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Chronic stimulation of alpha-2A-adrenoceptors with guanfacine protects rodent prefrontal cortex dendritic spines and cognition from the effects of chronic stress

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ABSTRACT

The prefrontal cortex (PFC) provides top-down regulation of behavior, cognition, and emotion, including spatial working memory. However, these PFC abilities are greatly impaired by exposure to acute or chronic stress. Chronic stress exposure in rats induces atrophy of PFC dendrites and spines that correlates with working memory impairment. As similar PFC grey matter loss appears to occur in mental illness, the mechanisms underlying these changes need to be better understood. Acute stress exposure impairs PFC cognition by activating feedforward cAMP-calcium- K^+ channel signaling, which weakens synaptic inputs and reduces PFC neuronal firing. Spine loss with chronic stress has been shown to involve calcium-protein kinase C signaling, but it is not known if inhibiting cAMP signaling would similarly prevent the atrophy induced by repeated stress. The current study examined whether inhibiting cAMP signaling through alpha-2A-adrenoceptor stimulation with chronic guanfacine treatment would protect PFC spines and working memory performance during chronic stress exposure. Guanfacine was selected due to 1) its established effects on cAMP signaling at post-synaptic alpha-2A receptors on spines in PFC, and 2) its increasing clinical use for the treatment of pediatric stress disorders. Daily guanfacine treatment compared to vehicle control was found to prevent dendritic spine loss in layer II/III pyramidal neurons of prelimbic PFC in rats exposed to chronic restraint stress. Guanfacine also protected working memory performance; cognitive performance correlated with dendritic spine density. These findings suggest that chronic guanfacine use may have clinical utility by protecting PFC gray matter from the detrimental effects of stress.

1. Introduction

The highly evolved prefrontal cortex (PFC) generates the mental representations needed to provide top-down regulation of behavior, thought and emotion (Arnsten, 2009a). These abilities are often tested in working memory tasks where representations of goals must be held "in mind" and used to guide choice of action. Understanding these PFC mechanisms has particular clinical significance, as deficits in PFC structure and function are common in mental illness. For example, patients with schizophrenia performing a working memory task show reduced activity in the dlPFC that correlates with symptoms of thought disorder (Perlstein et al., 2001). The onset of schizophrenia is accompanied by waves of gray matter loss in PFC (Cannon et al., 2014), and reduced PFC gray matter is a distinguishing characteristic of the illness (Cannon et al., 2002). Post-mortem studies of the brains of patients with schizophrenia have revealed that neuronal cell bodies are retained, but there is an extensive loss of dendrites and spines from layers III and V PFC pyramidal cells (Selemon and Goldman-Rakic, 1999; Glantz and Lewis, 2000; Black et al., 2004; Glausier and Lewis, 2013). In contrast, cortical areas such as the primary visual cortex show more subtle changes (Selemon and Goldman-Rakic, 1999; Glantz and Lewis, 2000).

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Studies of nonhuman primate dorsolateral PFC have shown that layer III pyramidal cells form microcircuits that generate the mental representations of visual space needed for spatial working memory (Goldman-Rakic, 1995; Arnsten, 2013). These networks interconnect via glutamatergic stimulation of NMDA receptor synapses on dendritic spines (Wang et al., 2013). Research in rodents has shown that exposure to chronic stress induces a marked loss of layer II-III dendritic spines that correlates with impaired working memory (Hains et al., 2009), emphasizing the importance of these synaptic connections to cognitive function.

Understanding the effects of stress on brain physiology has immediate clinical relevance, as mental illnesses such as schizophrenia are precipitated and/or exacerbated by stress exposure (Breier et al., 1991; Mazure, 1995). The PFC is particularly sensitive to stress exposure: acute stress exposure rapidly takes PFC "offline" through neurochemical actions, while repeated stress exposure leads to additional architectural changes (Arnsten, 2009b). Acute, uncontrollable stress has been shown to rapidly impair PFC function in monkeys (Arnsten and Goldman-Rakic, 1998), rodents (Murphy et al., 1996) and humans (Qin et al., 2009). In contrast, acute stress exposure often enhances the functioning of subcortical structures, allowing control of behavior to switch from slow, thoughtful PFC regulation to more rapid, reflexive and habitual responses (reviewed in Arnsten (2009a)). Acute stress rapidly impairs PFC function through a cascade of intracellular signaling events (Arnsten, 2009b): high levels of stress-induced catecholamine release in the PFC engage dopamine D1 and noradrenergic alpha-1 and beta receptors, which activate feedforward cAMP-calcium signaling in spines, which in turn open nearby K^+ channels that weaken NMDAR synaptic connections. This series of events reduces PFC neuronal firing and impairs working memory abilities. The effects of stress exposure can be mimicked by activating calcium-protein kinase C (PKC) (Birnbaum et al., 2004) or cAMP-protein kinase A (PKA) signaling (Taylor et al., 1999; Wang et al., 2007) in the PFC. Conversely, inhibiting cAMP signaling via post-synaptic alpha-2A receptors on PFC spines, strengthens connectivity and improves cognition through rapid closure of K^+ channels (Wang et al., 2007).

