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Antidepressant-relevant behavioral and synaptic molecular effects of long-term fasudil treatment in chronically stressed male rats

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ABSTRACT

Several lines of evidence suggest that antidepressant drugs may act by modulating neuroplasticity pathways in key brain areas like the hippocampus. We have reported that chronic treatment with fasudil, a Rho-associated protein kinase inhibitor, prevents both chronic stress-induced depressive-like behavior and morphological changes in CA1 area. Here, we examined the ability of fasudil to (i) prevent stress-altered behaviors, (ii) influence the levels/phosphorylation of glutamatergic receptors and (iii) modulate signaling pathways relevant to antidepressant actions. 89 adult male Sprague-Dawley rats received intraperitoneal fasudil injections (10 mg/kg/day) or saline vehicle for 18 days. Some of these animals were daily restraint-stressed from day 5–18 (2.5 h/day). 24 hr after treatments, rats were either evaluated for behavioral tests (active avoidance, anxiety-like behavior and object location) or euthanized for western blot analyses of hippocampal whole extract and synaptoneurosome-enriched fractions. We report that fasudil prevents stress-induced impairments in active avoidance, anxiety-like behavior and novel location preference, with no effect in unstressed rats. Chronic stress reduced phosphorylations of ERK-2 and CREB, and decreased levels of GluA1 and GluN2A in whole hippocampus, without any effect of fasudil. However, fasudil decreased synaptic GluA1 Ser831 phosphorylation in stressed animals. Additionally, fasudil prevented stress-decreased phosphorylation of GSK-3β at Ser9, in parallel with an activation of the mTORC1/4E-BP1 axis, both in hippocampal synaptoneurosomes, suggesting the activation of the AKT pathway. Our study provides evidence that chronic fasudil treatment prevents chronic stress-altered behaviors, which correlated with molecular modifications of antidepressant-relevant signaling pathways in hippocampal synaptoneurosomes.

1. Introduction

Stressful life experiences target the human brain and trigger the release of different stress mediators as adaptive responses, with consequent synaptic remodeling in several brain areas (McEwen et al., 2016). In contrast, chronic exposure to stressful experiences may produce maladaptive responses and increase the vulnerability of individuals to develop mental disorders, such as the highly comorbid depressive and anxiety disorders (Craske et al., 2017; Kendler et al., 1999; Mineka et al., 1998). Indeed, these disorders seem to share affected brain areas, including the hippocampus, amygdala and prefrontal cortex (PFC) (Craske et al., 2017; McEwen et al., 2016; Otte et al., 2016; Price and Drevets, 2010).

Several lines of evidence have revealed a strong association between

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alterations in mood and memory with a reduction of hippocampal volume (Malykhin and Coupland, 2015). These observations are in agreement with preclinical studies revealing that chronically stressed rodents have reduced hippocampal volume as a consequence of neuronal atrophy, dendritic arbor simplification (Pinto et al., 2015) and dendritic spine loss in CA1 pyramidal neurons (Castañeda et al., 2015). Functionally, hippocampal-dependent memory is affected by chronic stress in male rats (Luine, 2002). These evidences suggest that chronic stress triggers maladaptive plasticity of hippocampal synapses, which may participate in the pathology underlying depressive and anxiety disorders. Indeed, considering that the hippocampus is composed of intrinsic and extrinsic glutamatergic pathways, a dysfunctional glutamatergic synapse hypothesis for depression has arisen (Sanacora et al., 2012; Thompson et al., 2015). Glutamatergic receptors involved in fast excitatory neurotransmission include α-amino-3-hydroxyl-5-methyl -4-isoxazole-propionate (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors (AMPARs, NMDARs) (Traynelis et al., 2010). In the adult rat hippocampus, AMPARs are composed of GluA1-3 subunits (Wenthold et al., 1996). GluA1 can be phosphorylated at Ser831 and Ser845, which enhances AMPAR trafficking/channel conductance and AMPAR membrane insertion, respectively (Derkach et al., 2007). Additionally, GluA2 presence renders the AMPAR impermeable to Ca^{2+} (Derkach et al., 2007). Interestingly, changes in GluA1-phosphorylation and GluA2-presence modify synaptic efficacy (Derkach et al., 2007). On the other hand, NMDARs are composed of two GluN1 subunits and mainly two GluN2A or GluN2B subunits, for which the presence of GluN2A -in contrast to GluN2B- enhances open probability and channel deactivation, and lowers calcium conductance of the NMDAR (Sanz-Clemente et al., 2013).

Interestingly, various reports in rodents have demonstrated that chronic stress disturbs glutamatergic neurotransmission in the hippocampus (Kallarackal et al., 2013; Marrocco et al., 2014; Marsden, 2013; Sanacora et al., 2012; Tornese et al., 2019). On the other hand, antidepressants have also been shown to modify glutamatergic components in the hippocampus (Amidfar et al., 2019; Marrocco et al., 2014; Marsden, 2013; Martinez-Turrillas et al., 2002; Pittaluga et al., 2007; Tornese et al., 2019; Van Dyke et al., 2019; Zanos et al., 2016). For instance, chronic treatment with the selective serotonin-reuptake inhibitor (SSRI) fluoxetine or the selective noradrenaline-reuptake inhibitor reboxetine, increased the levels of GluA1 and GluA2 in the hippocampus (Barbon et al., 2011; Van Dyke et al., 2019). However, most studies assessing antidepressants effects are usually conducted in unstressed animals and only a few have evaluated their effects in a stress context. One report has shown that chronic stress increases GluN2A and GluN2B levels in the ventral hippocampus, while chronic treatment with the serotonin-noradrenaline reuptake inhibitor duloxetine restores only GluN2A levels in stressed rats (Calabrese et al., 2012). Further studies addressing antidepressant drugs in a stress context are mandatory to provide a higher translational value to new therapeutic agents against psychiatric disorders.

Additionally, antidepressants modulate signaling pathways that regulate neuroplasticity in the hippocampus (Duman and Voleti, 2012; Gould et al., 2019). These antidepressant-relevant pathways include the RAC serine/threonine protein kinase (AKT) (Park et al., 2014), the mechanistic target of rapamycin complex 1 (mTORC1) (Li et al., 2010; Park et al., 2014; Zhou et al., 2014), glycogen synthase kinase-3β (GSK-3β) (Beurel et al., 2011; Li et al., 2004) and the mitogen-activated protein kinase 1/extracellular signal-regulated protein kinase 2 (ERK-2) (Carrier and Kabbaj, 2011). Moreover, several antidepressants enhance cyclic AMP-responsive element-binding protein (CREB)-dependent transcription in the hippocampus (Blendy, 2006). These pathways converge in the control of protein synthesis and neuron survival, leading to an enhancement of synaptogenesis and connectivity (Gould et al., 2019). Combined, these findings support the idea that antidepressants may fine-tune glutamatergic components and synaptic plasticity.

We have previously reported that chronic treatment with fasudil, a Rho-associated protein kinase (ROCK) inhibitor, exerts several effects in chronically stressed rats, including antidepressant-like actions in the forced swimming test (FST) and prevention of stress-induced dendritic CA1 spine loss (García-Rojo et al., 2017). More recently, it has been described that fasudil also has antidepressant-like effects in unstressed adolescent mice (Shapiro et al., 2019) and physically stressed adult mice (Nakatake et al., 2019), according to FST analyses. Importantly, fasudil may impact antidepressant-relevant signaling pathways. For example, ROCK acts as a negative regulator of the AKT-mTORC1 pathway at two different levels. First, ROCK phosphorylates and activates PTEN, a negative regulator of AKT (Koch et al., 2018). Secondly, ROCK is capable of interacting with TSC2, which leads to the inhibition of mTORC1 (Koch et al., 2018). These findings suggest that ROCK inhibition by fasudil may enhance AKT-mTORC1 signaling.

