Dendritic complexity in the rat secondary motor cortex is not affected by chronic variable stress

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Chronic stress is a well-established risk factor for developing a substance use disorder. Some research suggests that stress may facilitate impaired behavioral control, resulting in an increased vulnerability to addiction. However, the specific mechanisms through which these two phenomena interact are not fully understood. Recent research supports the hypothesis that addiction is the result of a transition from voluntary substance use to compulsive, uncontrolled use. This is also often described as an increasing bias towards habitual action strategies over flexible, goal-directed ones. Because of this, several brain regions thought to be involved in either of these decision-making processes have been analyzed for morphological changes in dendritic complexity (a measure of dendritic branching) resulting from chronic stress exposure. The secondary motor cortex (M2) is implicated in flexible, goal-directed behavior with extensive connections to other regions involved in addiction (e.g., the striatum, ventral tegmental area, basolateral amygdala, and orbitofrontal cortex) but has not yet been investigated in the context of stress. Therefore, we examined the effects of chronic variable stress (CVS) on dendritic complexity in the rat M2 with the prediction that rats exposed to CVS would exhibit decreased dendritic complexity in this region compared to those in the control group. Pyramidal neurons in Layer 3 of the M2 were identified and manually reconstructed, and the dendritic complexity of each neuron was quantified using Sholl ring analysis. We found no significant difference in dendritic complexity between neurons from CVS and control rats, suggesting that cortical regions associated with goal-directed action strategies may not be as susceptible to chronic stress-induced changes in structural plasticity as observed in other regions.

Abbreviations: AGm – medial agranular cortex; BLA – basolateral amygdala; CMS – chronic mild stress; CON – control condition; CVS – chronic variable stress; DLS – dorsolateral striatum; dmPFC – dorsomedial prefrontal cortex; DMS – dorsomedial striatum; L3 – layer 3; L5 – layer 5; M2 – the secondary motor cortex; NAc – nucleus accumbens; OFC – orbitofrontal cortex; rmANOVA – repeated measures analysis of variance; SMA – supplementary motor area

Keywords: dendritic morphology; goal-directed behavior; habitual behavior; action strategies; addiction; pyramidal neurons

Introduction

The role of chronic stress as a risk factor for substance use disorders is well-documented (Sinha, 2001, 2008; Turner and Lloyd, 2003; Lloyd and Turner, 2008). Research findings in these areas have suggested several ways in which stress might increase vulnerability to addiction, but two in particular are relevant to our study. The first of these is through stress-induced changes to the mesolimbic pathway, which is involved in both the stress response and the rewarding effects of drugs (Sinha, 2008). The other is through the cognitive and behavioral effects of stress: a decrease in behavioral control and decision-making ability and an increase in impulsivity,

which together increase addiction vulnerability (Sinha, 2008). Nonetheless, researchers have yet to develop a complete picture of the neural mechanisms involved in addiction and the ways in which chronic stress acts as a contributor.

One hypothesis with a growing body of supporting literature is that addiction involves a transition from voluntary substance use to compulsive, uncontrolled use. This shift is also often described as an increasing bias toward habitual action strategies over flexible, goal-directed ones (Everitt et al., 2008; Everitt and Robbins, 2013). In the context of instrumental learning, which has historically been the conceptual basis for animal models of addiction, these behaviors are often referred to as the results of stimulus-response and action-outcome processes, respectively (Everitt and Robbins, 2013; Belin-Rauscent et al., 2016). Habitual behaviors, then, are reflexive or automatic responses to a conditioned stimulus, remaining unaffected by changes in reward value. In contrast, goal-directed behaviors are actions performed for the specific purpose of obtaining an associated reward or other desirable outcome and, as such, are sensitive to decrements in the value of this reward.

Researchers often study changes in the physical structure, or morphology, of neurons to understand aspects of their functioning. Dendrites are the parts of a neuron that receive signals from other neurons. One aspect of dendritic morphology is the amount of branching exhibited. For example, if a neuron has less branching, it might not be able to receive as many signals from other neurons nearby. This could, in turn, reduce the firing rate of that neuron, which could then lead to a decrease in behaviors associated with the brain region in which the neuron is located (McLaughlin et al., 2009).