With repeated stress exposure, the noradrenergic system grows stronger (Nestler and Alreja, 1999; Miner et al., 2006; Fan et al., 2013), while there is dendritic atrophy in PFC. Studies of pyramidal cells in layer II/III of rat medial PFC have found that repeated stress exposure produces a circuit-specific retraction of dendrites, and a marked loss of spines that is particularly evident in the distal apical tree (Hains et al., 2009; Seib and Wellman, 2003; Radley et al., 2006, 2008; Shansky et al., 2009). These dendritic and spine changes are associated with impaired attentional set-shifting (Liston et al., 2006), and impairment in working memory (Hains et al., 2009), emphasizing their functional significance. In young rats, dendritic atrophy is reversible if the stress exposure is stopped (Radley et al., 2005; Bloss et al., 2011), suggesting that plasticity remains. Similar architectural changes in PFC have been seen in humans, where brain imaging has revealed that repeated stress is associated with reduced PFC gray matter (Ansell et al., 2012) and weaker PFC connections (Liston et al., 2009).

What is causing spine loss in the PFC with repeated stress exposure? Given the immediate clinical relevance of this question, it is important to uncover the mechanisms that contribute to spine loss, and thus develop informed strategies for treatment. Previous research has shown that inhibiting calcium-PKC signaling rescues spine density and working memory from the effects of repeated stress exposure (Hains et al., 2009). Thus, it is possible that inhibition of cAMP signaling via alpha-2A receptor stimulation might also be protective. *In vitro* studies have shown that the application of alpha-2A-adrenoceptor agonists such as guanfacine enrich spinophilin at the cell surface (Brady et al., 2005) and promote spine growth (Hu et al., 2008; Ren et al., 2011) in cell cultures, suggesting that guanfacine may enhance or protect connections *in vivo* as well.

The current study utilized the chronic restraint stress paradigm previously shown to induce spine loss and cognitive impairment in rats (Hains et al., 2009; Radley et al., 2006), and examined whether chronic treatment with the alpha-2A-adrenoceptor agonist, guanfacine, prior to daily stress would protect PFC cognition and spine density from the detrimental effects of chronic stress exposure.

2. Methods

2.1. Overall experimental design

The research was approved by the Yale IACUC in accordance with the National Institute of Health guidelines for animal care. Male Sprague Dawley rats (n = 24, 250–350 g from Harlan, Indianapolis, IN), were pretrained on a spatial working memory delayed alternation task in a T maze to an equivalent level of baseline performance (overall mean of 74.3% ± 3% correct). They were then tested prior to drug/stress treatment each day in order to detect the accumulating effects of chronic stress exposure (schematically illustrated in Fig. 1). Rats were exposed to either a restraint stress paradigm (6 h/day) or control handling procedures for 21 days, and received either vehicle or guanfacine (0.1 mg/kg, s.c.), daily, prior to stress or control procedures. Thus, there were four treatment groups: no-stress + vehicle (n = 7), no-stress + guanfacine (n = 5), stress + vehicle (n = 7), or stress + guanfacine (n = 5). Rats were assessed for cognitive ability 10 times over the 21 days, and were rapidly anesthetized and sacrificed following the last test session. The prelimbic cortex was dissected and processed using the Rapid Golgi technique for analysis of dendritic morphology and spine density in layer II/III pyramidal cells.

2.2. Cognitive assessment

Rats were singly housed in ventilated cages with corncob bedding (Harlan Labs Teklad, 1/8″). They were maintained on a food-regulated diet of 16 g/day (Harlan Teklad Global Diets: 2018S 18% protein), which was sufficient to maintain a healthy growth curve. Rats were weighed on a weekly basis. Rats were trained and tested on the spatial delayed alternation task in a T maze during the

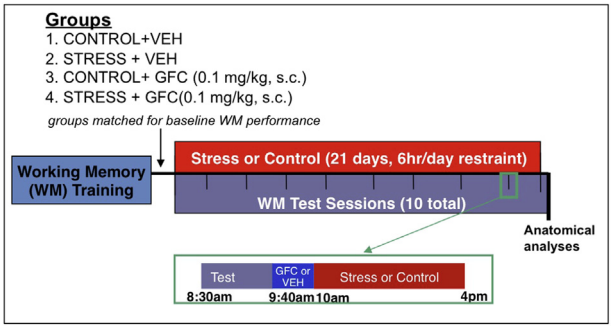

Fig. 1. The experimental design. Rats were trained on the delayed alternation task and divided into 4 groups with equivalent baseline performance. They then commenced 21 days of restraint stress or control treatment with vehicle or guanfacine pretreatment. Rats were assessed on the delayed alternation task prior to drug or vehicle treatment, which preceded 6/hr day of restraint stress or control treatment. Thus, cognitive assessments were performed ~23hr after the last drug treatment, and ~18.5 h after the last stress session in order to assess the effects of chronic treatment rather than acute exposure. At the end of the 21 days animals were sacrificed and the brains removed for assessment of layer II/III pyramidal cells in the prelimbic PFC.

animals' light cycle as described previously (Birnbaum et al., 2004). This task requires the animal to remember its previous choice of left or right arm, and choose the opposite direction on the ensuing trial. The task demands working memory as well as behavioral inhibition, and the ability to overcome distraction, and necessitates an intact medial prefrontal cortical function in rats (Dalley et al., 2004). After achieving a two-day (two test session) average of 68–78% correct, rats were separated into four groups with equivalent mean baseline performance.

