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REGULAR RESEARCH ARTICLE

The ROCK Inhibitor Fasudil Prevents Chronic Restraint Stress-Induced Depressive-Like Behaviors and Dendritic Spine Loss in Rat Hippocampus

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Abstract

Background: Dendritic arbor simplification and dendritic spine loss in the hippocampus, a limbic structure implicated in mood disorders, are assumed to contribute to symptoms of depression. These morphological changes imply modifications in dendritic cytoskeleton. Rho GTPases are regulators of actin dynamics through their effector Rho kinase. We have reported that chronic stress promotes depressive-like behaviors in rats along with dendritic spine loss in apical dendrites of hippocampal pyramidal neurons, changes associated with Rho kinase activation. The present study proposes that the Rho kinase inhibitor Fasudil may prevent the stress-induced behavior and dendritic spine loss.
Methods: Adult male Sprague-Dawley rats were injected with saline or Fasudil (i.p., 10 mg/kg) starting 4 days prior to and maintained during the restraint stress procedure (2.5 h/d for 14 days). Nonstressed control animals were injected with saline or Fasudil for 18 days. At 24 hours after treatment, forced swimming test, Golgi-staining, and immuno-western blot were performed.
Results: Fasudil prevented stress-induced immobility observed in the forced swimming test. On the other hand, Fasudil-treated control animals showed behavioral patterns similar to those of saline-treated controls. Furthermore, we observed that stress induced an increase in the phosphorylation of MYPT1 in the hippocampus, an exclusive target of Rho kinase. This change was accompanied by dendritic spine loss of apical dendrites of pyramidal hippocampal neurons. Interestingly, increased pMYPT1 levels and spine loss were both prevented by Fasudil administration.

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Significance Statement

This study examined the effects of a ROCK inhibitor, fasudil, on depressive-like behavior and neuronal changes associated with exposure to chronic restraint stress in a rodent model of depression. We observed that our stress model induced depressive-like behaviors in rats, which was prevented by Fasudil treatment. Furthermore, we observed that chronic stress decreased spine density in CA1 pyramidal neurons, modification that was related to an enhancement in phospho-MYPT1 levels, a known ROCK effector. These changes were prevented by Fasudil treatment, a ROCK inhibitor.

Conclusion: Our findings suggest that Fasudil may prevent the development of abnormal behavior and spine loss induced by chronic stress by blocking Rho kinase activity.

Keywords: behavior, dendritic spines, antidepressant, ROCK inhibitor Fasudil, stress

Introduction

Neuroplasticity, the ability of the brain to change and adapt in response to experience and fluctuating environment, involves several mechanisms, ranging from synaptic remodeling to functional modification of synapses and neural circuitries. Repeated exposure to unpredictable and uncontrollable stressors may result in brain modifications that decrease the capacity to appropriately respond to subsequent stressors, thus increasing the risk for developing mental disorders, including depression (McEwen and Gianaros, 2010). Furthermore, several studies have shown that functional modifications of the hippocampus, amygdala, prefrontal cortex, and other structures are responsible for depression symptoms (Drevets et al., 2008; Price and Drevets, 2010). The hippocampus, a stress-sensitive limbic structure, is crucial for episodic and spatial memory; these functions are impaired in depressive disorder (Austin et al., 2001; Drevets et al., 2008; Price and Drevets, 2010). Human brain imaging and preclinical studies in rodents have revealed that stress and depression are associated with reduced hippocampal volume, neuronal atrophy, and dendritic spine loss (McEwen and Seeman, 1999; Vyas et al., 2002; Fernandez-Guasti et al., 2012; Castaneda et al., 2015). In addition, the hippocampus participates in the intersection between cognition and emotion and plays a crucial in the pathophysiology of mood disorders (Campbell and Macqueen, 2004; Femenía et al., 2012). Similar to other studies (Bondi et al., 2008), we previously reported that chronic stress in rodents produces anhedonia, impairs associative learning, and increases immobility in the forced swimming test (FST) (Bravo et al., 2009; Ulloa et al., 2010; Castaneda et al., 2015). Notably, these behavioral modifications are accompanied by dendritic atrophy and spine density reduction of pyramidal neurons in the hippocampus and prefrontal cortex (McEwen and Seeman, 1999; Vyas et al., 2002; Fernandez-Guasti et al., 2012; Castaneda et al., 2015). Overall, this evidence suggests that the mechanisms involved in excitatory synapse formation and maintenance can be altered in stress-related mood disorders, contributing to the phenotypic alterations observed in depressive disorder.

The formation and elimination of dendritic spines are mechanisms by which neuronal connections can be shaped (Chklovskii et al., 2004). The growth of dendrites, filopodia, and dendritic spines occurs by protrusive forces of actin polymerization (Luo, 2002). Thus, it is plausible that the morphological alterations observed under chronic stress are produced by changes in signal transduction pathways that target the reorganization of the actin cytoskeleton. Members of the Rho-GTPase family regulate actin cytoskeleton dynamics and modulate axonal growth, dendritic arborization, and spine growth during development and adulthood (Luo, 2000; Nakayama et al., 2000; Tashiro and Yuste, 2004; Govek et al., 2005; Elia et al., 2006). For instance, activated RacGTP-ases stimulate the formation of thin spines (Nakayama et al., 2000) and through the activation of p21-activated kinase 1 trigger the activation of LIM-kinase, which phosphorylates and inhibits cofilin, a potent actin-depolymerizing molecule (Calabrese et al., 2006). In contrast, RhoA-GTPases promote neuronal dendritic arbor simplification and reduce spine length and number (Nakayama et al., 2000; Nakayama and Luo, 2000). RhoA signaling is mediated by Rho serine/threonine kinase (ROCK) isoforms 1 and 2, the latter being highly expressed in the brain and muscle tissue (Hashimoto et al., 1999). ROCK regulates cytoskeleton dynamics by phosphorylation of the myosin light chain (MLC) at Ser19, probably facilitating acto-myosin interaction (Hirose et al., 1998), which may produce fast neuronal remodeling, such as retraction of dendrites. Furthermore, ROCK can phosphorylate and inactivate the myosin phosphatase-targeting subunit 1 (MYPT1) of MLC phosphatase and may indirectly increase MLC phosphorylation state, favoring acto-myosin interaction (Amano et al., 1996).

