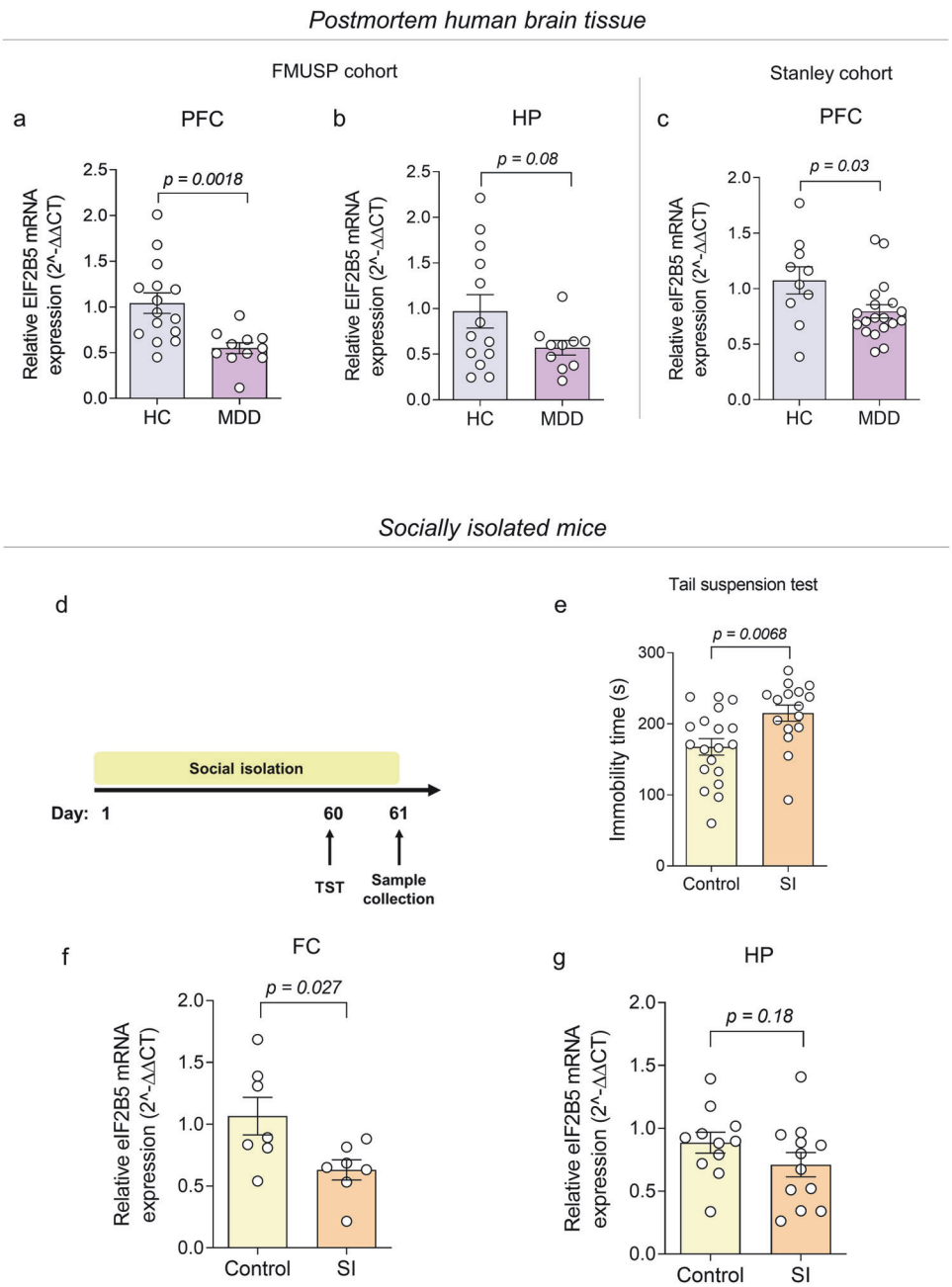

Fig. 1 eIF2B5 expression is reduced in the prefrontal cortex (PFC) of *post mortem* MDD brain tissues and in the frontal cortex (FC) of mice with depressive-like behavior. eIF2B5 expression in the PFC (**a**) and hippocampus (HP) (**b**) of MDD or healthy control individuals (HC) from FMUSP Brain Bank (two-tailed unpaired t-test, N = 11–15 individuals per group). **c** eIF2B5 expression in the PFC of MDD or HC individuals from Stanley Medical Research Institute Brain Bank (two-tailed unpaired t-test; N = 10 HC, N = 19 MDD). **d** Schematic protocol of social isolation. **e** Immobility time in tail suspension test (TST) in mice subjected to social isolation or controls (two-tailed unpaired t-test; N = 16 per group). eIF2B5 expression in FC (**f**) and HP (**g**) of mice (two-tailed unpaired t-test; N = 7 per group). All data are expressed as mean ± standard error of the mean (SEM).