Training and testing were conducted within the same 2-h time frame by a blinded experimenter. Food rewards were miniature chocolate chips. In the spatial delayed alternation task, rats were rewarded for entering either arm of the T maze on the first trial. Thereafter, for a total of 12 trials per session, rats were rewarded only if they entered the maze arm that was not previously chosen. Thus the animal had to maintain the previous selection "online" over a delay in order to guide the next choice. Four delay lengths (2, 5, 10, 15 s) were quasi-randomly distributed over the 12 trials to prevent ceiling effects. Consecutive errors were defined as the highest number of consecutive entries into one choice arm in a test session. The choice point of the maze was wiped with alcohol between trials to remove any olfactory clues. Testing occurred prior to daily stress to dissociate sustained from acute effects of the stress. Rats were tested 10 times (~every two to three days) throughout the 21-day stress period. The last two days of testing followed the 20th and 21st day of stress. Performance was evaluated as number of trials correct out of 12. Group differences were compared using one-way repeated measures ANOVAs, with test session as the repeated measure. Significant effects were evaluated with Tukey HSD post-hoc tests and performance over two consecutive test sessions was averaged and fit to a quadratic curve to illustrate performance over time. Correlations were analyzed with Pearson's correlation tests. $P < 0.05$ was considered statistically significant.

2.3. Chronic stress

Rats in the stress group were restrained in Plexiglas tubes daily, following testing and drug treatment, for 6 h (10:00AM–4:00PM) for 21 consecutive days, a paradigm known to reliably produce dendritic atrophy and spine loss in the prelimbic cortex (Radley et al., 2006). Animals were monitored to prevent undue distress. Animals in the no-stress group were handled daily. All animals were maintained on a restricted diet where food was offered after the cessation of stress to control for motivational effects.

2.4. Guanfacine treatment

Guanfacine (0.1 mg/kg; Tocris) was diluted in sterile saline and was administered by subcutaneous (s.c.) injection 20 min prior to stress onset. Injections occurred after behavioral testing, thus eliminating the confound of acute drug or stress treatment influencing task performance.

2.5. Morphological analyses

Immediately following the last spatial delayed alternation test session, rats were decapitated under isoflurane anesthesia. Tissue was prepared using the Rapid- Golgi kit (FD Neurotechnologies, Ellicott City, MD) according to manufacturer's instructions. Following a 14 day incubation period, tissue was sliced coronally (200 μm) and mounted on gelatin coated slides.

Pyramidal cell bodies ($n = 5$/rat) lying in layer II/III of prelimbic cortex (were reconstructed in three dimensions at 80–100× magnification (40–60× objective lens, with 1.0–2.0 turret magnification) using a microscope equipped with a motorized stage, video camera system, and Neurolucida morphometry software (MicroBrightField, Williston, VT). Neurons were reconstructed and spines were quantified by one of two blinded experimenters. The two experimenters achieved >90% reliability on spine number and dendrite reconstruction on reliability tests administered three times throughout the 18 months of reconstruction. In order for a neuron to be included in the analysis, it had to satisfy the following criteria: (1) have a cell body located within layer II/III of the prelimbic region as defined by cytoarchitectural characteristics; (2) demonstrate complete filling of dendritic tree, as evidenced by well-defined endings and dark and consistent filling; (3) demonstrate intact primary and secondary branches; (4) have an apical extent of at least 300 μm and a secondary branch emanating from the apical trunk between 20 and 70 μm from the soma; (5) have regions for spine quantification unobscured by neighboring branches.

Spines were quantified in four locations in each reconstructed neuron: (1) apical branch(es) lying 200 μm from the soma (30 μm segment, mean density was obtained if more than one branch met this criterion); (2) proximal apical spines on the first apical branch longer than 30 μm emanating from the apical trunk 20–70 μm from the soma (the first 30 μm segment of this branch); (3) proximal basal branch (first 30 μm segment from edge of soma); and (4) distal basal branch (30–60 μm the edge of the soma). All protrusions that were in direct continuity with the dendritic shaft, or spine heads located within 1.5 μm from the dendritic shaft, were identified as a spine. Spine density was expressed as number of spines per micron of dendrite. One animal in the vehicle control group and one animal in the vehicle stress group had only 4 neurons reconstructed for spine density. Dendritic length and branch intersections were evaluated by performing sholl analyses in 30 μm bins, as described in earlier studies (Radley et al., 2006). Total dendritic length and branch intersections represent the sum of the sholl output up to 300 μm radial distance from the soma, which was the greatest extent of the apical dendrite achieved by all neurons included in the analyses.

2.6. Statistical analyses

Group differences in spine density, dendritic length and dendritic branch intersections were compared using a mixed ANOVA design, with Neurons as the repeated, within-subject measure, and Group as the between subjects measure. Significant effects were evaluated with Tukey HSD post-hoc tests and a targeted model to test for differences between stressed animals with and without drug treatment. Apical dendritic length measurements were also analyzed with a fully between subjects analysis of variance to replicate methods used by earlier studies (Liston et al., 2006).

Correlations were analyzed with Pearson's correlation tests comparing mean spine density (the average of the 5 neurons within each animal), and mean performance over the last two days of testing. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Changes in weight

Exposure to either repeated stress or guanfacine treatment significantly reduced weight gain over the 3 week study (Fig. 2A; significant main effect of stress $F(1,20) = 5.43$, $p = 0.03$; significant main effect of guanfacine $F(1,20) = 6.153$, $p = 0.022$; no significant stress by guanfacine interaction $F(1,20) = 0.78$, $p = 0.783$). The reduction in weight gain with chronic stress exposure is similar to that seen in previous studies of restraint stress in rats (Radley et al., 2006).