Studies have shown high immunoreactivity of ROCK2 in several brain areas, including pyramidal neurons of the hippocampus (Hashimoto et al., 1999), suggesting a particular role of ROCK in these structures of the adult brain. Although ROCK2-deficient mice display normal brain anatomy, electrophysiological studies in hippocampal slices evidenced an impairment of both basal synaptic transmission and long-term potentiation (Zhou et al., 2009). These electrophysiological alterations were consistent with modifications in dendritic spine length and morphology, accompanied by an increase in activated cofilin (unphosphorylated form). Nonetheless, this study did not explore whether the deletion of ROCK2 modifies the behavior of animals (Zhou et al., 2009).

Considering that ROCK has several downstream effectors and that some of them are related to the regulation of cytoskeleton dynamics, studies have evaluated the effect of different ROCK inhibitors on brain function. For example, in vascular smooth cells, Fasudil increases cerebral blood flow after stroke by reducing vasoconstriction (Shin et al., 2007). Furthermore, systemic administration of hydroxyfasudil, an active metabolite of Fasudil, improves the cognitive deficits in aged rats and also improves learning and memory in a model of sporadic Alzheimer's disease (Hou et al., 2012). According to the relevance of the Rho-ROCK pathway in neuronal morphology in vitro and the effect of ROCK inhibitors on cognition, learning, and memory, it is particularly important to address whether RhoA signaling

is being altered under some pathological conditions, such as depressive disorders. Recently, in an animal model of depression induced by chronic restraint stress, we observed an increase in hippocampal phospho-MYPT1 levels, suggesting that ROCK2 is activated by this experimental paradigm (Castaneda et al., 2015). Interestingly, the rise in phospho-MYPT1 levels was associated with the spine loss observed in secondary dendrites of CA1 pyramidal neurons of the hippocampus (Castaneda et al., 2015). Moreover, it has been demonstrated that bilateral infralimbic administration of Y-27632, a specific ROCK inhibitor, promotes antidepressant-like effects in naive rats that are similar to those produced by the antidepressant fluoxetine (Inan et al., 2015).

Considering that stress increases immobility in the FST, an effect that is accompanied by a dendrite spine loss in CA1 pyramidal neurons and along with an increase in hippocampal phospho-MYPT1 levels, indicative of RhoA-ROCK pathway activation (Castaneda et al., 2015), we hypothesized that pharmacological inhibition of ROCK with Fasudil prevents both the increase in immobility in FST and dendrite spine loss in the hippocampus of rats under chronic restraint stress paradigm, an animal model of depression.

Materials and Methods

Animals

The Sprague-Dawley rats used in these experiments were obtained from the Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile. Efforts were made to minimize the number of animals and their suffering. The rats were handled according to guidelines outlined and approved by the Ethical Committee of the Faculty of Chemical and Pharmaceutical Sciences, Universidad de Chile and the Science and Technology National Commission (CONICYT), in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication, 8th Edition, 2011).

The adult male Sprague-Dawley rats (250–280 g) were housed in groups of 4/cage in a temperature (22–23°C) and humidity (55–65%) controlled room with a standard light: dark cycle (12 hours:12 hours). Food (standard rat chow) and water were freely provided, except when restraint stress was applied. The rats were handled once per day for 7 days prior to initiating the experimental procedures. The handling procedure consisted of picking up the rat by its body, weighing it, and finally returning it to its home cage.

Restraint Stress and Pharmacological Treatment

An animal model of chronic restraint stress was used to evaluate changes in ROCK activity and the effect of the ROCK inhibitor Fasudil on behavior. A dose of 10 mg/kg i.p. was selected based on the observation that i.p. administration of 5 to 15 mg/kg of Fasudil was previously reported to induce neuroprotection in the CNS (Wu et al., 2012; Song et al., 2013; Wei et al., 2014). The rats were randomly assigned to weight-matched groups that received one of the following treatments prior to the restraint protocol: (1) unstressed control animals injected every day for 18 days with saline (0.9% NaCl) (CONTROL, n = 16) or with 10 mg/kg Fasudil (LC Laboratories, Woburn, MA) (FASUDIL, n = 13), and (2) stressed animals injected for 18 days with saline (STRESS, n = 16) or 10 mg/kg Fasudil (STRESS-FASUDIL, n = 15). All injections were carried out i.p. 15 minutes prior to the stress protocol. To examine the preventive action of Fasudil on chronic stress effects, the stress procedure was initiated 4 days after the first drug or saline was administered. In this study, we used restraint stress as previously described, which consisted of placing the rats in Plexiglas tubes (25 x 8 cm) that were wide enough to allow comfortable breathing but restricting the movement of the animals for 2.5 h/d for 14 consecutive days. Every stress session was performed between 9:00 AM and 12:00 PM to avoid any effects due to changes in circadian rhythms. After the procedure, the animals were returned to their respective cages. After vehicle or Fasudil administration, unstressed animals were maintained in their home cages and left in another room. Twenty-four hours after the last treatment, animals were evaluated in a behavioral test (described below) or were killed to obtain either brain tissue to conduct morphological analyses, or the hippocampus for protein level determinations by immuno-western blot.

FST

The behavioral test consisted of blinded observations, which was carried out 24 hours after the last restraint procedure, in a quiet room. This test was performed as previously described (Lucki, 1997). A transparent Plexiglas cylinder (50 cm high x 20 cm wide) was filled up to a depth of 30 cm with water at 24°C. Four hours after the last stress session and Fasudil treatment on day 14, rats were trained for 15 minutes by placing them in the water-filled cylinder. On day 15 (24 hours after cessation of stress and Fasudil treatment), the rats were subjected to 5 minutes of forced swimming and escape behaviors (climbing and swimming) that were registered by trained observers who were blind to the treatments. Climbing was defined as upward-directed movements of the forepaws along the side of the swim chamber, while swimming consisted of movements throughout the swim chamber. Duration of immobility was defined as the time the animal was not actively involved in escape responses (i.e., total time of the test minus the time the animal spent climbing or swimming).