~40% the expression of eIF2B5 in the FC ($p = 0.027$; two-tailed Student's t-test) (Fig. 1f). Hippocampal expression of eIF2B5 was unaltered by SI ($p = 0.26$; two-tailed Student's t-test) (Fig. 1g). Together, results in human and mice point to eIF2B5 expression as a potential common pathological feature of depressive-like states.

Pharmacological inhibition of protein synthesis induces depressive-like behavior in mice

As a proof-of-principle approach to the correlation between depressive behavior and impaired translational control, we determined whether global protein synthesis inhibition by anisomycin promoted depressive-like behavior in mice. Anisomycin was administered (100 mg/kg, i.p.), and TST or novelty-suppressed feeding test (NSF) were performed 3 h later (Fig. 2a). Mice that received anisomycin showed higher immobility in TST (167 ± 13.7 s control vs. 229.9 ± 12.6 s anisomycin; $p = 0.003$, two-tailed Student's t-test; Fig. 2b) and latency to feed in NSF (239 ± 26.6 s control vs. 332.5 ± 18.5 s anisomycin, $p = 0.009$; two-tailed Student's t-test; Fig. 2c). Anisomycin did not induce changes in locomotion (open field test: 12.3 ± 3.0 m control vs. 10.6 ± 1.4 m anisomycin, $p = 0.16$) or in home cage feeding behavior (home cage latency to feed: 35.9 ± 22.8 s control vs. 39.7 ± 18.8 s