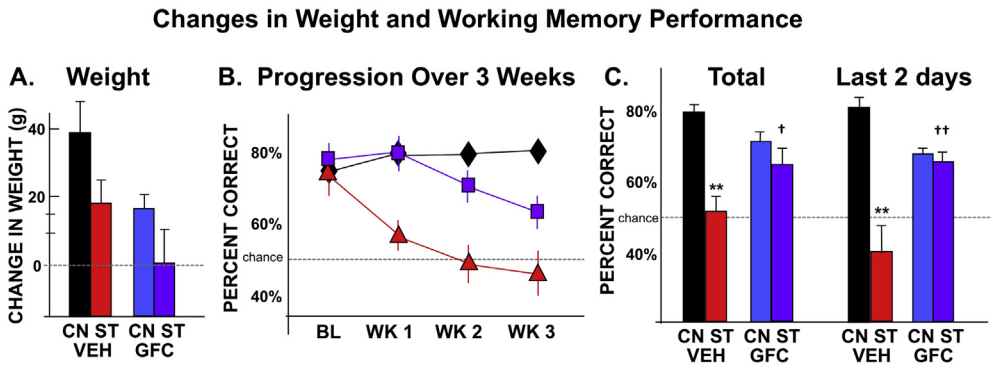

Fig. 2. Guanfacine treatment ameliorates stress-induced working memory impairment, but not weight loss. **A.** Changes in weight from baseline compared to the end of the 3-week study. Vehicle-treated control rats gained weight, but exposure to repeated stress or guanfacine treatment lessened weight gain. **B.** Mean spatial delayed alternation (working memory) performance over the 3 weeks of chronic daily restraint stress. All groups showed similar performance at baseline, and vehicle control animals (black diamonds) maintained stable performance. In contrast, vehicle stress animals (red triangles) showed a progressive impairment in performance over time. Daily guanfacine treatment (purple squares) prevented impairment in working memory performance. Please note that the guanfacine control group is not shown for the sake of clarity. **C.** Average performance on the delayed alternation task for the total session, or for the last 2 days was significantly impaired by daily restraint stress; guanfacine treatment significantly protected performance from stress. Different from nonstress + vehicle: **$p < 0.0001$; different from stress + vehicle: † $p < 0.05$; †† $p < 0.006$, error bars denote s.e.m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Performance of the spatial working memory task

Replicating our previous results (Hains et al., 2009), exposure to stress induced a dramatic and progressive loss of delayed alternation performance in vehicle-treated rats, growing more pronounced as the 3 weeks of stress exposure proceeded (Fig. 2B). The groups showed similar performance at baseline, but diverged as the stress sessions continued. Vehicle-treated stressed animals showed a progressive impairment in performance over time (significant effect of group $F(3,20) = 6.802$, $p = 0.002$; vehicle control vs. stress for baseline: $F = 0.324$, $p = 0.81$; the first week of testing: $F = 3.375$, $p = 0.039$; the second week of testing: $F = 4.07$, $p = 0.021$; the third week of testing: $F = 21.9$, $p < 0.001$). As animals were tested prior to the daily stress session, these impairments reflect the accumulating effects of chronic stress rather than an immediate response to an acute stress. Treatment with daily guanfacine protected delayed alternation performance in stressed rats compared to vehicle controls ($F = 5.81$, $p < 0.05$). Average performance for the entire study and for just the last 2 days of cognitive testing were both significantly greater for guanfacine-treated than vehicle-treated stressed rats (Fig. 2C; whole study: $p < 0.03$; last 2 days: $p < 0.006$). It should be remembered that guanfacine was administered immediately before the stress session, i.e. approximately 23 h before delayed alternation assessment; thus it is unlikely that drug was present during cognitive testing. There was no correlation between cognitive performance and weight of the animal ($r = 0.16$, $p = 0.45$).

3.3. Morphological assessments

Spine density (Fig. 3) and dendritic morphology (Fig. 4) were evaluated in five layer II/III pyramidal neurons from the prelimbic cortex of each cognitively characterized animal. The apical and basal dendrites were completely reconstructed to allow for measurements of dendritic length and branching. Dendritic spines were quantified in four regions of the dendrites −the distal and proximal portions of the apical dendrite, and the distal and proximal portions of a basal dendrite-as illustrated in Fig. 4A–D. The effects of chronic stress in vehicle-treated groups replicated previous studies, with reductions in both spine density and dendritic length being particularly prominent for the distal portion of the apical dendritic tree (Fig. 3A–D; (Hains et al., 2009; Radley et al., 2006; Liston et al., 2006; Radley et al., 2005; Cook and Wellman, 2004).

3.3.1. The effects of chronic stress and guanfacine on spine density

Comparisons of spine density across all four groups indicated a highly significant effect of group for distal apical spines ($F(3,18) = 20.14$, $p < 0.001$). Repeated restraint stress significantly reduced spine density on the distal apical tree in vehicle-treated animals (Fig. 3D; vehicle control vs. stress: $p = 0.016$). Daily guanfacine treatment protected spine density: guanfacine-treated stressed rats had a significantly higher density of distal apical dendritic spines 200 μm from the center of the soma than did vehicle-treated stressed rats (Fig. 3D; $p < 0.001$). Unexpectedly, guanfacine also increased distal apical spine density in nonstressed rats compared to vehicle controls (Fig. 3D; $p = 0.004$).