Golgi Staining and Evaluation of Dendritic Spine Density

After decapitation, one brain hemisphere was used for morphological studies and in the other, the hippocampus was dissected and rapidly frozen in liquid nitrogen and stored at -80°C. The FD Rapid GolgiStain kit (FD Neuro Technologies, Baltimore, MD) was used as recently described (Castaneda et al., 2015). Secondary apical dendrites of pyramidal neurons from the CA1 region were selected for spine analyses (bregma -2.3 to -4.3) (Paxinos and Watson, 1982). The criteria for the selection of Golgi-impregnated neurons for morphological analyses were recently described (Castaneda et al., 2015). Confocal z-stacks of identified intersections were captured on a Zeiss LSM 700 confocal laser scanning microscope. Each designated segment was located in the microscope field, and confocal stacks of 15 to 30 digital images, separated by a z-step of 0.5 to 1 µm, were captured using a Plan-Apochromat 40 × 1.4 NA Zeiss oil-immersion objective. Protrusions in direct connection with the dendritic shaft, irrespective of their morphological characteristics, were considered as spines. A mushroom spine type was identified when its head diameter exceeded 0.6 µm; the remaining spines (filopodia, stubby and other protrusions) were classified as "non-mushroom." Spines were counted in segments of 8 µm, starting from the origin of the branch, along a distance of 80 µm of the secondary dendrite. The number of spines at a given distance from the origin of the branch were then averaged using all of the neurons from the same animal (at least 6 cells/animal), and

these data were pooled with the mean of the other animals belonging to the same experimental group. The total number of spines corresponded to the sum of spines along the dendritic length of 80 µm.

Immunoblot Analysis

Whole hippocampus was homogenized in a glass-glass homogenizer in the presence of 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5 mM EGTA, 0.5 mM EDTA, 0.5 mM DTT, 0.125 mM Na_3VO_4, 0.2 mM PMSF, 2 µg/mL leupeptin, 2 µg/mL aprotinin, 2 mM NaF, 0.25 mM $Na_2P_2O_7$, and 1% Triton X-100 and then sonicated on ice for 5 minutes. After centrifuging lysates at 17 860 x g for 30 minutes, the supernatant was collected and a sample was saved for protein determination using the bicinchoninic method (Pierce BCA Protein Assay Kit ThermoFisher Scientific). The remaining supernatant was boiled immediately in sample loading buffer. A total of 25 to 50 µg of each protein extract was resolved on 12% SDS-polyacrylamide gels and then blotted onto 0.2 µm nitrocellulose (for detection of total and phosphorylated form of LIMK and MYPT1) or 0.2 µm PVDF membranes (for determination of total and phosphorylated form of cofilin). After blocking, membranes were incubated overnight with the appropriate primary antibodies diluted in blocking solution and then, with their corresponding secondary antibody (Table 1), as we described previously (Castaneda et al., 2015). All membranes were then incubated with enhanced chemiluminescent substrate (Perkin Elmer Life Sciences, Boston, MA) and detected by a chemiluminescence imager (Syngene). Band intensities were determined and analyzed using the UN SCAN IT software. The levels of β-actin were used to verify equivalent protein loading. For detection of total MYPT1 and LIMK, blots were stripped in Ponceau S solution for 1 hour, then incubated with the appropriate dilution of the antibodies (Table 1). For detection of total cofilin, blots were stripped with ReBlotPlus Mild Antibody Stripping Solution during 10 minutes and were then incubated overnight with an appropriate dilution of anti-cofilin (Table 1). After rinses, the procedure continued as described above.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Data are expressed as mean ± SEM and were analyzed by 2-way ANOVA followed by Tukey's post-hoc test.

Results

Effect of Stress and Fasudil Treatment on Body Weight Gain

Because basic studies have shown that stress affects physiological parameters related to hypothalamic-pituitary-adrenal activation, including energy mobilization mediated by glucocorticoid action, we tested whether mild restraint and Fasudil led to changes in body weight gain. We determined the percentage of body weight gain for each day either prior (days -4 to 0) or during the stress procedure (day 1 to day 14) compared with the initial weight at the beginning of the stress procedure (day 1), expressed as daily weight x 100/start weight at day 1. We observed no difference in weight gain prior to the beginning of the stress procedure, in which rats were injected with saline (CONTROL) or Fasudil (FASUDIL) (Figure 1A). Nonetheless, 2-way ANOVA analysis performed with the daily variation of weight gain during the 14 days of stress and Fasudil administration showed that treatment (or experimental groups) ($F_{3,56}$ = 7.45, P < .0003), time (days) ($F_{13,56}$ = 132.2, P < 0.0001), and the interaction between these factors ($F_{39,56}$ = 5.54 P < .0001) had a significant effect on body weight gain. Moreover, Tukey's post-hoc analysis indicated that stressed rats injected either with saline solution (STRESS) or Fasudil (STRESS-FASUDIL) showed a reduction in weight gain observable after 7 days of stress (Figure 1A). Additionally, 2-way ANOVA analysis of both factors at the end point of treatment showed that stress ($F_{1,56}$ = 15.93, P = 0.0002), but not Fasudil treatment ($F_{1,56}$ = 0.8979, P = 0.3474), had a significant effect on body weight gain (Figure 1B). The interaction between both factors was not significant ($F_{1,56}$ = 0.03771, P = .8467). Moreover, no significant change in adrenal weight was observed by restraint stress (CONTROL, 28.9 ± 1.3 mg vs STRESS, 26.23 ± 2.2 mg) and Fasudil, compared with control (24.1 ± 1.4 mg) and stressed rats (24.0 ± 1.3 mg). Thus, the restraint stress, but not Fasudil, produced a reduction in weight gain, but this was not related to changes in adrenal weight, suggesting that our stress model is mild in terms of HPA activation.