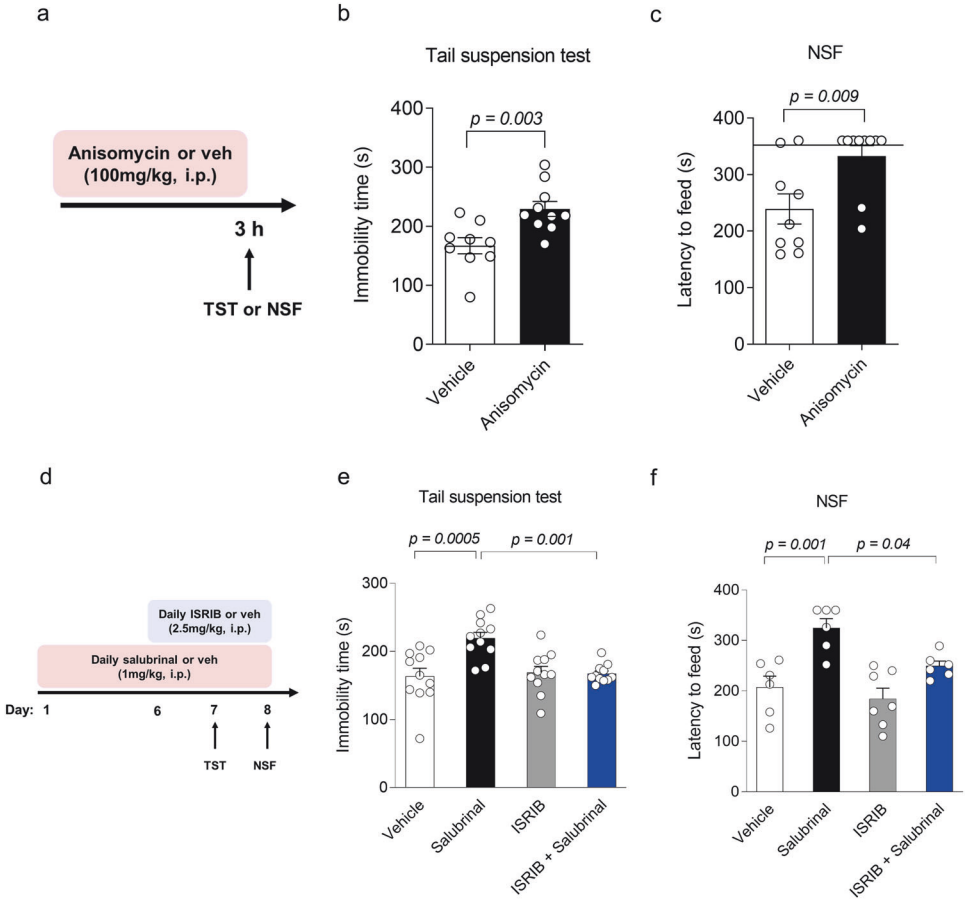

Fig. 2 Inhibition of protein synthesis induces depressive-like behavior in mice which is recovered by ISRIB. a Schematic protocol of anisomycin injection. **b** Immobility time in tail suspension test (TST) (two-tailed unpaired t-test, N = 9–10 per group) (**c**) and latency to feed in the novelty-suppressed feeding test (NSF) (two-tailed unpaired t-test, N = 9–10 per group) 3 h after anisomycin (100 mg/kg, i.p.) injection in mice. **d** Schematic protocol of salubrinal and ISRIB injection. **e** Immobility time in TST (two-tailed two-way ANOVA; N = 7–12 per group) (**f**) and latency to feed in NSF (two-tailed two-way ANOVA; N = 6 per group) assessed in animals injected with salubrinal (1 mg/kg/day, i.p.) and/or ISRIB (2.5 mg/kg/day, i.p.). Data are expressed as mean ± standard error of the mean (SEM).

anisomycin, $p = 0.55$) (Supplementary Fig. 2). Thus, we can rule out that the effects of anisomycin on depressive-like behavior paradigms were due to alterations in locomotion or feeding patterns.

We next tested the hypothesis that translational repression by eIF2α-P promotes depressive-like behavior in mice. Mice received 1 mg/kg (i.p.) salubrinal, an inhibitor of the eIF2α phosphatase, GADD34, daily for 8 days [19, 25, 34]. On days 7 and 8, mice were subjected to TST and NSF (Fig. 2d). We found that salubrinal increased both the immobility time in TST (163.3 ± 11.9 s for vehicle-treated vs. 219.3 ± 8.7 s for salubrinal-treated mice; $p = 0.0005$; Fig. 2e) and latency to feed in NSF (207.2 ± 22.1 s for vehicle-treated vs. 324.7 ± 18.4 s for salubrinal-treated mice; $p = 0.001$; Fig. 2f).

Administration of ISRIB (2.5 mg/kg/day, i.p.), a compound that directly activates eIF2B even in the presence of elevated eIF2α-P, reverted the behavioral alterations induced by salubrinal [6, 35, 36] (Fig. 2e, f) (TST: 167.2 ± 4.0 s, $p = 0.001$; NSF: 249 ± 9.2 s, $p = 0.04$, two-way ANOVA with Holm-Sidak correction). Control experiments revealed that neither salubrinal nor ISRIB caused changes in motor performance or anxiety-like behaviors, as assessed by the rotarod test (vehicle: 242.3 ± 32.7 s; salubrinal: 190.8 ± 19.2 s; ISRIB: 175.8 ± 22.5 s; $F_{(1,\ 32)} = 0.91$, $p = 0.34$), total distance traveled in an open field arena (vehicle: 12.4 ± 1.18 m; salubrinal: 11.2 ± 1.30 m; ISRIB: 13.1 ± 1.64 m; $F_{(3,\ 32)} = 0.35$, $p = 0.78$), and center-periphery measures (time in center area: 9.02 ± 1.59 s vehicle; 13.4 ± 2.98 s salubrinal; 14.3 ± 4.08 s ISRIB, $F_{(3,30)} = 2.04$, $p = 0.12$; time in periphery: 270.4 ± 3.96 s vehicle; 273.8 ± 5.97 s salubrinal; 285.7 ± 4.08 s ISRIB, $F_{(3,32)} = 0.74$, $p = 0.10$) (Supplementary Fig 3a-d). We further measured the home cage latency to feed after salubrinal and/or ISRIB administration. We did not observe differences among the groups analyzed (26.1 ± 15.5 s vehicle; 24.7 ± 22.4 s salubrinal; 28.3 ± 7.3 s salubrinal + ISRIB; 33.8 ± 25.6 s ISRIB. $p = 0.35$) (Supplementary Fig 3e). These results demonstrate that salubrinal is sufficient to trigger depressive-like behavior in mice and that direct stimulation of eIF2B by ISRIB corrects such depressive-like phenotypes. Therefore, inhibition of eIF2 may have a relevant role in the pathophysiology of depression.