Similar to previous studies, stress did not alter spine density in the proximal portion of the apical dendrite (Fig. 3C; $F(3,18) = 1.36$, $p = 0.287$), and had only subtle effects on the spine density of basal dendrites, producing very small, nonsignificant reductions (Fig. 3A–B; $p > 0.5$). However, guanfacine treatment increased spine density on basal dendrites in stressed rats compared to their vehicle-treated counterparts for both the proximal region of basal dendrites (Fig. 3A; effect of group on basal proximal spine density: $F(3,18) = 7.559$, $p = 0.002$; guanfacine stress vs. vehicle stress: $p = 0.004$), and the distal region of basal dendrites (Fig. 3B; effect of group on basal distal spine density: $F(3,18) = 12.904$, $p < 0.001$; guanfacine stress vs. vehicle stress: $p = 0.022$). Guanfacine also significantly increased spine density in the distal portion of basal dendrites in nonstressed rats compared to vehicle-treated control animals ($p = 0.001$).

3.3.2. The effects of chronic stress and guanfacine on dendritic morphology

The main analysis of apical dendritic length showed a borderline effect of group ($F(3,20) = 3.046$, $p = 0.053$), whereby stressed, vehicle-treated animals tended to have reduced apical dendritic length compared to vehicle control animals (Fig. 4A). If a between subjects analysis was used, with each neuron as an independent entity as done in previous studies (Liston et al., 2006), the reduction in dendritic length following stress in vehicle-treated rats was significant, replicating earlier results (significant effect of group: $F(3,115) = 3.705$, $p = 0.014$; vehicle control vs. vehicle stress $p = 0.015$). Guanfacine treatment partially normalized apical dendritic length in stressed rats; i.e. dendritic length in guanfacine-treated stressed rats was not statistically different from either vehicle-treated controls ($p > 0.4$) or vehicle-treated stressed rats