Effects of Fasudil on Activity in the FST

The antidepressant effect of Fasudil was examined in the FST, which is a highly reliable behavioral assay for the detection of antidepressant activity exerted by pharmacological agents (Cryan et al., 2002). As shown in Figure 2, control animals spent

Table 1. Western-Blot Conditions and Antibodies

Antibody	Host, Isotype	Source /Catalog No.	Blocking Solution	Primary Antibody	Secondary Antibody
β-Actin	Mouse	Sigma-Aldrich/A5316	3% nonfat milk -TBS 0.1% Tween-20	1:10 000; 1 h	1:10 000; 2 h
pThr508-LIMK	Rabbit	Sigma-Aldrich/SAB 4504460	3% nonfat milk -TBS 0.1% Tween-20	1:500; overnight	1:10 000; 2 h
LIMK 1	Rabbit	Sigma-Aldrich/L2290	1% nonfat milk -TBS 0.1% Tween-20	1:4000; overnight	1:10 000; 2 h
pSer3-cofilin	Rabbit	Cell Signaling/77G2	5% BSA -TBS 0.1% Tween-20	1:1000; overnight	1:10 000; 2 h
cofilin	Rabbit	Cytoskeleton/ACFL02	3% nonfat milk -TBS 0.1% Tween-20	1:8000; overnight	1:10 000; 2 h
pThr853-MYPT1	Rabbit	Cell Signaling/4563	5% nonfat milk -TBS 0.1% Tween-20	1:500; overnight	1:10 000; 2 h
MYPT1	Rabbit	Cell Signaling/2634	5% nonfat milk -TBS 0.1% Tween-20	1:250; overnight	1:10 000; 2 h

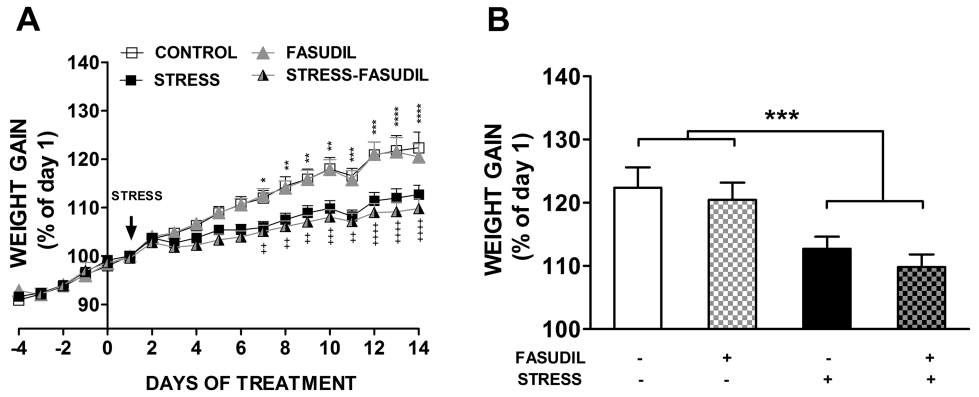

Figure 1. Effect of chronic restraint on body weight gain. (A) The graph represents mean ± SEM of the change in body weight as a percentage of the initial weight for control animals that were left untreated (CONTROL, n = 16) or treated with Fasudil (FASUDIL, n = 13), untreated animals subjected to chronic restraint stress (STRESS, n = 16) and Fasudil-treated animals subjected to chronic restraint (STRESS-FASUDIL, n = 15). Two-way ANOVA followed by Tukey's post-hoc analysis. CONTROL vs STRESS *P < .05, **P < .01, ***P < .001, ****P < .0001. FASUDIL vs STRESS-FASUDIL ++P < .01, +++P < .001, ++++P < .0001. (B) Variation of body weight gain was evaluated at the endpoint of treatments. Two-way ANOVA, ***P < .001.

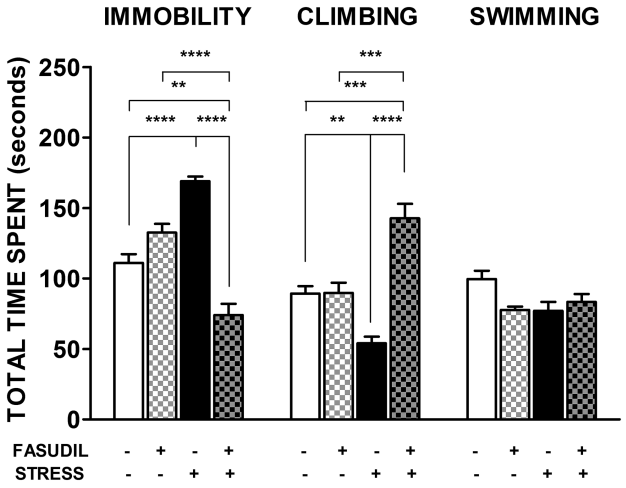

Figure 2. Effects of Fasudil administration on immobility and active response duration in the forced swimming test (FST). Bar graph indicates mean ± SEM of total time spent in immobility and climbing behaviors. Two-way ANOVA followed by Tukey's post-hoc analysis indicated that Fasudil-treated animals (FASUDIL, n = 5) showed similar durations of immobility and active behaviors as animals injected with saline (CONTROL, n = 7). Stressed animals (STRESS, n = 7) spent more time in an immobile posture and significantly reduced climbing behavior. Chronic treatment with Fasudil (STRESS-FASUDIL, n = 6) prevented the stress-induced immobility by increasing active behaviors, mainly in the form of climbing. **P < .01; ***P < .001; ****P < .0001.