As an extension to these findings, here we explored further antidepressant-relevant behavioral and molecular effects of fasudil, by employing a rat chronic restraint stress model -useful for studying depressive-like behaviors and antidepressants (Bravo et al., 2009; Ulloa et al., 2010)- to delve into the preventive effects of fasudil against chronic stress. We aimed to determine whether stress-triggered behav ioral alterations could be prevented by concomitant chronic fasudil treatment, and whether these changes correlate with molecular modi fications of hippocampal glutamatergic components and antidepressant -relevant signaling pathways.

2. Materials and methods

2.1. Treatments

Chronic stress and fasudil treatments were performed as described (García-Rojo et al., 2017). Briefly, 89 adult male Sprague-Dawley rats randomly received one of the following treatments: (i) unstressed animals injected intraperitoneally every day for 18 days with saline (0.9% NaCl; Control) or with 10 mg/kg fasudil (LC Laboratories, Woburn, MA, USA; Fasudil), or (ii) stressed rats treated every day with saline (Stress) or 10 mg/kg fasudil (Stress-Fasudil) for 18 days, but daily submitted to restraint stress as described (Pacheco et al., 2017), from the 5th day of injections for 14 consecutive days. Three different cohorts of animals were used: two for behavioral testing and one for molecular analyses, in order to avoid any biases induced by behavioral tests exposure. Fig. S1 provides detailed timelines for each cohort. Efforts were made to minimize both the number of animals and their suffering. All procedures were approved by the Ethical Committee of the Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile, in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publication, 8th edition, 2011). Female subjects were excluded from the present study since our model of chronic restraint stress failed to trigger significant behavioral alterations (data not shown) and we focused primarily in the preventive effects of fasudil against chronic stress.

2.2. Active avoidance conditioning (AAC)

This test was performed as described (Bravo et al., 2009; Castañeda et al., 2015; Ulloa et al., 2010). Briefly, 24 h after the last treatment of the first cohort of animals (Fig. S1A), each rat was individually placed in a two-way shuttle box (Lafayette Instrument Co., Lafayette, IN, USA). After habituation for 5 min, rats were subjected to 5 sets of 10 trials each (intertrial interval: 30 s). Each trial consisted in the presentation of a tone (2800 Hz) and following 5 s an electric foot shock (0.20 mA) was overlapped to the chamber where the animal was located. Each shock lasted until the animal escaped onto the opposite chamber (maximum shock duration: 10 s). A conditioned avoidance response (CAR) was defined as the crossing onto the opposite chamber within the first 5 s of the tone, before the shock was applied. If no escape response to the shock occurred throughout shock duration, both the shock and tone

were discontinued and an escape failure (EF) was registered.

2.3. Object location test (OLT)

Rats of a second cohort were habituated to a 60 × 60 cm square arena in two daily sessions of 10 min, during days 17 and 18 of injections (Fig. S1B). 24 hr after the last treatment, the acquisition and testing phases were performed as described (Aguayo et al., 2018b). Each animal was allowed to explore two identical objects located in adjacent corners for 3 min. After a delay of 5 min, one object was placed in its original position and the other in one diagonal corner. The time that the rat explored each object was registered during 3 min and a discrimination index (DI) was calculated as the difference between times spent exploring the object in the novel location and the object in the familiar location, as a percentage of total exploration time.

2.4. Elevated plus maze (EPM)

One hour after assessing the OLT activity, each rat was tested in the EPM (Fig. S1B), as described (Ulloa et al., 2010). During a 5 min period, the number of entries and time spent in the open and closed arms were registered. The results correspond to the percentage of open arm entries and of time spent in open arms, regarding the total number of entries and the total time spent in both open and closed arms, respectively.

2.5. Western blot of synaptoneurosome-enriched and whole homogenate fractions from rat hippocampus

24 h after treatments, animals of a third cohort not tested for behavioral analyses (Fig. S1C) were euthanized to obtain protein extracts from hippocampal whole homogenates and synaptoneurosome-enriched fractions, as we have reported (Aguayo et al., 2018a). Western blot was performed as described (Aguayo et al., 2018b, 2018a). Briefly, 30 μg or 15 μg of protein of whole hippocampal homogenates or synaptoneurosomes, respectively, were resolved in 10% SDS-polyacrylamide gels. Proteins were then electroblotted onto 0.2 μm nitrocellulose or PVDF membranes. Membranes were finally processed for western blot, according to the conditions depicted in Table S1. Blots were then incubated with the appropriate HRP-conjugated secondary antibody: anti-rabbit IgG (Cell Signaling Technology, cat #7074) or anti-mouse IgG (Merck, cat #402335). Membranes were developed by incubation with enhanced chemiluminescent substrate (EZ-ECL, Biological Industries, Israel) and imaged with Syngene (Cambridge, UK). Bands were quantified with ImageJ (https://imagej.nih.gov/ij), relativized to an internal control sample (loaded equally across all gels) and normalized to β-actin immunoreactivity as a loading control.

2.6. Statistical analyses

Statistical analyses were performed using Prism 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as mean ± standard error of the mean (SEM). The number of acquired CARs along time points (*i.e.,* trial sets) was analyzed by three-way analysis of variance (ANOVA) with matching by trial set, followed by Tukey's multiple comparisons test. The effects of fasudil, stress and their interaction (stress × fasudil) in other parameters were evaluated by two-way ANOVA and differences between groups were determined with Tukey's test. Differences between two groups that differed only by one factor were analyzed with the Mann-Whitney *U* test, where indicated. Sample sizes (*n*) are indicated beneath figure legends. Full detail of test statistics and sample sizes for figures in the main text and for figures in the supplementary material are shown in Table S2 and Table S3, respectively.

3. Results

3.1. Chronic fasudil treatment prevents stress-induced impairments in active avoidance conditioning, anxiety-like behavior and novelty preference

Given that chronic restraint stress impairs active avoidance conditioning (Bravo et al., 2009; Castañeda et al., 2015), we evaluated whether this impairment was sensitive to fasudil. We found a significant stress × fasudil × trial set interaction ($p = 0.0239$; Table S2) and post-hoc comparisons showed that from the 3rd trial set forward, control rats started to acquire conditioned responses (CARs), while saline-treated stressed rats were unable to achieve conditioned avoidance (Fig. 1A). Interestingly, fasudil treatment completely avoided the stress-provoked impairment in CARs acquisition (Fig. 1A). When the overall CARs were considered, a significant stress × fasudil interaction was found ($p = 0.0041$; Table S2) and saline-treated stressed rats showed reduced percentage of CARs vs controls, which fasudil completely prevented (Fig. 1B). Accordingly, the percentage of escape failures (EFs) also displayed a significant stress × fasudil interaction ($p = 0.0014$; Table S2) and were significantly increased in saline-treated stressed rats, compared to controls (Fig. 1C), an effect also fully prevented by fasudil (Fig. 1C). Fasudil did not exert any effect on CARs and EFs in unstressed animals (Fig. 1B and C). These results indicate that fasudil was able to completely prevent chronic stress-induced impairments of AAC, but without any effect in unstressed rats.

Since chronic stress may cause anxiety disorders in humans (Craske et al., 2017) and anxiety-like behaviors in rodents (Bondi et al., 2008), we used the EPM test to explore whether fasudil had any effects in anxiety-like behavior in both unstressed and stressed animals. We found that the percentage of time spent in the open arms exhibited a significant stress × fasudil interaction ($p = 0.0047$; Table S2) and was decreased in saline-treated stressed rats, compared to controls, which could be partially prevented by fasudil (~54% of control group; Fig. 1D). Similarly, the percentage of entries intro open arms showed a significant stress × fasudil interaction ($p = 0.0047$; Table S2), for which saline-treated stressed rats displayed less percentage of entries into open arms vs controls, while fasudil partially prevented this effect (~56% of control group; Fig. 1E). Notably, fasudil did not evoke any effect in unstressed animals (Fig. 1D and E). These findings suggest that chronic stress induces an anxiogenic-like effect on rats, which could be partially prevented by fasudil, while fasudil has not neither anxiogenic- nor anxiolytic-like effects in unstressed animals.