DISCUSSION

Dysregulation of the eIF2-eIF2B axis and attenuation of global brain protein synthesis due to elevation of eIF2α-P have been demonstrated in several neurological disorders, including AD, Parkinson's disease, amyotrophic lateral sclerosis and prion-mediated neurodegeneration [6, 20, 37]. We also demonstrated that bolstering eIF2B activity with ISRIB [36, 38] alleviates synapse dysfunction and memory defects in mouse models of AD [25]. However, the participation of the eIF2-eIF2B in mood control remains elusive.

Previous studies reported that ISR and eIF2α-P are elevated in brains of mice susceptible to chronic stress (in contrast to stress-resilient ones) [39] and in mice exposed to corticosterone

(compared to controls) [40]. Likewise, chronic social defeat stress increases eIF2α-P and endoplasmic reticulum stress-related kinase activation in the hippocampus of mice [41]. Another study reported evidence for the suppression of eIF2 signaling in somatostatin-positive neurons in stressed mice [42]. Here, we show that the mRNA expression of eIF2B5 in the PFC is reduced in two different cohorts of MDD individuals, as well as, in the frontal cortex of a mouse model of depressive-like behavior induced by social isolation. It is noteworthy that the FMUSP and Stanley cohorts differ substantially both in terms of ethnicity, with most FMUSP cases being of Latinos whereas most cases from the Stanley Brain Bank were white, and in terms of age, with the FMUSP cohort comprised of geriatric depression cases. Moreover, the Stanley cohort include suicidal and psychotic individuals, who are absent from the FMUSP cohort. Thus, our finding of similar results regarding eIF2B5 expression in the two cohorts underlines the potential relevance of this mechanism for depressive states. Current results in humans hint at a new mechanism of eIF2B regulation and further confer clinical relevance to previous experimental findings.

Interestingly, our results indicate clearer effects in the PFC than in the hippocampus. The PFC is notably morphologically and functionally impaired in MDD and in animal models of depressive-like states [43, 44]. A previous mouse study by our group suggested that the frontal cortex was more sensitive than the hippocampus to modulation of Fndc5 mRNA (coding for the exercise-induced hormone irisin) by chemical stress or antidepressants [45]. Notably, a recent single-nucleus RNAseq study identified clusters of genes related to "cellular response to stress" and cellular stress in interneurons and microglia in the PFC of MDD donors [46]. This leads us to believe that the PFC might be more responsive to stressful stimuli, and therefore, more vulnerable to dysfunction in MDD. The specific reduction of PFC eIF2B5 is an interesting mechanism linking PFC function and mood disorders that warrants further investigation.

Recent evidence has implicated other pathways that mediate translational control as key players in depressive-like behavior and in the actions of known antidepressants. For example, reduced phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) leads to increased inflammatory responses and reduced serotonin levels, thus modulating depressive-like behavior in mice [47, 48]. eIF4E phosphorylation increases after chronic fluoxetine administration and is required for its antidepressant effects [47, 49]. In addition, interaction of eIF4E with its binding partners eIF4E binding protein 1 (4E-BP1) and 4E-BP2 is essential for the fast-acting antidepressant responses of ketamine and 2 R,6R-hydroxynorketamine [50, 51]. A limitation of our study is the reduced power to investigate the effects of antidepressant treatment on brain eIF2B5 expression in humans. We believe that this issue needs to be addressed in the future, using animal models and additional human cohorts. Certainly, investigating the potential recovery of eIF2B5 levels after effective antidepressant treatment would shed light on the association between the control of brain protein synthesis and mood disorders.

Finally, altered systemic responses to endoplasmic reticulum stress, a cellular condition known to promote eIF2α-P, have been proposed in MDD [52] and in bipolar disorder [53]. Along with these previous findings, our current results support the notion that an adequate control of mRNA translation is essential to ward off depressive-like behavior and MDD.