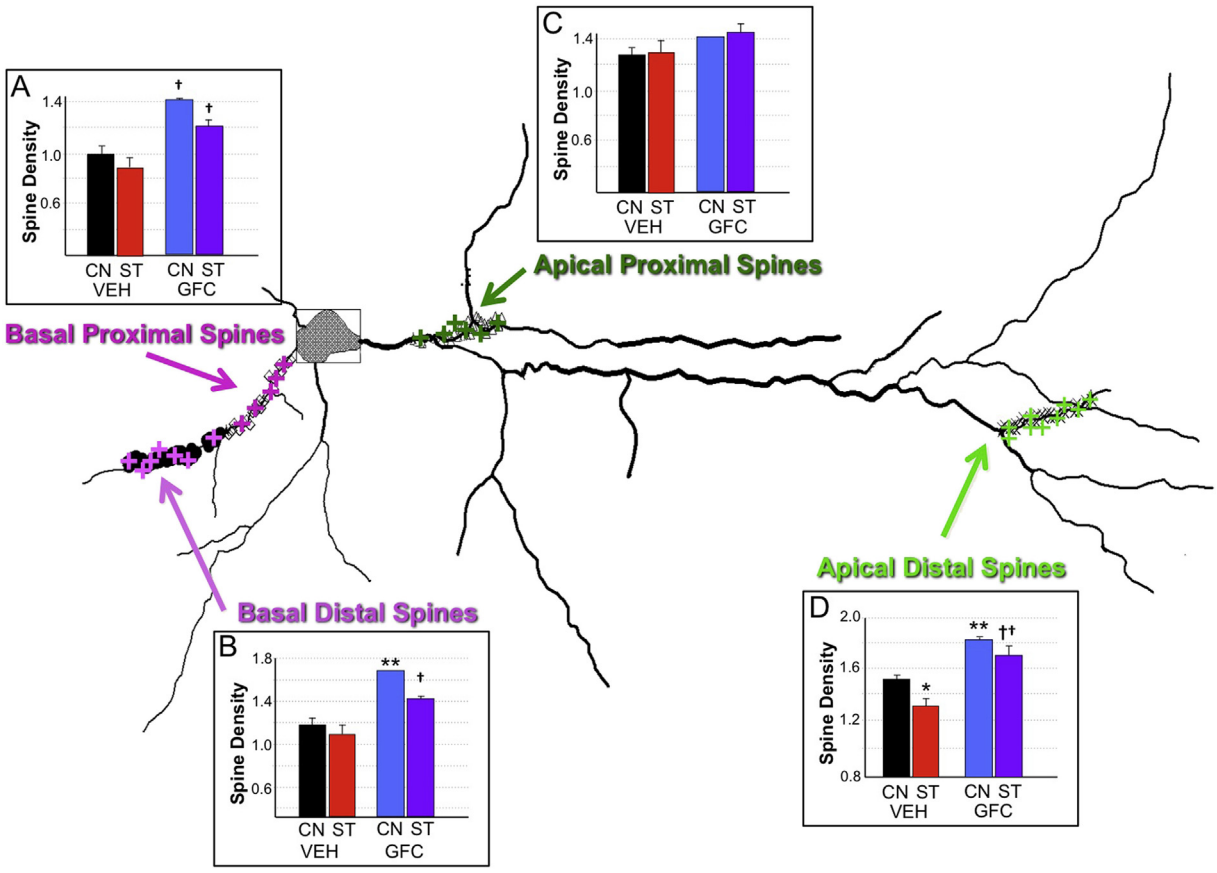

Fig. 3. The effects of chronic restraint stress and/or daily guanfacine treatment on the spine density of layer II/III pyramidal cells from the prelimbic cortex. As seen in previous studies, chronic stress had no significant effect on spine density in the proximal (A) or distal (B) portion of the basal dendritic tree, nor in the proximal portion of the apical tree (C), but significantly reduced spine density in the distal apical dendritic tree (D). Daily guanfacine treatment significantly protected distal apical dendritic spine density from the detrimental effects of stress, as well as increasing distal apical spine density in nonstressed animals. Guanfacine also increased spine density in nonstressed animals in distal basal dendrites. Different from nonstress + vehicle: $^{*}p < 0.05$, $^{**}p < 0.001$; different from stress + vehicle: †† $p < 0.001$, error bars denote s.e.m.

($p > 0.6$). Stress effects on apical dendritic branching was not significant (Fig. 4B; $F(3,20) = 2.824$, $p = 0.065$).

Consistent with previous findings (Liston et al., 2006; Radley et al., 2005), chronic restraint stress did not alter the length.or branching of basal dendrites in vehicle-treated rats (Fig. 4C–D; $p > 0.5$). However, guanfacine produced an unexpected and striking increase in basal dendritic length (Fig. 4C; effect of group: $F(3,20) = 4.79$, $p = 0.011$; guanfacine control vs. vehicle control: $p = 0.02$) and branching (Fig. 4D; effect of group: $F(3,20) = 4.94$, $p = 0.01$; guanfacine control vs. vehicle control: $p = 0.02$) in non-stressed rats.

3.3.3. *Correlations between cognitive performance and spine density in PFC*

Pearson's tests were used to determine whether there was a correlation between spine density and cognitive performance. We observed a significant correlation between mean apical dendritic spine density (averaged across all 5 neurons, within one animal) and mean delayed alternation performance over the last two days of the study (Fig. 5A; $r = 0.4908$, $p = 0.015$). Stressed rats with the lowest spine density had the worst cognitive performance, while those receiving guanfacine and the non-stressed rats had higher spine density and better cognitive performance. Proximal basal spine density also showed a significant, albeit weaker relationship with spatial delayed alternation performance (Fig. 5B; $r = 0.4293$, $p = 0.039$). These correlations are similar to what was seen in our previous study, suggesting these two dendritic regions have particular relevance to cognitive performance.

4. Discussion

4.1. *Summary and evaluation of data*

The current study replicated previous research showing that exposure to repeated restraint stress caused a progressive impairment in spatial working memory performance and a loss of dendritic spines in the distal apical dendritic tree of layer II-III pyramidal cells in the prelimbic PFC of rats (Hains et al., 2009; Liston et al., 2006). Daily administration of the alpha-2A adrenoceptor agonist, guanfacine, prior to stress exposure protected both working memory performance and spine density from the detrimental effects of stress exposure. There was a significant correlation between cognitive performance and spine density on the distal apical and proximal basal portions of the dendritic trees, supporting a relationship between dendritic integrity in the PFC and cognitive abilities. There was no correlation with weight, suggesting that alterations in motivation for food reward was not a factor in protecting cognitive performance. As guanfacine is FDA-approved for safe use in humans (the immediate release formulation for treating hypertension in adults, and the extended release formulation, Intuniv™, for the treatment of pediatric ADHD), these data suggest it may be especially useful in treating stress-related PFC clinical disorders.