It is well known that chronic stress impairs hippocampal-dependent memory in male rats (Luine, 2002; Pinto et al., 2015). Therefore, we determined whether this impairment was sensitive to fasudil using the object location test (OLT), which relies strongly on the hippocampus (Mumby et al., 2002). The OLT measures the ability of rats to distinguish between a novel location and familiar location of an object. A significant stress × fasudil interaction was found for the discrimination index ($p = 0.0047$; Table S2), which was significantly reduced in saline-treated stressed rats compared to controls, and chronic fasudil treatment fully prevented this reduction, with no effect in unstressed rats (Fig. 1F). Total exploration time was not affected by treatments (Fig. 1G; Table S2). These results indicate that chronic stress induced an impairment in novel location preference, which was completely prevented by fasudil.

Overall, our behavioral analyses provide evidence that fasudil was able to fully prevent the chronic stress-induced impairments in active avoidance and novel location preference, while it only partially prevented the stress-increased anxiety-like behavior.

3.2. Chronic stress decreases activating phosphorylations of ERK-2 and CREB, while fasudil only partly prevents the reduction in CREB phosphorylation

Since chronic stress reduces the signaling of the ERK-CREB pathway

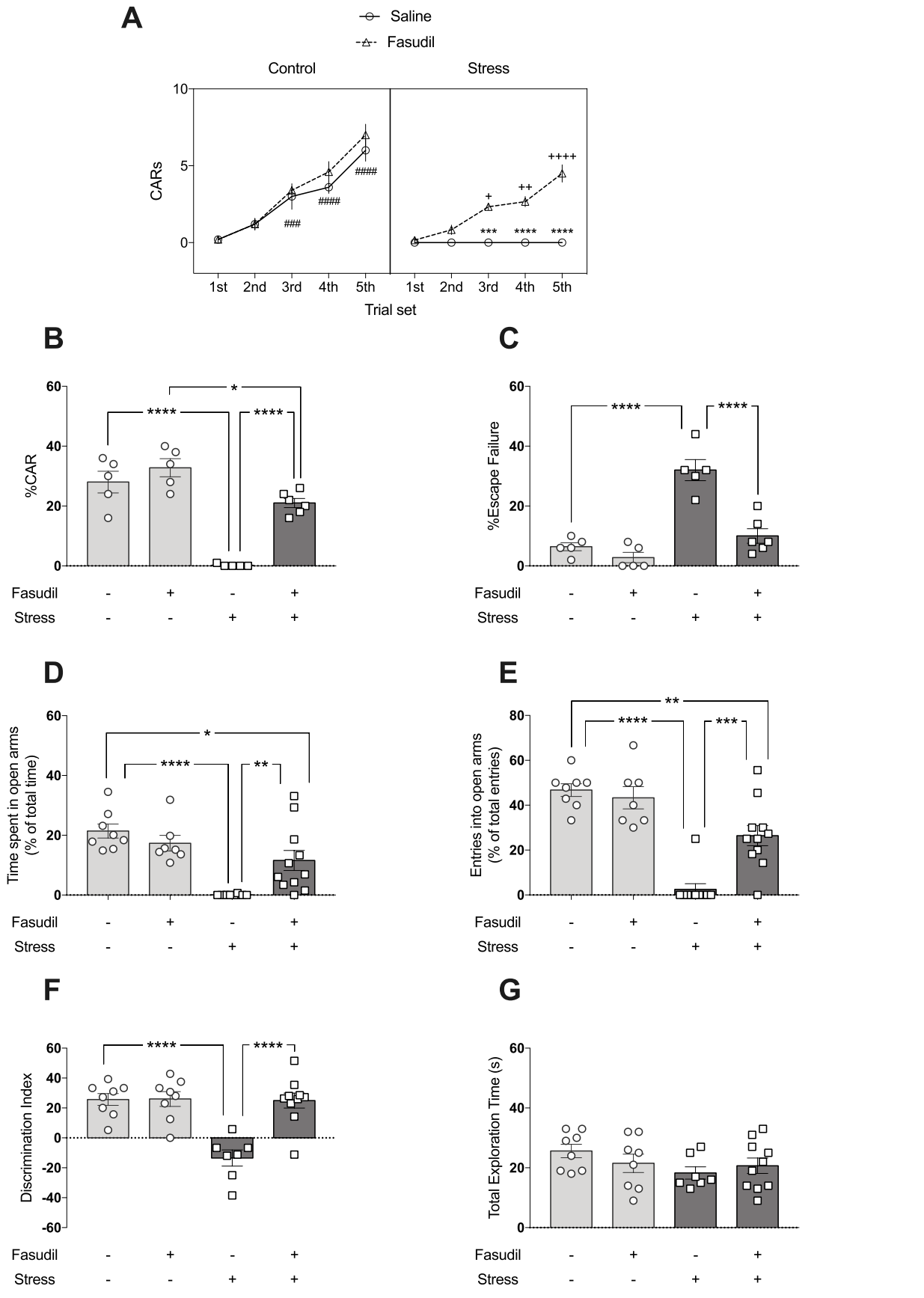

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Fig. 1. Chronic fasudil treatment prevents stress-induced impairments in active avoidance conditioning, anxiety-like behavior and novelty preference. The number of conditioned avoidance responses (CARs) and escape failures (EFs) were determined in the active avoidance conditioning test. **(A)** Number of acquired CARs along each trial set (3-way ANOVA followed by Tukey's test: ###$p < 0.001$ and ####$p < 0.0001$ vs the 1st trial set of control saline; ***$p < 0.001$ and ****$p < 0.0001$ vs control saline; ++++ $p < 0.0001$, ++ $p < 0.01$ and + $p < 0.05$ vs stress-saline; points represent mean ± SEM; $n = 5$–6 per condition). Graphs representing the overall percentage of **(B)** CARs and **(C)** EFs ($n = 5$–6 per condition). The percentage of **(D)** time spent in open arms and **(E)** entries into open arms, regarding total time and entries, were measured in the elevated plus maze ($n = 7$–11 per condition. **(F)** The discrimination index, a measure of novelty preference, calculated in the object location test. **(G)** Total exploration time of rats during the object location test ($n = 7$–10 per condition). **(B-G)** Graph bars represent mean ± standard error of the mean and were analyzed by two-way ANOVA. Multiple comparisons were calculated with Tukey's test: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$ and * $p < 0.05$. Table S2 shows detailed sample sizes and test statistics.

in the hippocampus (Dwivedi and Zhang, 2016; Laifenfeld et al., 2005), we then evaluated whether our model recapitulates these observations and if fasudil could prevent altered ERK-CREB signaling in hippocampal homogenates. We found a significant main effect of stress in pThr185/Tyr187 ERK-2 ($p = 0.0014$; Table S2). Indeed, these activating phosphorylations were reduced by stress, but with no preventive effect of fasudil (Fig. 2C). In contrast, we found a stress × fasudil interaction ($p = 0.0091$; Table S2) for the CREB-activating phosphorylation at Ser133, and post-hoc analysis showed that pSer133-CREB levels were decreased in stressed rats by 40% compared to controls (Fig. 2D). Notably, fasudil