Here we show that depressive-like behavior induced by salubrinal, which promotes eIF2α-P, was rescued by treatment with the small-molecule compound ISRIB, which favors eIF2B activity, in mice. eIF2α-P is known to control synaptic plasticity and memory [13, 54, 55]. We have previously demonstrated that ISRIB corrects hippocampal eIF2α-P-induced signaling and memory impairments in mice [25]. An interesting study by Lin and Sibille (2015) demonstrated that inhibition of the eIF2α kinase, PERK, with GSK2606414 attenuated anxiety and depressive-like behavior in mice exposed to chronic unpredictable stress [42]. Together, these observations suggest that impaired ISR resolution may lead to multiple forms of brain dysfunction, including memory loss and depressive-like behavior.

Finally, we demonstrate that acute inhibition of protein synthesis with anisomycin, a well-known inhibitor of protein synthesis in eukaryotic cells through binding to 60S ribosomal subunits, is sufficient to generate depressive-like behavior in mice in a timeframe consistent with attenuation of brain protein synthesis, as demonstrated [56, 57]. It is noteworthy that some studies have demonstrated that anisomycin may modulate cell signaling cascades other than translation itself [58–60]. Nonetheless, anisomycin administration has been consistently shown to cause brain impairments, such as memory defects in animal models, in a manner that depends on protein synthesis [61–64]. Additionally, a more recent study showed that attenuation of translational rate by anisomycin impairs antidepressant effects mediated by elevated synaptic expression of the AMPAR subunit GluA1 in CaMK2α excitatory neurons [65]. Thus, it is likely that alterations in protein synthesis rates impair mood control. Taken together, results suggest that impaired the eIF2-eIF2B axis participates in the pathophysiology of MDD, and underscore eIF2-linked translational control as a potential target for novel therapeutic approaches in MDD and related mood disorders.

Although different animal models can reproduce certain behavioral and physiological aspects related to depression, the complexity and heterogeneity of this pathology in humans confer certain limitations on its study in animal models [66, 67]. Therefore, evaluating the mechanisms associated to the regulation of brain protein synthesis in different experimental models should also be considered. Nevertheless, our results in animal models and in postmortem human brains constitute a starting point for detailed exploration of the roles of the eIF2-eIF2B axis in mood control.

Postmortem intervals (PMI) of human brain samples could significantly impact mRNA stability, potentially introducing bias into the results. However, it is important to note that PMI did not differ significantly between groups in each cohort and that similar results were observed in two cohorts with different average PMI. Thus, reduced eIF2B5 mRNA in the PFC seems to be a consistent feature of MDD.

MDD is a disorder with complex pathophysiology, and a large proportion of patients are refractory to available therapeutic options. Here we identify a new process associated with MDD by demonstrating the association between defective eIF2 and eIF2B translation factors and depressive-like behavior. Future studies are warranted to dissect the specific mechanisms linking impairments in translational control to molecular and functional consequences in MDD.

METHODS

Ethics approval and consent to participate

All procedures involving animals in the present study were carried out in accordance with the "Principles of Laboratory Animal Care" (U.S. National Institutes of Health) guidelines and approved by the Ethics Committee for Animal Research of the Federal University of Rio de Janeiro under protocols A02/22-135-18 and 029-23. Human samples were used in compliance with the ethical guidelines of the Ethics Committee for Human Research of the Federal University of Rio de Janeiro and approved under protocol 4.730.934. Samples from both human cohorts were collected with the consent of each individual's closest relatives, in compliance with the ethical guidelines of both brain banks. Written informed consent for postmortem brain tissue collection and use in research was obtained from donors or relatives by each brain bank team.

Animals

Male C57BL/6 mice (2.5–3 months old) were obtained from the animal facility at the Federal University of Rio de Janeiro. Animals were housed in

groups of up to five per cage at 22 ± 2 °C with ~60% relative humidity and 12 h light/dark cycle (lights on at 07:00) with access to water and food *ad libitum*. Animals were randomly assigned to each group/treatment used.