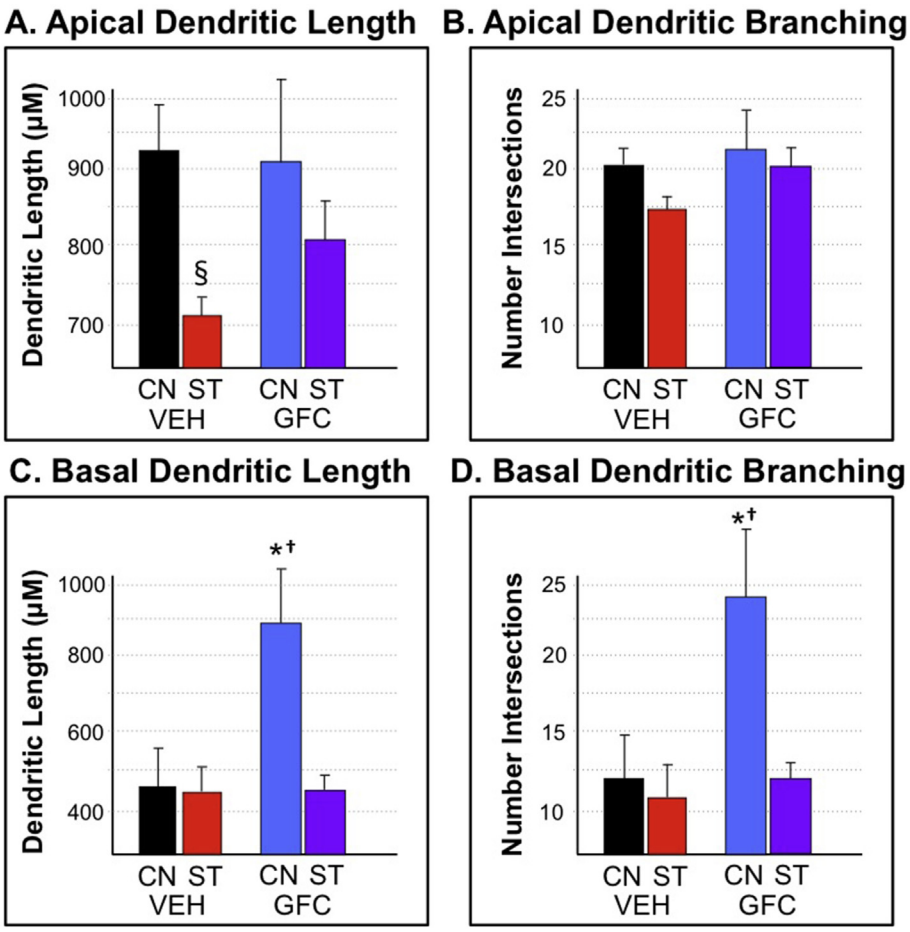

Fig. 4. The effects of chronic restraint stress and/or daily guanfacine treatment on the dendritic morphology of layer II/III pyramidal cells from the prelimbic cortex. Results represent mean ± S.E.M. **A.** The effects of chronic stress and guanfacine treatment on the average total length of the apical dendrite. **B.** The effects of chronic stress and guanfacine treatment on the average number of intersections (measure of dendritic branching) of the apical dendrite. **C.** The effects of chronic stress and guanfacine treatment on the average total length of the basal dendrites. **D.** The effects of chronic stress and guanfacine treatment on the average number of intersections (measure of dendritic branching) of the basal dendrites. Similar to that seen in previous studies, chronic stress reduced the length of apical dendrites, but did not significantly alter basal dendritic length in vehicle-treated animals. Guanfacine treatment produced an unexpected increase in basal dendritic length and branching in control animals. * significantly different from vehicle controls, $p < 0.05$; † significantly different from vehicle stressed rats, $p < 0.05$; § significantly different from vehicle controls using a fully between subjects analysis, $p = 0.015$.

Although guanfacine treatment significantly improved cognitive performance compared to vehicle-treated stressed animals, it did not completely normalize performance, especially as stress progressed over the 21 days. It may be that a higher dose or repeated dosing would be needed to optimize performance as stress exposure accumulated. It is important to remember that guanfacine was administered 23 h prior to cognitive testing each day, and given the rapid metabolism of the drug in young rats (Kiechel, 1980), it was likely eliminated from the system by the time of testing each day; indeed, rats may even have been in drug withdrawal at this time. As alpha-2A-adrenoceptors can desensitize by reducing their expression in the membrane in response to chronic agonist stimulation (Heck and Bylund, 1998), and as alpha-2A-adrenoceptor stimulation on spines plays a large role in permitting network connectivity (Wang et al., 2007), it is possible that some spines were rendered ineffective at the time of cognitive testing due to reduced alpha-2A-adrenoceptor expression in the spine membrane, leading to suboptimal behavioral performance. Thus, a superior dose regimen would likely be more effective for clinical use, e.g. multiple daily dosing to maintain a higher level of receptor stimulation. However, the current study shows that even under suboptimal guanfacine dosing conditions (once daily administration of a modest dose, cognitive performance tested 23 h after administration during likely drug withdrawal), the treatment still demonstrated significant protection of PFC spine density and cognitive performance. Combination with other mechanisms, e.g. inhibition of PKC signaling or stimulation of growth factors (see below), may maximize protection, especially as the stress accumulates.

Interestingly, there was an unexpected, small but significant increase in dendritic extensions and spine density in control rats treated with guanfacine. However, these animals did not perform better than vehicle controls. It is possible that the new dendritic spines were not useful, or at least not within the short timeframe (21 days) of this study. If this is true, guanfacine may serve to protect and optimize cognition, but may not be a cognitive enhancer, i.e. that at least with this dose regimen it does not produce supranormal performance in otherwise healthy and engaged young animals. Alternatively, it may be that as cognitive performance was assessed 23 h after last guanfacine treatment when rats were in “drug withdrawal”, the new spines may have been less effective due to reduced alpha-2A receptor expression at the time of cognitive assessment as described above. Thus, their cognitive performance may not have been a true reflection of their capabilities. Either way, cognitive performance in stressed animals

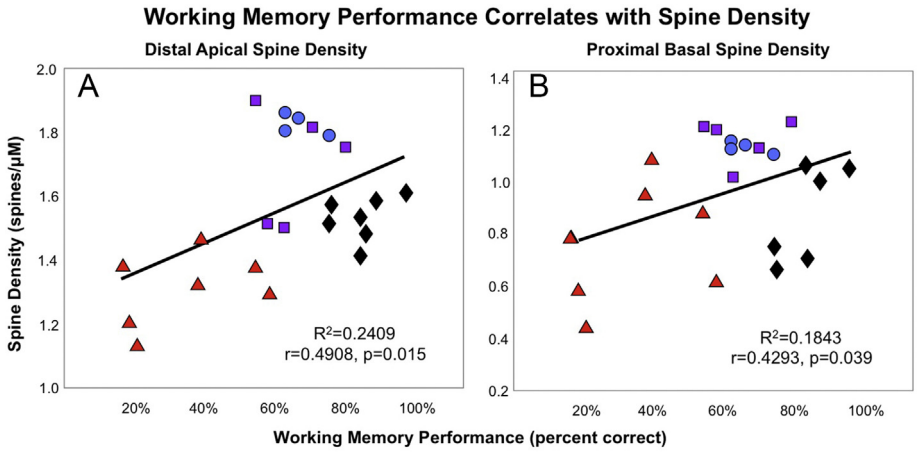

Fig. 5. Performance of the delayed alternation task (mean of the last 2 testing sessions) showed a significant correlation with distal apical spine density (**A**) and to a lesser extent, with proximal basal spine density (**B**). Vehicle control = black diamonds; vehicle stress = red triangles; guanfacine control = blue circles; guanfacine stress = purple squares.

receiving guanfacine was significantly better than stressed animals receiving vehicle.