approximately 60% of the time in active response, that is, swimming and climbing. The 2-way ANOVA analysis showed a significant main effect of Fasudil treatment ($F_{1,21}$ = 36.59, P < .0001) but not for stress ($F_{1,21}$ = 0.004, P = .95) on spent time in immobility. Nonetheless, the interaction between these factors was significant ($F_{1,21}$ = 91.75, P < .0001). Tukey post-hoc analysis showed that stressed animals increased their time in immobility (P < .0001), which was prevented by Fasudil (P < .0001), suggesting an antidepressant-like effect of Fasudil. In addition, the 2-way ANOVA analysis on spent time in climbing showed a significant main effect of Fasudil treatment ($F_{1,21}$ = 40.11, P < .0001) but not of stress ($F_{1,21}$ = 1.559, P = .226). We found a significant interaction between stress and Fasudil treatment ($F_{1,21}$ = 39.19, P < .0001), evidencing that Fasudil treatment in stressed animals induces an increment in the spent time in climbing, indicating that this response is higher than control rats (P < .001). In the case of the swimming behavior, we observed that neither stress ($F_{1,21}$ = 1.45, P = .1578) nor Fasudil treatment ($F_{1,21}$ = 1.850, P = .1882) showed a significant effect.

Effects of Stress and Fasudil on Dendritic Spine Number of CA1 Pyramidal Cells

We analyzed the effect of stress and Fasudil on dendritic spine number of secondary apical dendrites from pyramidal neurons of CA1. We evaluated whether treatments modified the number of spines regardless of their morphological features. Figure 3A shows an example of a CA1 pyramidal neuron, and the magnification shows the segment of a secondary dendrite used to count the number of spines along 80 µm. Figure 3B shows the magnification of a segment of a secondary dendrite, where the effect of treatments on the density of protrusions along the dendrite can be observed. When the total number of spines counted in 80 µm was analyzed by the 2-way ANOVA test, we observed a significant main effect of stress ($F_{1,15}$ = 5.666, P < .03) but not of Fasudil treatment ($F_{1,15}$ = 1.67, P < .3); nonetheless a significant interaction was detected ($F_{1,15}$ = 10.99, P < .005). Post-hoc analysis revealed that stress induced a reduction in spine density (P < .01) and that Fasudil prevented the stress-induced reduction in spine density (Figure 3C). We segregated the spines according to their morphology to visualize a differential effect of treatments. We observed that neither stress ($F_{1,15}$ = 0.06797, P = .7979) nor Fasudil treatment ($F_{1,15}$ = 0.9393, P = .3478) induced variation in the number of mushroom spines (Figure 3D). Nonetheless, we found a significant effect of stress ($F_{1,15}$ = 9.750, P < .007), Fasudil treatment ($F_{1,15}$ = 4.863, P = .0435), and interaction between these factors ($F_{1,15}$ = 6.324, P = .0238) on the number of nonmushroom spines in 80 µm (Figure 3E). Furthermore, Fasudil administration prevented the stress-induced reduction in spines (Figure 3C), mainly in nonmushroom spines (Figure 3E).

Effect of Stress and Fasudil on Phosphorylation Levels of MYPT1, LIMK, and Cofilin in Hippocampus Extract

To evaluate whether chronic stress and Fasudil modulate ROCK activity, we evaluated the phosphorylation state of myosin phosphatase targeting protein 1 (MYPT1, also known as Myosin Binding Subunit) in its Thr853 residue, an exclusive target of ROCK. We observed a significant main effect of stress ($F_{1,19}$ = 21.19,