The preceding points are considered here together because chronic stress affects neuron morphology in regions associated with both of these behaviors (habitual and goal-directed) and with addiction. The striatum is one such region that plays a major part in addiction and addiction-like behaviors, and various striatal subregions are differentially involved in either goal-directed or habitual behavioral strategies. The dorsomedial striatum (DMS) plays a role in both goal-directed behavior (Yin et al., 2005) and, along with the nucleus accumbens (NAc) shell, the reinforcing effects of drug use and consequent acquisition of drug-taking behavior (Everitt et al., 2008). The dorsolateral striatum (DLS), on the other hand, is implicated in habitual behavior (Yin et al., 2004) and, with the NAc core, the transition to compulsive drug-taking (Ito et al., 2004; Everitt and Robbins, 2013). To our knowledge, two studies (Dias-Ferreira et al., 2009; Taylor et al., 2014) have analyzed each of these subregions for changes in dendritic complexity—a measure of dendritic branching—as a result of chronic mild and chronic variable stress (CMS and CVS, respectively). They found increased complexity in the DLS and NAc core, decreased complexity in the NAc shell, and no change in the DMS. This is for the most part in line with what one would predict based on a shift from the voluntary to more compulsive drug use associated with addiction (Everitt et al., 2008; Everitt and Robbins, 2013) in that, as Schwabe et al. (2011) proposed, chronic stress can increase an individual's susceptibility to addiction through a shift in dominant action strategy from goal-directed to habitual.

The secondary motor cortex (M2; also called the supplementary motor area [SMA], medial agranular cortex [AGm], or dorsomedial prefrontal cortex [dmPFC]) is another region which has been implicated in goal-directed (and not habitual) behavior in several studies. Sul et al. (2011) found that M2 lesions made choice behavior less value-dependent; that is, rats continued to press a lever for a reward even after the value of the reward was decreased. This behavior is consistent with a shift away from goal-directed decision-making (and presumably toward habitual action, though this was not specifically tested). Similarly, Gremel and Costa (2013a) observed a bias in action strategy toward habitual rather than goal-directed behavior after giving rats lesions in this same region.

The M2 also has major connectivity to other regions involved in goal-directed decision-making. This includes projections to the DMS (Yin et al., 2005; Dias-Ferreira et al., 2009; Gremel and Costa, 2013b), inputs from the mediodorsal thalamic nucleus (Corbit et al., 2003; Yin et al., 2005), and reciprocal connections with the orbitofrontal cortex (OFC; Schoenbaum and Shaham, 2008; Gremel and

Costa, 2013b). Though there is little to no research linking the M2 to addiction directly, it does project to the dorsal striatum (Taylor et al., 2014) and NAc (Everitt et al., 2008; Everitt and Robbins, 2013; Taylor et al., 2014); receives inputs from the substantia nigra pars compacta (Wise, 2009), ventral tegmental area (Taylor et al., 2013), and dorsal raphe nucleus (Valentino et al., 2010); and has reciprocal connections with the basolateral amygdala (BLA; Ito et al., 2004; Taylor et al., 2013) and OFC (Schoenbaum and Shaham, 2008). All of these regions that make connections with the M2 are implicated in addiction to varying extents.

Similarly, there is no significant body of literature about the effects of chronic stress on dendritic morphology in the M2 despite its connections to other regions affected by chronic stress, which include the BLA (Vyas et al., 2002), OFC (Liston et al., 2006; Dias-Ferreira et al., 2009), and striatum (Dias-Ferreira et al., 2009; Taylor et al., 2014). To address this gap in the literature and further our understanding for how stress increases addiction vulnerability, we examined the effects of CVS on dendritic complexity in the M2 region of the same rat brain tissue used by Taylor et al. (2014). Based only on the findings discussed above about other regions involved in goal-directed or habitual behavior, it might be unclear what effects we would expect to see. However, we also considered that the M2 is part of the medial prefrontal cortex (mPFC). Two more ventral subregions of the mPFC (the prelimbic and anterior cingulate cortices) located on the same anterior-posterior planes as the M2 have shown dendritic retraction as a result of chronic stress (Radley et al., 2004; Liston et al., 2006). This consideration, coupled with the proposal by Schwabe et al. (2011) mentioned earlier, led us to predict that the rats subjected to CVS would exhibit a decrease in dendritic complexity in the M2 compared to those in the control group.