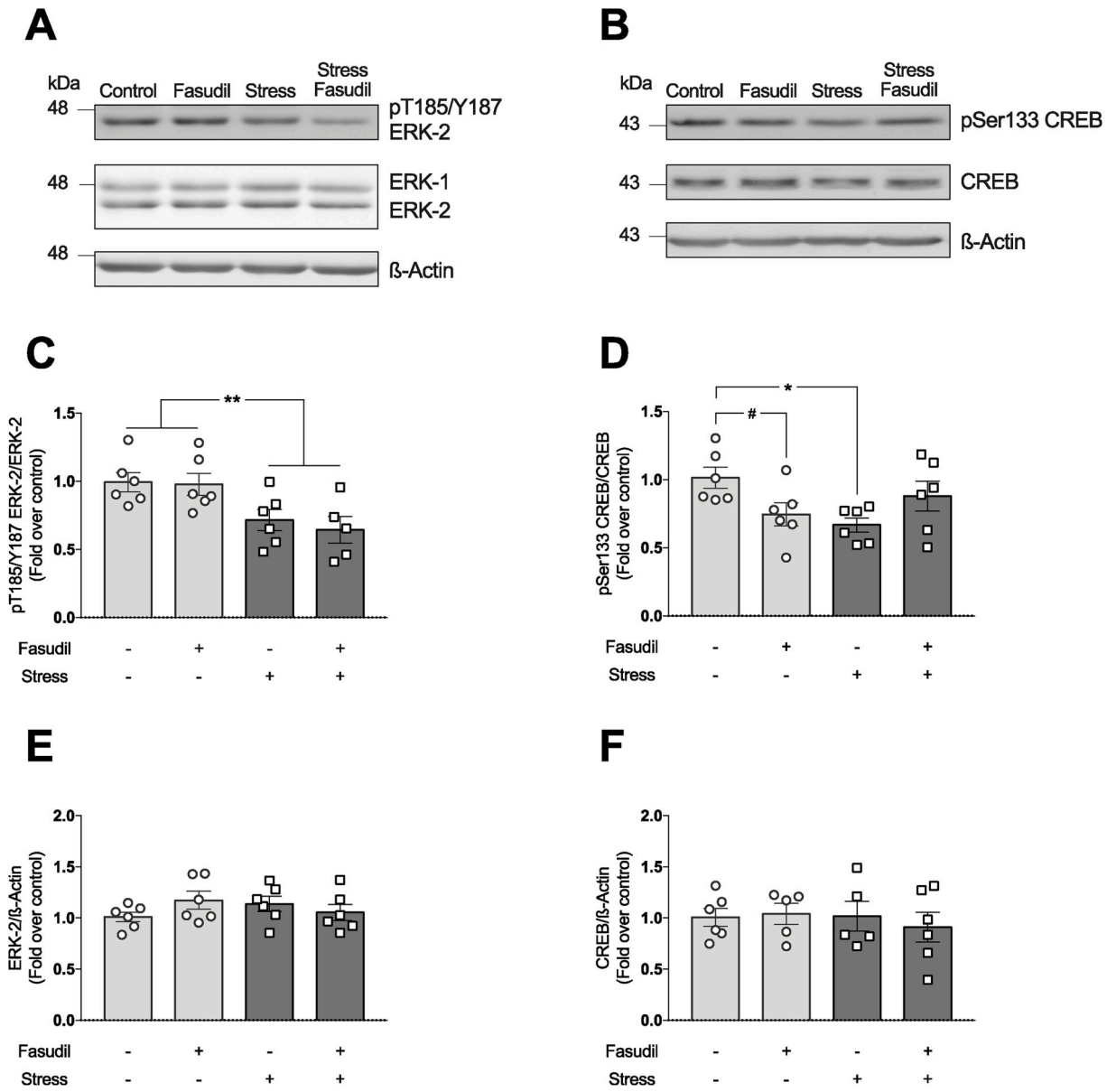

Fig. 2. Chronic stress decreases phosphorylation levels of ERK-2 and CREB in hippocampal homogenates of adult male rats. (A) Representative western blots of phosphorylated and total ERK-2 levels. **(B)** Phosphorylation of ERK-2 at Thr185/Tyr187 was decreased by chronic stress despite fasudil treatment. **(C)** Total ERK-2 levels remained unchanged. **(D)** Representative western blots of phosphorylated and total CREB levels. **(E)** Ser133 phosphorylation of CREB was reduced in chronically stressed rats, while fasudil did not fully prevent this decrease. **(F)** Total CREB levels were not affected by treatments. Bars represent mean ± SEM. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test. *$p < 0.05$, **$p < 0.01$. Two-tailed Mann-Whitney test: #$p < 0.05$. Table S2 shows detailed sample sizes and two-way ANOVA details. $n = 5$–6 per condition.

reduced pSer133-CREB levels in unstressed rats (two-tailed Mann-Whitney test: $U = 4$, $p = 0.0260$; Fig. 2D). Total ERK-2 and CREB levels were not modified by treatments (Fig. 2E and F). Overall, these results suggest that the ERK-CREB pathway is impaired in the hippocampus of male rats following our chronic restraint stress model, although with no significant improvement elicited by fasudil.

3.3. Chronic stress decreased GluA1 and GluN2A levels regardless of fasudil treatment in whole hippocampal homogenate

To explore whether chronic stress and fasudil had any impact on glutamatergic components in the hippocampus, we evaluated AMPAR and NMDAR subunits -and some of GluA1 functional phosphorylations- in whole hippocampal homogenates. Our rationale was that fasudil-normalized behaviors, which are integrated or dependent on hippocampal circuitry, may involve molecular changes of hippocampal glutamatergic components. We observed that GluA1 levels were decreased by stress in hippocampal homogenates, despite fasudil treatment (main effect of stress, $p = 0.0053$; Fig. 3A, Table S2). On the contrary, neither GluA1 phosphorylations at Ser845 and Ser831 nor GluA2 levels were modified by treatments in hippocampal homogenates (Fig. 3B, C, D, respectively). Regarding NMDAR subunits, GluN1 and GluN2B levels were unchanged by stress and fasudil in hippocampal homogenates (Fig. 3E and G). However, chronic stress triggered a reduction of GluN2A levels (main effect of stress, $p = 0.0003$; Fig. 3F, Table S2), which was insensitive to fasudil treatment.

3.4. Chronic fasudil treatment increases synaptic GluA1 and GluA2 in unstressed rats, while it reduces synaptic GluA1 Ser831 phosphorylation in stressed rats

This far we have noted noxious effects of chronic stress in some glutamatergic components and ERK-CREB signaling in hippocampal homogenates, without major intervening effects of fasudil. To further address whether fasudil triggers molecular changes in the hippocampus -that may be in agreement with the observed behavioral outcomes- we used a well-characterized synaptoneurosome-enriched fraction (Aguayo et al., 2018b, 2018a) to analyze more precisely the variation of synapse-located proteins. Despite that neither fasudil nor chronic stress exerted main variance effects for GluA1 synaptic levels, a pairwise comparison revealed that fasudil increased synaptic GluA1 levels by 40% in unstressed rats vs controls (two-tailed Mann-Whitney test: $U = 4$, $p = 0.0260$; Fig. 4A), while GluA1 phosphorylation at Ser845 was unaffected by treatments (Fig. 4B). Interestingly, phosphorylation of GluA1 at Ser831 displayed a stress × fasudil interaction ($p = 0.0245$; Table S2) and was significantly decreased by 40% in fasudil-treated stressed rats vs controls (Fig. 4C). Additionally, GluA2 levels also displayed a significant stress × fasudil interaction ($p = 0.0101$; Table S2) and were increased by fasudil by 60% in unstressed animals, vs controls (Fig. 4D). Regarding NMDAR subunits in synaptoneurosomes, we observed that GluN1, GluN2A and GluN2B levels were insensitive to treatments (Fig. 4E–G). These results suggest that fasudil triggers particular changes in glutamatergic components in hippocampal synaptoneurosomes depending on the presence/absence of a stressful context.