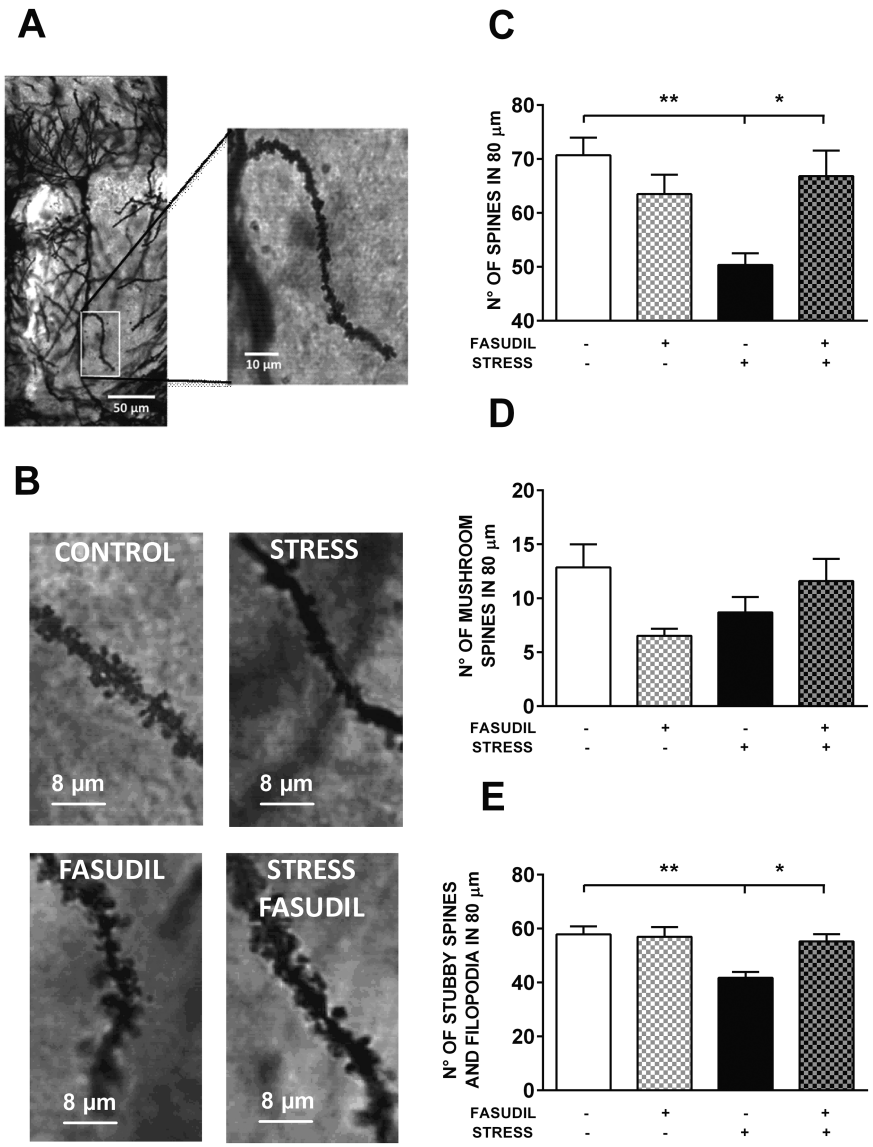

Figure 3. Effects of stress and Fasudil treatment on spine number along apical dendrites of pyramidal neurons in the CA1 hippocampal region. (A) The photograph shows a representative isolated Golgi-stained pyramidal neuron of the CA1 hippocampal region of control animals, and a magnification of a secondary dendritic branch in the stratum radiatum area, which was used to count spines. (B) Photographs show the qualitative effects of stress and Fasudil treatment on spine density from a secondary dendrite. (C) Effect of treatments on total spine number in a dendritic segment of 80 µm. Two-way ANOVA followed by Tukey's post-hoc analysis revealed that stress induced a reduction in spine density and that Fasudil prevented the stress-induced reduction in spine density. (D) Effect of treatments on mushroom spine number in a dendritic segment of 80 µm. (E) Effect of treatments on nonmushroom spine number in a dendritic segment of 80 µm. Two-way ANOVA followed by Tukey's post-hoc analysis indicated that stress induced a reduction in spine density and that Fasudil prevented the stress-induced reduction in spine density. Values are the mean ± SEM (CONTROL, n = 5; FASUDIL, n = 4; STRESS, n = 5; STRESS-FASUDIL, n = 5). *P < .05; **P < .01.

$P = .0002$), Fasudil treatment ($F_{1,19} = 13.74$, $P = .0015$), and strong interaction between factors ($F_{1,19} = 10.49$, $P = .0043$) on the levels of p-MYPT1, relative to MYPT1 (Figure 4B). Post-hoc analysis showed that stress induced an increase of 50% in p-MYPT1 levels ($P < .0001$), variation that was prevented by Fasudil ($P < .001$) in the hippocampus of stressed animals, an effect probably related to ROCK inhibition.

We also evaluated changes in the phosphorylation state of LIMK, which is a common effector of Rho and RAC GTPases in vitro. We observed that neither stress ($F_{1,14} = 0.955$, $P = .345$) nor Fasudil treatment ($F_{1,14} = 1.028$, $P = .328$) influenced p-LIMK levels, relative to total LIMK (Figure 4C). Accordingly, there were no significant effect of stress ($F_{1,18} = 3.09$, $P = .095$) and Fasudil ($F_{1,18} = 0.87$, $P = .361$) on p-cofilin levels relative to total cofilin (Figure 4D).

Discussion

Alteration in neuronal morphology related to dendritic arbor and spines are hallmarks of several psychiatric disorders, including major depressive disorders (Licznerski and Duman, 2013). Stress exposure may contribute to the development of depression (Kendler et al., 1999), and several studies support the idea that stress-induced brain atrophy might be responsible, in part, for the phenotypic characteristics of depressive disorders (Pittenger and Duman, 2008). The observed morphological changes include dendritic tree simplification accompanied by a reduction in the number of dendritic spines (Lin and Koleske, 2010). We have recently described that dendritic spine loss in the hippocampus of chronically stressed rats is related to an increase in phospho-MYPT1, a well-known downstream