4.2. Speculation on mechanisms

Guanfacine likely acts through a number of inter-related mechanisms to reduce the detrimental effects of stress exposure. Stimulation of post-synaptic alpha-2A-adrenoceptors strengthens PFC connectivity by inhibiting cAMP opening of K^+ channels (Wang et al., 2007). Alpha-2A adrenoceptor stimulation also weakens amygdala function (DeBock et al., 2003), reduces stress-induced DA release in the PFC (Morrow et al., 2004), and reduces the tonic firing of LC neurons and related NE release (Nestler and Alreja, 1999; Cedarbaum and Aghajanian, 1977; Quintin et al., 1986; Engberg and Eriksson, 1991). As the noradrenergic system sensitizes with repeated stress (Miner et al., 2006; Finlay et al., 1995; Jedema and Grace, 2003), these regulatory actions on NE neurons may be especially helpful under conditions of chronic stress exposure. Guanfacine may also prevent spine loss by reducing inflammation in brain. Evidence suggests that synapses can be phagocytized by reactive astroctyes and activated microglia (Stephan et al., 2012; Chung et al., 2013). Microglia and astroctyes are activated by beta adrenoceptor stimulation (Sutin and Griffith, 1993; Gyoneva and Traynelis, 2013), and activated microglia are deactivated by alpha-2A-adrenoceptor stimulation (Gyoneva and Traynelis, 2013). Guanfacine may also reduce inflammation by inhibiting cAMP signaling in spines, providing "replacement therapy" for the reduced actions of the phosphodiesterase PDE4A5, whose activity and proper anchoring by DISC1 are weakened by the inflammatory mitogen-activated protein kinase signaling cascade (MacKenzie et al., 2011). Thus, guanfacine may protect PFC gray matter by reducing the neurochemical and inflammatory stress response, and by strengthening PFC connectivity.

In addition to PKA and PKC intracellular signaling, mTor signaling plays an important role (Ota et al., 2014), where stress increases the expression of REDD1, reducing mTor signaling and reducing synaptogenesis. Interestingly, in the immune system, adrenergic stimulation of beta receptor-cAMP signaling increases the expression of REDD1 in macrophages (Yanagawa et al., 2014). Similar events in PFC neurons could provide a bridge between current catecholamine cAMP mechanisms and the mTor signaling pathway. REDD1 is induced by hypoxic stress (Katiyar et al., 2009), whereas guanfacine protects PFC dendritic spines and cognitive performance from the detrimental effects of hypoxia (Kauser et al., 2013), suggesting that this may be a fruitful arena for future research. Taken together, these data suggest that guanfacine protects the PFC from both physiological (hypoxia) and psychological (restraint) stressors.

4.3. Clinical implications

Many mental disorders are associated with both a loss of PFC synapses and increased signs of neuroinflammation e.g. (Pace and Heim, 2011; Raison and Miller, 2013; Yoshiyama et al., 2007; Schizophrenia Working Gro, 2014; Sharma et al., 2014; Riedel et al., 2014; Masi et al., 2014). Given that guanfacine can protect working memory (Birnbaum et al., 2000) and PFC dendritic spines, as well as reducing inflammation, it may be helpful across a wide spectrum of mental disorders. Indeed, guanfacine is already in widespread use (off-label) to treat trauma in children and adolescents, where signs of PFC dysfunction such as impaired regulation of emotion (e.g. impulsive aggression) and attention are common and problematic (Connor et al., 2013; Arnsten et al., 2015). Two studies of guanfacine in adults with long-established PTSD (i.e. for many decades) have not shown benefit (Neylan et al., 2006; Davis et al., 2008); the spine substrate for guanfacine actions may be lost under these conditions. Guanfacine has also been shown to be helpful in treating patients with traumatic brain injury (McAllister et al., 2011), stroke and encephalitis (Malhotra et al., 2006; Singh-Curry et al., 2011), and substance abuse (Fox and Sinha, 2014; McKee et al., 2014). The current results suggest that guanfacine might also be useful in the treatment of prodromal schizophrenia, as it is FDA-approved for the treatment of adolescents, and might lessen the wave of PFC gray matter loss and inflammatory response that are associated with the descent into illness (Cannon et al., 2014).

5. Conclusion

Daily treatment with the alpha-2A agonist, guanfacine, was shown to protect cognitive performance and PFC spine density in rats exposed to chronic restraint stress. As guanfacine has been shown to be safe for long-term use in humans, it may be a helpful treatment for a variety of stress-related cognitive disorders.

Disclosures

AFTA and Yale University receive royalties from the sales of extended release guanfacine (Intuniv™) for the treatment of pediatric ADHD from Shire Pharmaceuticals. They do not receive royalties from immediate release guanfacine or generic versions of Intuniv, available starting December 2014.

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