Since we have reported that fasudil prevents stress-induced dendritic spine loss in the CA1 subfield of the hippocampus (García-Rojo et al., 2017), we decided to evaluate modifications in the levels of key pre- and post-synaptic scaffolding proteins. The levels of presynaptic protein synaptophysin, and postsynaptic markers PSD-95 and Homer-1, were not affected by treatments in hippocampal synaptoneurosomes (Fig. S2).

3.5. Fasudil modulates antidepressant-relevant signaling pathways in the hippocampus

The changes observed in hippocampal glutamatergic components may involve modifications of hippocampal synaptic plasticity. Hence, we wondered whether antidepressant-relevant pathways involved in synaptic plasticity could be sensitive to stress and fasudil in hippocampal synaptoneurosomes. We found that phosphorylation of AKT at Ser473 was not affected by treatments (Fig. 5A). However, AKT-mediated GSK-3β phosphorylation at Ser9 was decreased in saline-treated stressed rats, which was prevented by fasudil treatment (main effect of fasudil: $p = 0.0119$; Fig. 5B). Additionally, phosphorylation of the AKT effector mTOR at Ser2448 was increased by fasudil in both unstressed and stressed rats (main effect of fasudil: $p = 0.0210$; Fig. 5C). Accordingly, mTORC1-mediated phosphorylations of the eukaryotic translation initiation factor 4E-binding protein (4E-BP1) at Thr37/46 were increased by fasudil in both unstressed and stressed rats (main effect of fasudil: $p = 0.0359$; Fig. 5E). However, the mTORC1-mediated phosphorylation of S6 kinase (S6K) at Thr389 was not modified by treatments (Fig. 5D). Total protein levels of the studied phosphorylations remained unchanged by treatments (Fig. S3).

Since mTORC1/4E-BP1 signaling controls local protein synthesis at the synapses, we evaluated the synaptic levels of LIM domain kinase-1 (LIMK-1), an actin-remodeling protein that can be synthesized *de novo* in the post-synapse (Schratt et al., 2006). We found that LIMK-1 levels were sensitive to chronic stress (main effect of stress: $p = 0.0114$), with a close fasudil effect ($p = 0.1012$), and post-hoc analysis showed increased LIMK-1 in hippocampal synaptoneurosomes of fasudil-treated stressed rats (Fig. 5F). Overall, these results suggest that fasudil regulates antidepressant-relevant signaling pathways that may explain its actions on behavior.

4. Discussion

Our observations have proven that fasudil prevents noxious effects of chronic stress in associative learning, anxiety-like behavior and novel location preference, accompanied with particular molecular modifications in hippocampal synaptic fraction. Some of these molecular changes were produced by fasudil either in stressed or unstressed animals, while others were observed in both groups.

It is well-described that several stress paradigms trigger profound effects in behaviors related to emotionality, learning and memory (McEwen et al., 2016). In the present study, we evaluated animals in a shuttle-box AAC paradigm, which provides a measure of associative learning and memory. Some studies have evaluated the effects of drugs with antidepressant-like effect in this kind of active avoidance. For example, in unstressed animals, acute administration of different tricyclic antidepressants (TCAs) or the SSRI fluoxetine impaired avoidance behavior (Lucki and Nobler, 1985). Likewise, chronic treatment with the SSRI sertraline in unstressed rats also impaired active avoidance (Ulloa et al., 2010); however, chronic treatment with the monoamine oxidase inhibitor (MAOI) moclobemide enhanced the acquisition of CARs in active avoidance in unstressed animals (Getova et al., 2003). These findings suggest that no singular effect of antidepressants is observed in AAC; nevertheless, fasudil had no effect in AAC in unstressed rats. In this and previous studies (Bravo et al., 2009; Castañeda et al., 2015), we have found that chronic stress impairs the acquisition of CARs and increases EFs. Interestingly, fasudil completely prevented these stress-affected parameters, similarly to the TCA desipramine (Bravo et al., 2009), but contrarily to sertraline (Ulloa et al., 2010).

Several antidepressants are useful for the treatment of anxiety disorders (Ravindran and Stein, 2010). A recent report revealed that oral administration of 10 mg/kg/day fasudil for 30 days had an anxiogenic-like effect in elderly mice tested in the EPM (Greathouse et al., 2019), a well validated test for studying anxiety-modulating drugs (Pellow and File, 1986). However, we did not observe any effect of fasudil in unstressed rats, which could be related to the duration of fasudil treatment; *i.e.*, 18 days (this study) vs 30 days (Greathouse et al., 2019). Analogously, another report showed that acute fasudil treatment in unstressed adult mice did not affect latency to approach food in the