Pharmacological modulation of protein synthesis

Salubrinal (Sigma-Aldrich #SML0951), a selective inhibitor of the eIF2α phosphatase, GADD34, that promotes eIF2α-P accumulation, was initially dissolved in DMSO and further diluted in saline (0.9% NaCl). Male C57BL/6 mice received salubrinal (1 mg/kg/day) or vehicle intraperitoneally (i.p.) for 8 days, as described [19, 25], in the presence or absence of ISRIB (integrated stress response inhibitor; Sigma-Aldrich #SML0843; 2.5 mg/kg/day i.p.), a small-molecule compound that directly activates eIF2B even in the presence of elevated eIF2α-P [36, 38], from the sixth to the eighth day. The tail suspension test and novelty-suppressed feeding test were performed on days 7 and 8, respectively. For inhibition of global protein synthesis, male C57BL/6 animals were injected with anisomycin (Research Products International—RPI #A50100; 100 mg/kg, i.p.), and 3 h later, they were subjected to behavioral testing. Control animals in both protocols were injected with vehicle solution (NaCl 0.9% with DMSO at the same concentration as used in drug solutions).

Social isolation protocol

Male C57BL/6 mice were individually housed (social isolation—SI) or group-housed (5 mice per cage—control group) in standard mouse cages for 60 days, a protocol adapted from previous reports [68, 69]. Food and water were available *ad libitum* for both groups. The tail suspension test was performed on day 61. Mice were then euthanized, and cerebral tissues were collected for analysis.

Behavioral testing

Tail suspension test (TST). Mice were suspended by their tails at the height of 1 m, so that they were unable to escape or touch any surfaces. They were kept in this position for 6 min, and immobility time was assessed as a measure of helplessness. Total immobility time during the 6 min of the test was compared between the groups [70].

Novelty-suppressed feeding test (NSF). This paradigm evaluates hyponeophagia, in which exposure to a novel environment suppresses feeding behavior and has been used as an indicator of anxiety-related behavior in animals [71]. Mice were fasted for 24 h before the test and then placed in an arena (460 × 300 × 160 mm) containing a food pellet positioned in the center. The latency to feed was measured. The maximum duration of the test was 6 min. To evaluate whether the drugs impaired the latency to feed in the home cage, the home-cage latency to feed test was performed as a standard control. After the NSF test, the animals were returned to their respective cages, and the latency to feed a food pellet was measured.

Rotarod test. To evaluate sensorimotor coordination and motor performance of animals injected with salubrinal and/or ISRIB, a specific group of mice was subjected to the rotarod apparatus (EFF 411, Insight, Brazil) on days 7 and 8 of the protocol. First, mice were subjected to two training sessions (maximum time: 6 min), with a 1-h interval between sessions. Animals were placed on a rod constantly rotating at 16 rpm and latency to fall was measured. During training sessions, the animals were returned to the rod if they fell before 60 s. Twenty-four hours after training, animals were placed in the rotating rod with increasing acceleration from 16 rpm to 37 rpm during a maximum of 6 min (stage 1–2: 16 rpm; stage 3–4: 20 rpm; stage 5–6: 25 rpm; stage 7–8: 28 rpm; stage 9–10: 37 rpm). Latency to fall was measured.

Open field (OF). Animals were placed in an arena measuring 350 × 400 × 170 mm and allowed to freely move for 5 min while being recorded. The total distance traveled, and time spent in the center or periphery zones, respectively, were evaluated as measures of general locomotor activity or anxiety-like behavior.

Human samples

Postmortem human brain samples from individuals diagnosed with MDD and healthy controls were donated from two well-established cohorts and used in the present study. Cohort 1: Prefrontal cortex (PFC) and hippocampus (HP) samples were donated from the Biobank for Aging Studies from the University of São Paulo Medical School (FMUSP, Brazil). The cohort comprised 20 participants diagnosed with major depression (MDD) and 16 healthy controls (HC), of both sexes. Clinical evaluation consisted of information about the clinical and functional status of the subject in the 3 months prior to death and lifetime history of MDD. A semi-structured clinical interview assessed demographics (age, sex, and education), conditions related to death, past clinical and surgical medical history, treatments, smoking habits, alcohol consumption, functional status and neuropsychiatric symptoms [72]. Mean ages were 65.69 ± 14.42 (HC, mean ± SD) and 66.05 ± 14.11 (MDD, mean ± SD) years old. The mean postmortem interval (PMI) was 13.44 ± 2.89 h for HC and 14.45 ± 3.31 h for MDD (Table 1). Cohort 2: RNA samples from postmortem human dorsolateral prefrontal cortex (PFC) were obtained from the Stanley Medical Research Institute Brain Bank (USA). The cohort comprised 36 patients diagnosed with MDD with or without psychotic features, or healthy control subjects (HC). As indicated by the SMRI, diagnoses of MDD were performed by two senior psychiatrists, using DSM-IV criteria and based on medical records and, when necessary, telephone interviews with family members. Diagnoses of unaffected controls were based on structured interviews by a senior psychiatrist with family members to rule out Axis I diagnoses. The exact number of samples from each group used here is represented in Table 2. In this study, the patients with MDD with or without psychotic features were grouped. Patients were mostly white (58% males), and the mean ages varied between 46.4 and 42.8 years for the two groups. The PMI was 29.9 h for MDD patients and 23.5 h for HC (Table 2). More detailed demographic information on the Stanley cohort can be found at https://www.stanleyresearch.org/brain-research/depression-collection. Postmortem hippocampal samples from these subjects were not available at the time of this study. For both cohorts, tissues from patients who had been diagnosed with other neurological or neuropsychiatric diseases were not included in this study.