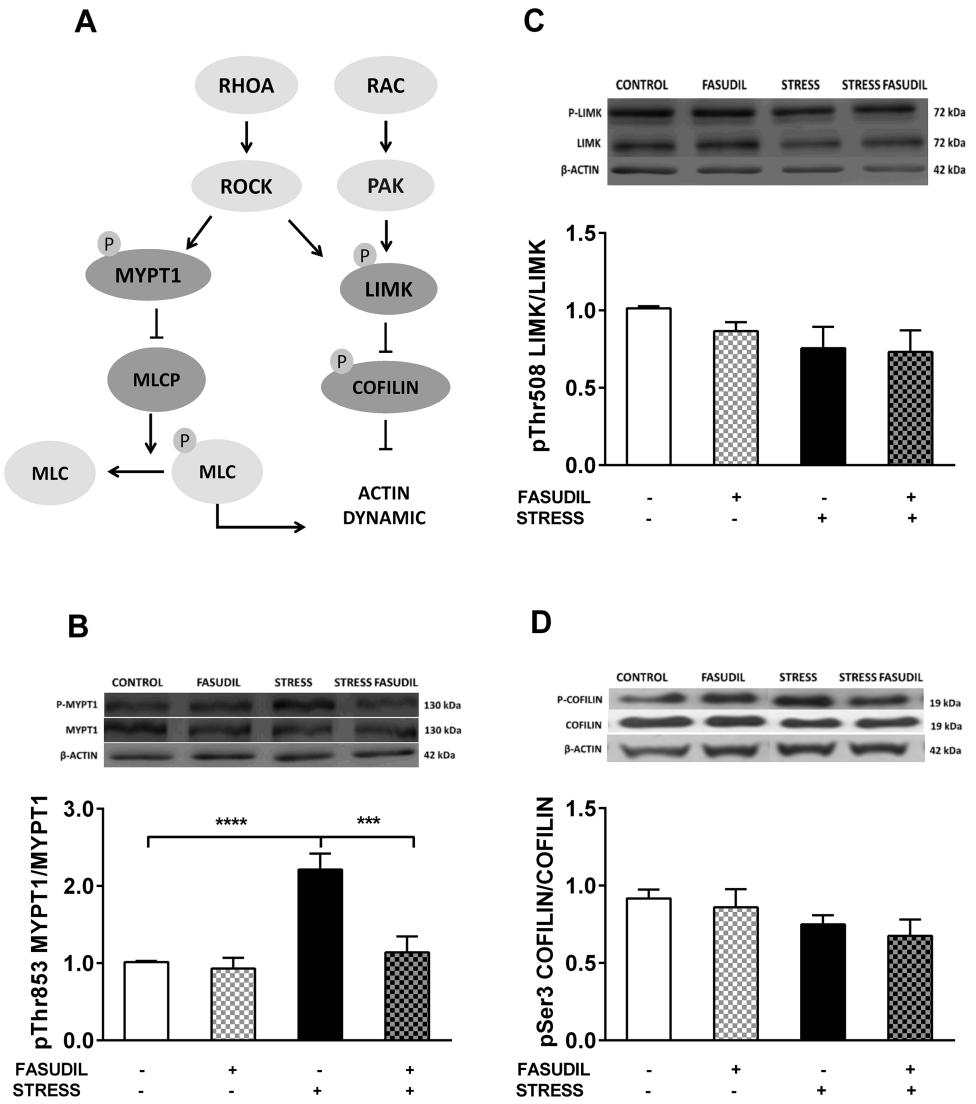

Figure 4. Effect of stress and Fasudil on the phosphorylation state of proteins related to actin dynamics, myosin phosphatase targeting subunit 1 (MYPT1), LIMK, and cofilin. (A) Pathways related to RAC and RhoA activation and their downstream effectors. (B) phospho-MYPT1 expressed as a ratio of phospho-MYPT1 relative to MYPT1. Two-way ANOVA followed by Tukey's post-hoc analysis indicated an increase in p-MYPT1 levels that was prevented by Fasudil. (CONTROL n = 7; FASUDIL n = 5; STRESS n = 6 and STRESS-FASUDIL n = 5). ***P < .001, ****P < .0001 (C) phospho-LIMK expressed as a ratio of phospho-LIMK relative to LIMK. (CONTROL n = 5; FASUDIL n = 4; STRESS n = 5 and STRESS-FASUDIL n = 4). (D) phospho-cofilin expressed as a ratio of phospho-cofilin relative to cofilin (CONTROL n = 6; FASUDIL n = 5; STRESS n = 6 and STRESS-FASUDIL n = 5). β-Actin was used as the loading control. Values are the mean ± SEM.

target of ROCK, suggesting that stress promotes the activation of the RhoA-ROCK pathway and therefore induces changes in cytoskeleton dynamics (Castaneda et al., 2015). Our main findings in the present study performed in rats can be summarized as follows: Fasudil, a ROCK inhibitor, prevents the stress-induced immobility observed in the FST, spine loss in CA1 neurons of the hippocampus, and increase in pMYPT1 levels.

By using a chronic restraint stress paradigm in rats, we have previously reported anhedonic behavior accompanied by a significant reduction in escape-directed behavior (Bravo et al., 2009; Castaneda et al., 2015). In the present study, we tested the effect of Fasudil in the FST, a test widely used to assess antidepressant activity of pharmacological agents (Porsolt et al., 1979; Cryan et al., 2005). It has been described that all antidepressant drugs reduce behavioral immobility. However, those antidepressants that increase serotonergic neurotransmission predominantly increase swimming behavior, whereas those that increase catecholaminergic neurotransmission increase climbing behavior (Cryan et al., 2005). We demonstrated that Fasudil treatment reduced stress-induced immobility through an increase in active responses, mainly in climbing behavior, to a level that was even higher than in control rats. Notably, the effect of Fasudil in stressed animals resembles the action of the antidepressant DMI (Bravo et al., 2009), which blocks the norepinephrine transporter. However, in our model, we do not know whether chronically administered Fasudil modifies noradrenergic neurotransmission. Furthermore, a recent study demonstrated that acute bilateral microinjection of the ROCK inhibitor Y-27632 (0.25 μg) into the infralimbic cortex of naïve rats increased active behavior during the FST (swimming and climbing) (Inan et al., 2015), similarly to drugs with dual effects on norepinephrine and serotonin transporters that extend the duration of both active behaviors (Detke et al., 1995, 1997). Thus, it seems plausible that drugs that do not share the same pharmacological profile, that is, antidepressant molecules and

ROCK inhibitors, may converge into a common intracellular pathway that underlies the action of antidepressants. The specificity of the FST for screening antidepressant-like agents has been widely questioned (Petit-Demouliere et al., 2005), because a large variety of nonantidepressant drugs have been shown to increase the locomotion that can be erroneously interpreted with an antidepressant-like effect (for review, see Yin et al., 2016). For this reason, some studies that evaluate the effects of antidepressant drugs in FST also evaluate potential hyperactivity in the open field arena. Although we did not use parallel test to exclude drug-induced locomotor false positives, there is a report indicating that Fasudil did not change locomotor activity in the open field test (Yoshimi et al., 2010). Additionally, we have observed that the total arm visits in elevated plus-maze was similar in control and stressed animals with or without Fasudil administration, suggesting that treatments did not alter locomotor activity (data not shown).