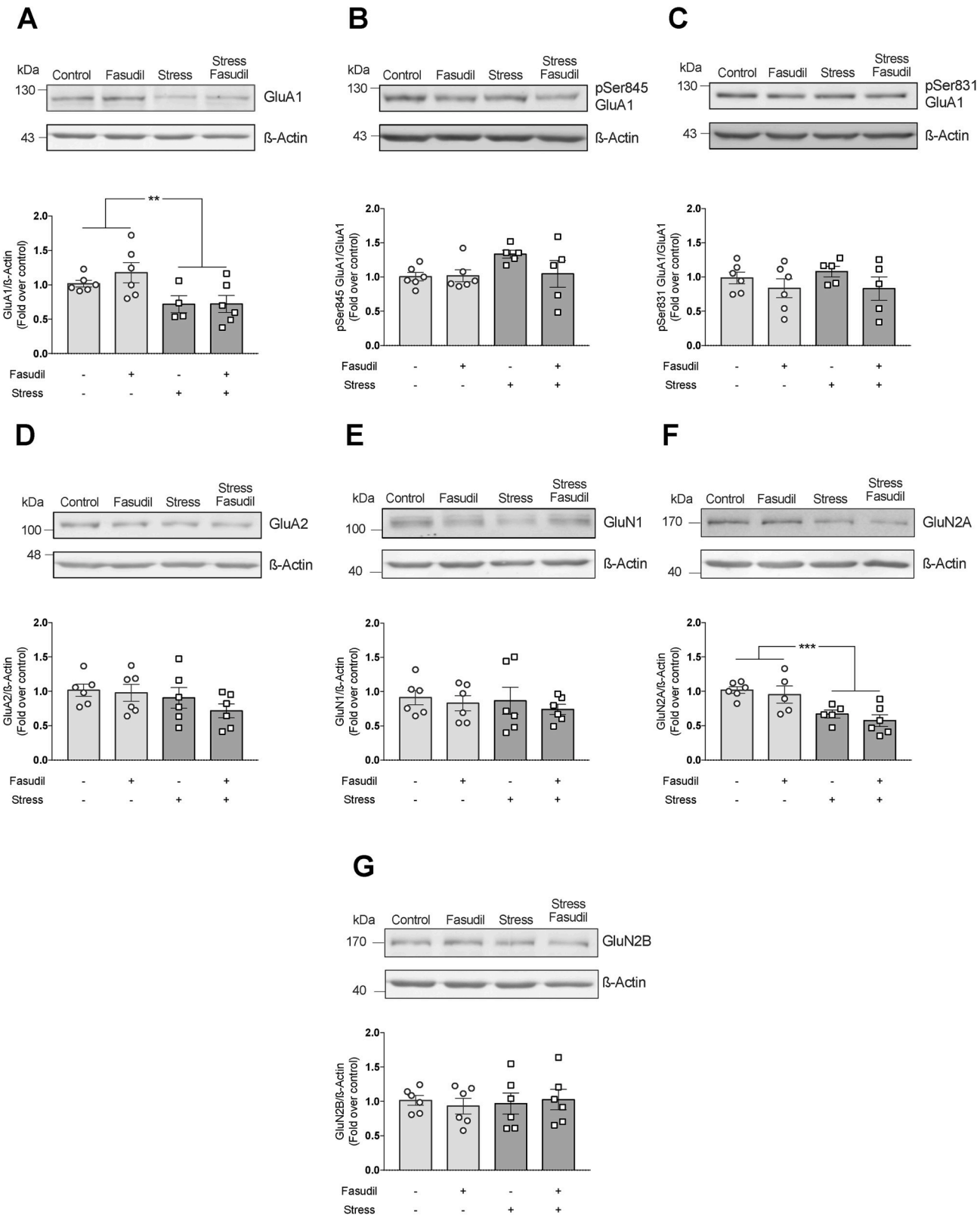

Fig. 3. GluA1 and GluN2A levels were decreased by chronic stress regardless of fasudil treatment in whole hippocampal homogenates. Western blot analyses of **(A)** GluA1, **(B)** pSer845 GluA1, **(C)** pSer831 GluA1, **(D)** GluA2, **(E)** GluN1, **(F)** GluN2A and **(G)** GluN2B levels in whole hippocampal homogenates. Bars represent mean ± SEM. Data were analyzed by two-way ANOVA, main effect of stress: $*p < 0.05$. Table S2 shows sample sizes and two-way ANOVA details. $n = 5–6$ per condition.

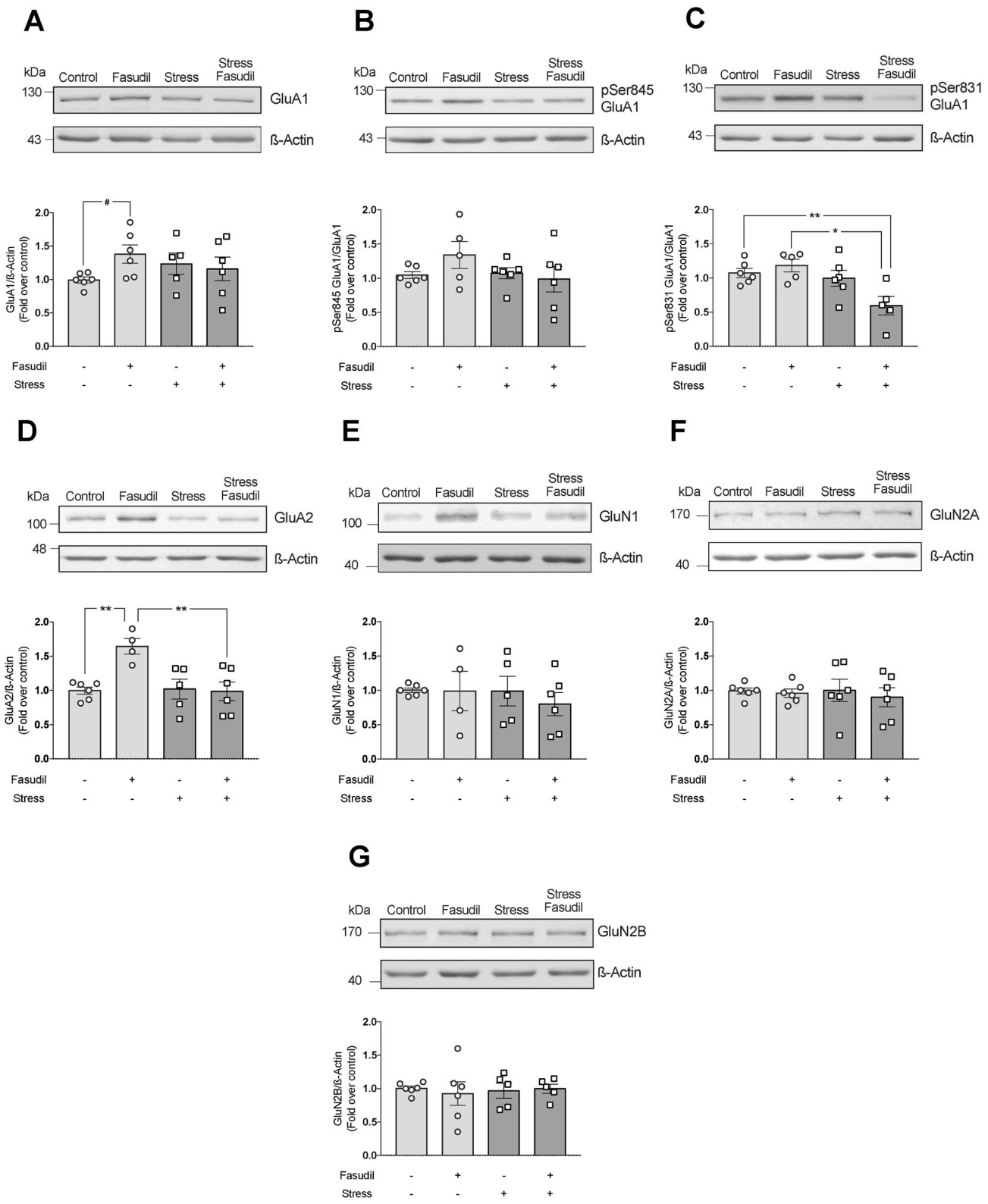

Fig. 4. Long-term fasudil treatment reduces Ser831 phosphorylation of GluA1 in stressed rats and increases GluA1 and GluA2 levels in unstressed animals in hippocampal synaptoneurosomes. Western blot analyses of synaptoneurosomes from the hippocampus of rats treated with fasudil and/or chronic stress. **(A)** GluA1 was increased by fasudil in unstressed rats. **(B)** Phosphorylation of GluA1 at Ser845 was unaffected by treatments, **(C)** while phosphorylation at Ser831 was decreased in fasudil-treated stressed rats. **(D)** GluA2 levels were increased by fasudil in unstressed rats. Synaptic levels of NMDAR subunits **(E)** GluN1, **(F)** GluN2A and **(G)** GluN2B were not modified by treatments. Data were analyzed by two-way ANOVA, followed by Tukey's post-hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$. Two-tailed Mann-Whitney test: $^{\#}p < 0.05$. Table S2 shows detailed sample sizes and two-way ANOVA details. $n = 4$–6 per condition.