RNA extraction and qRT-PCR

Total RNA was extracted from human and mouse cerebral tissues using RNeasy Lipid Tissue Mini Kit (Qiagen) or Trizol (Invitrogen), respectively, following manufacturer instructions. RNA purity and integrity were assessed using the 260/280 absorbance ratio determined by Nanodrop and only preparations with values ≥1.8 were used, thus, low-quality mRNA samples were excluded from analysis. For quantitative real-time reverse transcription PCR (qRT-PCR) 1 µg of RNA was used for the synthesis of complementary DNA (cDNA) using the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific). Quantitative expression analysis of target genes was performed on a 7500 Real-Time PCR system (Thermo Fisher Scientific) with PowerUp SYBR Green PCR Master Mix (Thermo Fisher Scientific), as described [45, 73, 74]. For mouse samples, β-actin (*actb;* Fw: TGTGACGTTGACATCCGTAAA; Rv: GTACTTGCGCTCAGGAGGAG) was used as an endogenous reference gene. For human samples, the geometric mean of β-actin (*actb;* Fw: GCACCCAGCACAATGAAG; Rv: CTTGCTGATCCACAT) and GAPDH (*gapdh;* Fw: CCTGTTCGACAGTCAGCCG; Rv: CGACCAAATCCGTTGACTCC) was used as endogenous reference. Specific mouse *EIF2B5* (Fw: CACTGCACCGTCTGTCCTTAG; Rv: CCTGCCGAGTGTCTGTTTAG) and human *EIF2B5* (Fw: TGTCCTCCGTGATGTTGATG; Rv: CCTCAAGGGCTCTGGTGATA) primers were used. qRT-PCR was performed in 20 µL reactions, in accordance with manufacturer instructions. Cycle threshold (C_t) values were used to calculate fold change in expression relative to controls using the $2^{-\Delta\Delta Ct}$ method [75] .

Statistical analysis

All data were analyzed with GraphPad Prism software version 8.0 for Windows (San Diego, CA, USA). Data were expressed as mean ± standard error of the mean (SEM). Data were tested for Gaussian distribution and outliers were identified (ROUT 1%) before statistical tests and excluded from analysis. Sample size for each experiment (including animal experiments) was estimated by performing pilot studies and by previous experience with different experiments. The rationale was to obtain robust measurements according to the type of experiment, taking variability into consideration. RT-qPCR assays on human samples were performed with the experimenter blind to the groups. The behavioral tests were not made blindly; however, all experiments were carried out by more than one experimenter. Statistical significance was carried out using two-way ANOVA followed by Holm-Sidak multiple comparison corrections or Student's t-test, as indicated in figure legends. Differences between the groups were considered statistically significant when $p \leq 0.05$. p-values and data dispersion are shown in the bars.

DATA AVAILABILITY

All data produced in this study is shown in manuscript and supplementary information, and unprocessed data are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

ARI designed the study, performed research, analyzed data, discussed results. MGC performed research. RLF performed research. BAW performed research. BGC performed research. and MVL designed the study. REPL contributed reagents, materials and analysis tools. CKS contributed reagents, materials and analysis tools. PVN contributed reagents, materials and analysis tools, and discussed results. FGDF contributed reagents, materials, and analysis tools. STF contributed reagents, materials and analysis tools, and discussed results. MVL designed the study, contributed reagents, materials and analysis tools, and discussed results. ARI and MVL wrote the manuscript with inputs from all authors.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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