On the other hand, studies have used FST to visualize the "depressogenic effect" of various types of stress; however, their effects seem to be dependent on the stress paradigm used (for review, see Bogdanova et al., 2013). Some authors have proposed that the immobility observed during the test session of the FST indicates that the animal "learned" a strategy to save energy during the FST training session. Thus, in this scenario, the increase in the time spent in immobility does not seem to be related to "behavioral despair" or depressive-like symptoms (for review, see de Kloet and Molendijk, 2016). Considering that in our stress model Fasudil increases the time spent in climbing in the FST, the increment in this active response may be indicative of changes in the activity of some neural circuits related to this behavior promoted by the ROCK inhibitor. In the context of depressive behavior, motivation has been evaluated by the exposure of rodents to an inescapable stressor, such as the tail-suspension test or FST, and quantifying the proportion of time spent performing escape-related behavior (struggling) relative to time spent immobile. Recently, and by using an optogenetic approach, acute and selective inhibition of ventral tegmental area (VTA) dopaminergic neurons was shown to promote several depression-like behaviors, such as anhedonia (sucrose consumption) and reduced struggling behavior in the tail-suspension test (Tye et al., 2013). Interestingly, this study also showed that the phasic activation of VTA dopaminergic neurons reverts the reduction in escape behavior (struggling) induced by chronic mild stress (Tye et al., 2013). Furthermore, other optogenetic approaches revealed that a selective population of neurons in medial prefrontal cortex (PFC) is implicated in the active response to behavioral challenges (Warden et al., 2012). More recently, optogenetic approaches have also shown that activation of the ventral-hippocampus-medial PFC pathway is required for the sustained action of ketamine antidepressant action, observed as an increase in climbing in the FST (Carreno et al., 2016). Although a similar mechanism probably operates in our stress model, the suggested Fasudil antidepressant action in the FST must be interpreted with caution, and further studies are required to prove its action on specific circuits that are sensitive to stress, such as those related to motivated behavior.

Structural neuronal changes within the hippocampus and the prefrontal cortex are increasingly recognized as key to the pathophysiology of depression (Pittenger and Duman, 2008). A reduction in spine densities in CA1 neurons has been associated with depression-like behaviors in several animal models of depression induced by restraint stress or by chronic exposure to light at night, suggesting an altered glutamatergic excitatory neurotransmission in the hippocampus (Bedrosian et al., 2012; Fernandez-Guasti et al., 2012; Castaneda et al., 2015; Huang et al., 2015). Some studies have reported that antidepressant drugs with different primary mechanisms of action such as imipramine and fluoxetine (Bessa et al., 2009) revert both behavioral deficits and spine loss in CA3 (Bessa et al., 2009) caused by stress. Accordingly, we next analyzed the potential contribution of Fasudil on dendritic spine density that may explain its stress preventive effect on depression-like behaviors. Thus, in the present study, we found that stress-induced spine loss (non-mushroom spines) in secondary dendrites of CA1 pyramidal neurons was prevented by Fasudil treatment, suggesting that ROCK inhibition and perhaps other kinases can modify spine density. Recently, it was shown that primary hippocampal neurons acutely exposed to Y-27632, a ROCK inhibitor, specifically increased the number of filopodia and thin spines (Swanger et al., 2015). Hence, this evidence suggests that ROCK inhibition promotes variation in the density of spines, mainly the immature forms.

The effect of Fasudil may be related to modulation of the cytoskeleton in vivo that favors spine stability, an effect that correlates well with the observed antidepressant-like action of this drug in stressed animals. In accordance with this, ROCK directly phosphorylates MLC, at least in vitro (Amano et al., 1996), and probably favors actomyosin interaction and contraction. In addition, some studies have indicated that ROCK activates the LIMK-cofilin pathway (Maekawa et al., 1999), but we found that phosphorylation levels of this kinase are insensitive to stress and Fasudil treatment. Moreover, ROCK2 activity may indirectly increase the level of phospho-MLC by phosphorylating the Thr853 residue of the MLC phosphatase regulator MYPT1 (Hartshorne et al., 1998; Somlyo and Somlyo, 2000; Somlyo et al., 2000), resulting in a decrease in MLC phosphatase activity (Kimura et al., 1996). Recently, we reported a rise in phospho-MYPT1 levels in the hippocampus of stressed animals and considering that this protein is an exclusive target of ROCK (Grassie et al., 2011), we suggested that chronic stress activates ROCK (Castaneda et al., 2015). The present study showed that both factors, increased phospho-MYPT1 levels and reduced spine density triggered by chronic stress, are prevented by Fasudil, suggesting that this drug may mediate those effects by inhibiting ROCK activity.

Overall, this evidence suggests that the disruption of the Rho-ROCK pathway and/or inhibition of other kinases by Fasudil seem to exert antidepressant-like actions, probably by preventing spine loss in some areas (e.g., hippocampus). However, further studies are necessary to confirm whether Fasudil acts on similar substrates in different brain areas. We should also consider that Fasudil, through the inhibition of ROCK, may affect several transduction pathways. In vivo reports have shown that i.p. administration of Fasudil in a dose similar to the present study (10 mg/kg) implicated ROCK in the negative regulation of PTEN activity and the enhancement of AKT activity, a neuroprotective transduction pathway (Wu et al., 2012). Moreover, many new effects of Fasudil have been described, particularly in the CNS. It has been shown that systemic administration of Fasudil protects against ischemia (15 mg/kg) (Wei et al., 2014) and attenuates neuronal apoptosis and proinflammatory cytokine production (5–10 mg/kg) (Song et al., 2013) in animals models of neurodegeneration. Interestingly, diverse evidences have situated neuroinflammation and proinflammatory cytokines as principal players in depressive disorder pathogenesis and recurrence (Slavich and Irwin, 2014). Thus, it remains to be elucidated

whether Fasudil, through its antidepressant-like actions and preventive effect on dendritic spine loss, are related to ROCK inhibition or to other kinases sensitive to this drug and/or are associated with modulatory effects on glial and inflammatory cells in the CNS. However, considering that systemic administration of Fasudil may act as a potent vasodilator, it will be important to consider this action in vivo, which may mediate not only effects on peripheral organs but also cause protective effects in the brain.

Conclusion

The present study suggests that Fasudil could prevent both increase in immobility in the FST and spine loss induced by chronic restraint stress in the rat hippocampus. Although further experiments are necessary to reveal the molecular mechanisms underlying the Fasudil-induced improvement in activity level in the FST and spine loss prevention, the present results can be considered as an initial step by providing support for the hypothesis that ROCK inhibition initiates a cascade of events that can prevent stress-induced behavior and spine loss. Finally, our findings offer new perspectives on pharmacological intervention in depressive disorders.

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Statement of Interest

None.

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