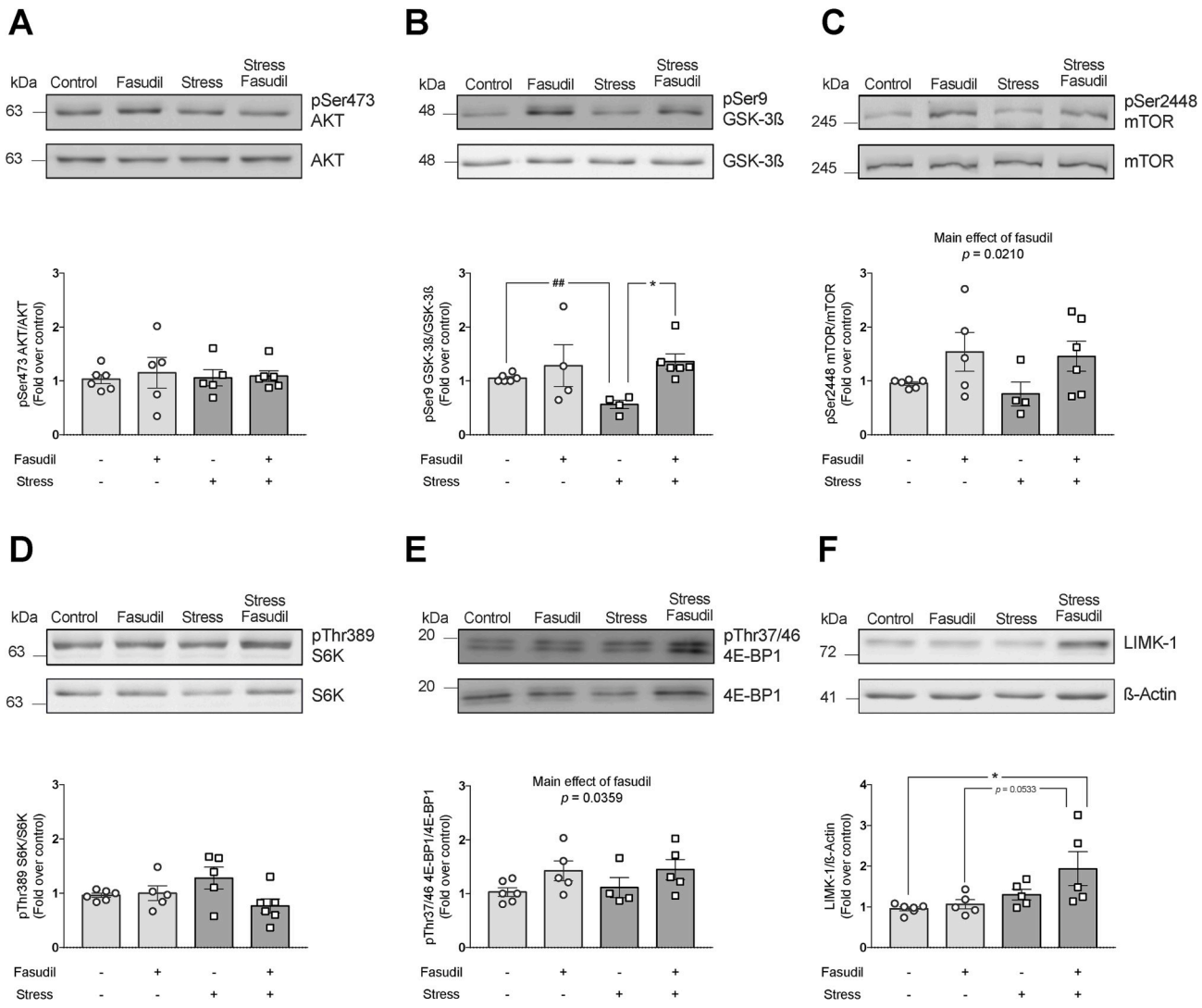

Fig. 5. Fasudil modulates antidepressant-relevant signaling pathways in hippocampal synaptoneurosomes. Western blot analyses from hippocampal synaptoneurosomes of phosphorylated proteins of different antidepressant-relevant signaling pathways involved in neuroplasticity. **(A)** phospho-Ser473 AKT, **(B)** phospho-Ser9 GSK-3β, **(C)** phospho-Ser2448 mTOR, **(D)** phospho-Thr389 S6K, **(E)** phospho-Thr37/46 4E-BP1 and **(F)** total LIMK-1 levels. Bars represent mean ± SEM. Data were analyzed by two-way ANOVA followed by Tukey's post-hoc test ($^{*}p < 0.05$). Two-tailed Mann-Whitney test: $^{\#\#}p < 0.01$. Table S2 shows detailed sample sizes and two-way ANOVA details. $n = 4$–6 per condition.

novelty-suppressed feeding test (Shapiro et al., 2019), which relies on drugs with anxiolytic-like effects (Ramaker and Dulawa, 2017). Nonetheless, here we demonstrate that chronic fasudil treatment partially averted the chronic stress-induced anxiety-like behavior in the EPM. Interestingly, similar observations were described by Bondi et al. using chronic desipramine treatment, which could prevent stress-increased anxiety-like behavior (Bondi et al., 2008).

Both associative memory and anxiety-like behavior may comprise hippocampal circuitries, especially those involving the ventral hippocampus (Bannerman et al., 2003; Moustafa et al., 2013; Wang et al., 2015). However, the dorsal hippocampus is also sensitive to chronic stress (Luine, 2002) and antidepressants (Liu et al., 2012). Therefore, we also evaluated animals in the OLT, which depends strongly on dorsal hippocampal encoding, consolidation and retrieval (Mumby et al., 2002). Some studies have evaluated the effect of antidepressants in this test. For instance, treatment of unstressed adult rats with fluoxetine for 7 days enhanced animal performance in the OLT (Casarotto et al., 2020). Another report showed that after 30 days of a single dose fasudil (10 mg/kg, ip.) in male rats had no effect in the discrimination index in novel object and location tests (He et al., 2017). Similarly, we did not observe any effect in OLT performance by chronic fasudil treatment in unstressed rats, suggesting that in our model, fasudil may not have a cognitive-enhancing effect on hippocampal-dependent behavior in unstressed rats. In contrast, it is well known that chronic stress impairs novelty preference in male rats (Luine, 2002). In our study, fasudil completely prevented stress-induced loss in novel location preference, suggesting that fasudil has a hippocampal-protective effect against chronic stress-induced impairment on this behavior. Similarly, chronic treatment with fluoxetine reversed the loss of novel location preference induced by chronic corticosterone administration (Orrico-Sanchez et al., 2019), a model that partly recapitulates depressive-like behaviors (Sterner and Kalynchuk, 2010; Ulloa et al., 2010). Interestingly, chronic fasudil treatment has also been described to improve hippocampal-dependent learning and memory following various kind of insults, such as cerebral ischemia (Yan et al., 2015), Parkinson's disease (Tatenhorst et al., 2016) and Alzheimer's disease (Yu et al., 2018) animal models.

Both clinical and preclinical studies have shown that ERK signaling is impaired in the hippocampus of suicide subjects (Dwivedi et al., 2006) and stressed animals (Dwivedi and Zhang, 2016). Therefore, we

explored whether ERK and CREB phosphorylations were affected by chronic stress and fasudil in whole hippocampal homogenate. As expected, we found that ERK-2 activating phosphorylations were downregulated by chronic stress, despite fasudil treatment. The ERK-2 downstream effector ribosomal protein S6 kinase/p90RSK can phosphorylate CREB at Ser133, enhancing its transcriptional activity (Finkbeiner et al., 1997). We observed that CREB Ser133 phosphorylation was decreased in saline-treated stressed rats, which is in compliance with another report showing that chronic stress decreases pSer133-CREB in the hippocampus (Laifenfeld et al., 2005). However, fasudil-treated stressed rats displayed pSer133-CREB levels that were not different from neither controls nor stressed rats. This suggest that fasudil may partially prevent the stress-induced decrease in pSer133-CREB. Although this proposal does not seem to comply with the fact that fasudil did not prevent the stress-induced decrease in pThr185/Tyr187 ERK-2, several other protein kinases acting independently of ERK-2 are able to phosphorylate CREB at that same position (Ehrlich and Josselyn, 2016), which may be enhanced by fasudil.

Structural plasticity plays a pivotal role in brain limbic areas and is demonstrated to be impaired in mood disorders (Pittenger and Duman, 2008; Price and Drevets, 2010). We have previously reported that chronic stress decreases dendritic spine density of hippocampal CA1, which was prevented by chronic fasudil treatment (García-Rojo et al., 2017). In the present study, there were no variations in the levels of synaptic scaffolding proteins synaptophysin, PSD-95 and Homer. This agrees with *in vitro* evidence, where PSD-95 dendritic levels remain unchanged after spine loss (Woods et al., 2011). However, these findings associated with dendritic spine remodeling may involve modifications in glutamatergic receptors abundance. Indeed, we observed that GluA1 and GluN2A levels were reduced by chronic stress in whole hippocampal homogenates. However, these changes were insensitive to fasudil treatment. To this extent, our results indicate that the changes in glutamatergic components in whole hippocampal homogenate seems to be dissociated from the behavioral effects of chronic stress and fasudil treatments. Therefore, we next explored whether synapse-located glutamatergic components and/or antidepressant-relevant signaling pathways are somehow associated to the behavioral outcomes. In fact, we observed reduced pSer831-GluA1 levels in synaptoneurosomes of fasudil-treated stressed rats, a modification related to decreased AMPAR-mediated conductance (Derkach et al., 2007) and synaptic depotentiation (Lee et al., 2000).

Several antidepressants modulate signaling pathways in key brain areas such as the hippocampus (Duman and Voleti, 2012). For instance, antidepressants activate the AKT pathway in rat primary hippocampal neurons in a dose-response manner (Park et al., 2014). Although we did not find variations in pSer473-AKT, we found significant effects of fasudil in two AKT downstream nodes: GSK-3β and mTOR. More precisely, we report that fasudil prevented the chronic stress-induced decrease in the inhibitory phosphorylation of GSK-3β at Ser9. This agrees with other reports, where increased GSK-3β activity is associated with chronic stress and depressive-like behavior (Liu et al., 2012). Conversely, several antidepressants and mood stabilizers inhibit GSK-3β activity by increasing Ser9 phosphorylation (Beurel et al., 2011; Gould and Manji, 2005; Li et al., 2004). On the other hand, fasudil increased mTOR Ser2448 phosphorylation -which is required for mTOR kinase activity (Navé et al., 1999)- in both unstressed and stressed rats. Even though both GSK-3β and mTOR phosphorylations were dissociated from Ser473 phosphorylation of AKT, this may be accounted by the fact that pSer473 of AKT is dispensable for GSK-3β and mTORC1 modulation (Jacinto et al., 2006) and its only essential to achieve full AKT kinase activity. Regardless of the phosphorylation status of AKT, our results strongly suggest the activation of the AKT pathway by fasudil.

Interestingly, evidence supports that mTORC1 signaling underlies the long-lasting antidepressant-like actions of ketamine (Li et al., 2010). This is in compliance with the fact that ROCK acts as an inhibitor of mTORC1, either by activating PTEN or interacting with the tuberous sclerosis complex 2 (TSC2) (Koch et al., 2018). Hence, ROCK inhibition by fasudil may increase mTORC1 signaling, as displayed by our results. Downstream of mTORC1, we found that pThr389 S6K was unaltered by chronic stress and/or fasudil, but mTORC1-dependent phosphorylations of 4E-BP1 were increased by fasudil in hippocampal synaptoneurosomes from both stressed and unstressed animals. Since phosphorylation of 4E-BP1 at Thr37/46 relieves translational repression (Gingras et al., 1999), our findings suggest that fasudil may enhance mTORC1-dependent protein synthesis at the synapse. Interestingly, we detected that fasudil upregulates synaptic LIMK-1 levels in stressed rats. Despite that fasudil-treated unstressed rats also showed increased mTOR and 4E-BP1 phosphorylations, but invariant LIMK-1 levels, other stress-sensitive post-transcriptional mechanisms may be involved in regulating synaptic LIMK-1 levels. The findings described in our study, combined with previous evidence, propose that fasudil may activate the AKT pathway, which in turn may lead to neuroplastic changes in the hippocampus, that might translate onto some of the observed behavioral effects. Despite that our study has focused on the hippocampal synapse, combined effects of fasudil in other circuits and brain areas relevant to antidepressant actions (Gould et al., 2019), might account for its preventive activity against chronic stress in behaviors. Indeed, chronic stress also affects brain areas other than the hippocampus, such as the amygdala, prefrontal cortex and ventral striatum (Pittenger and Duman, 2008). Moreover, these brain areas are also relevant for associative learning and anxiety-like behavior (Price and Drevets, 2010; Ramirez et al., 2015). Since fasudil only partially prevented the stress-induced anxiety-like behavior, some brain areas may be more sensitive to fasudil than others. Further studies are needed to assess if these brain areas display similar sensitivity to fasudil under a chronic stress paradigm. In a similar vein, the lack of a topographical perspective certainly establishes a limitation to the present study, since distinct hippocampal synapses may be differentially sensitive to chronic stress and antidepressant treatment (Kallarackal et al., 2013; Van Dyke et al., 2019). In fact, this may explain why some molecular changes -especially those related with glutamatergic components- do not correlate with the behavioral outcomes.

Unlike many studies, our report focused on exploring the behavioral and molecular effects of fasudil under a chronic stress paradigm, serving as a model that recapitulates some psychiatric conditions (Nestler and Hyman, 2010). Even though fasudil did not produce any behavioral effects in unstressed rats, it triggered some molecular modifications. Fasudil increased synaptic GluA1 and GluA2 levels, with no effect in synaptic GluA1 phosphorylations and NMDA receptor subunits. Since we did not observe this increase in GluA1 and GluA2 in whole homogenates, we speculate that fasudil may somehow trigger trafficking/synthesis of AMPARs to the synapses. This increase in synaptic GluA1 and sometimes GluA2 has been observed with several slow- (Martinez-Turrillas et al., 2002; Van Dyke et al., 2019) and fast-acting antidepressants; for example, acute treatment with ketamine or *(2R, 6R)*-HNK increases both GluA1 and GluA2 levels in hippocampal synaptoneurosomes (Zanos et al., 2016). Altogether, these evidences suggest that antidepressants increase AMPAR abundance in the hippocampal synapses.

Since fasudil might inhibit other protein kinases, at least *in vitro* (Ono-Saito et al., 1999), the observed effects may not be exclusively mediated by ROCK inhibition. However, fasudil is believed to act as a prodrug, since it is readily metabolized to hydroxyfasudil, a more potent and selective ROCK inhibitor (Koch et al., 2018). In line with this, we have previously established that our model of fasudil treatment effectively inhibits phosphorylation of myosin phosphatase target subunit 1 (MYPT1) -an exclusive ROCK target-which was increased by chronic stress in the hippocampus (García-Rojo et al., 2017). Also, a recent report revealed that ventromedial PFC-targeted ROCK-2 silencing -the major ROCK isoform found in the brain-reduced immobility in the FST in adolescent mice, similar to fasudil (Shapiro et al., 2019). Whether the preventive behavioral and molecular effects of fasudil against chronic

stress rely upon ROCK inhibition remains elusive. Nonetheless, these and our findings provide ROCK as an interesting target for the treatment of stress-related disorders.

5. Conclusion

Altogether, our results indicate that fasudil prevents behavioral impairments induced by chronic stress. These protective effects of fasudil were dissociated from its effects on glutamatergic components in the hippocampus, but they were correlated with an activation of the AKT pathway in hippocampal synaptoneurosomes. Further studies addressing the specificity and mechanism of fasudil are needed to understand how this drug may regulate neural circuits that are involved in chronic stress-induced altered behaviors.

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Declaration of competing interest

None.

CRediT authorship contribution statement

Luciano Román-Albasini: Conceptualization, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Gabriela Díaz-Véliz:** Methodology, Investigation, Formal analysis. **Felipe Antonio Olave:** Methodology, Investigation. **Felipe Ignacio Aguayo:** Investigation, Validation, Writing - review & editing. **Gonzalo García-Rojo:** Conceptualization, Methodology. **Wladimir Antonio Corrales:** Validation. **Juan Pablo Silva:** Validation. **Ana María Ávalos:** Writing - review & editing. **Paulina S. Rojas:** Validation. **Esteban Aliaga:** Validation, Writing - review & editing. **Jenny Lucy Fiedler:** Conceptualization, Methodology, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2020.100234.

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