Materials and Methods

Animals and chronic variable stress

The present study used the same brain tissue that was obtained for a previous study (Taylor et al., 2014) from eighteen male Sprague-Dawley rats acquired from Charles River Laboratories (Wilmington, MA, USA), assigned to either a chronic variable stress (CVS) or control (CON) condition. CVS rats were exposed to two variable stressors (e.g., restraint, overnight crowding, and footshock) every day for 14 consecutive days, while CON rats remained in the vivarium and were handled daily. All stress and handling procedures for these animals and further methodological details can be found in Taylor et al. (2014). All procedures with animals and tissue processing were conducted at Arizona State University according to federal guidelines outlined in the Guide for Care and Use of Laboratory Rats (Institute of Laboratory Animal Resources on Life Science, National Research Council) and were approved by the Institutional Animal Care and Use Committee at Arizona State University. Analysis of M2 dendritic complexity was conducted at Hendrix College.

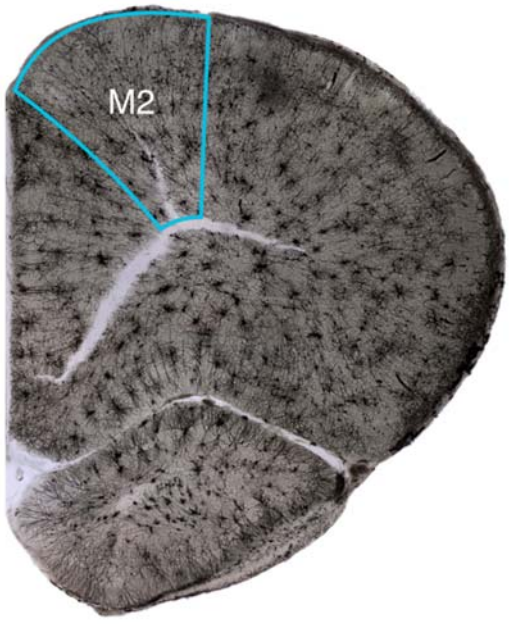

Figure 1. Location of the rat secondary motor cortex (M2).

Dendritic complexity

Shortly after completion of the two-week CVS exposure period, all rats were euthanized and their brains extracted. The brains were then stained using FD Rapid Golgistain™ Kits (FD NeuroTechnologies, Baltimore, MD, USA), cut into 200-µm coronal sections, and permanently mounted onto slides. See Taylor et al. (2014) for additional procedural information.

Pyramidal neurons were identified in Layer 3 (L3) of the M2 in 10 CVS and 8 CON rats according to the boundaries used by Sul et al. (2011), between 2.70 and 4.20 mm anterior to bregma, as delineated in a rat brain atlas (Figure 1; Paxinos and Watson, 1998). Neurons were selected for reconstruction if they were located in the region of interest, structurally complete, fully stained, and sufficiently distinct from surrounding neurons (Figure 2B). We focused on L3 because there was not a large enough quantity of neurons meeting the inclusion criteria in any other layer of the M2.

Selected neurons were manually reconstructed at 400x magnification using an Olympus BX51 microscope with an affixed camera lucida attachment. All drawings were checked by a second experimenter to confirm the accuracy and completeness of the reconstructions, after which point dendritic complexity was quantified using Sholl ring analysis (Figure 2A; Uylings and van Pelt, 2002; Taylor et al., 2014). Dendritic intersections were measured at 20-µm increments from the soma. Throughout all stages of data collection, all experimenters were blinded to experimental condition.

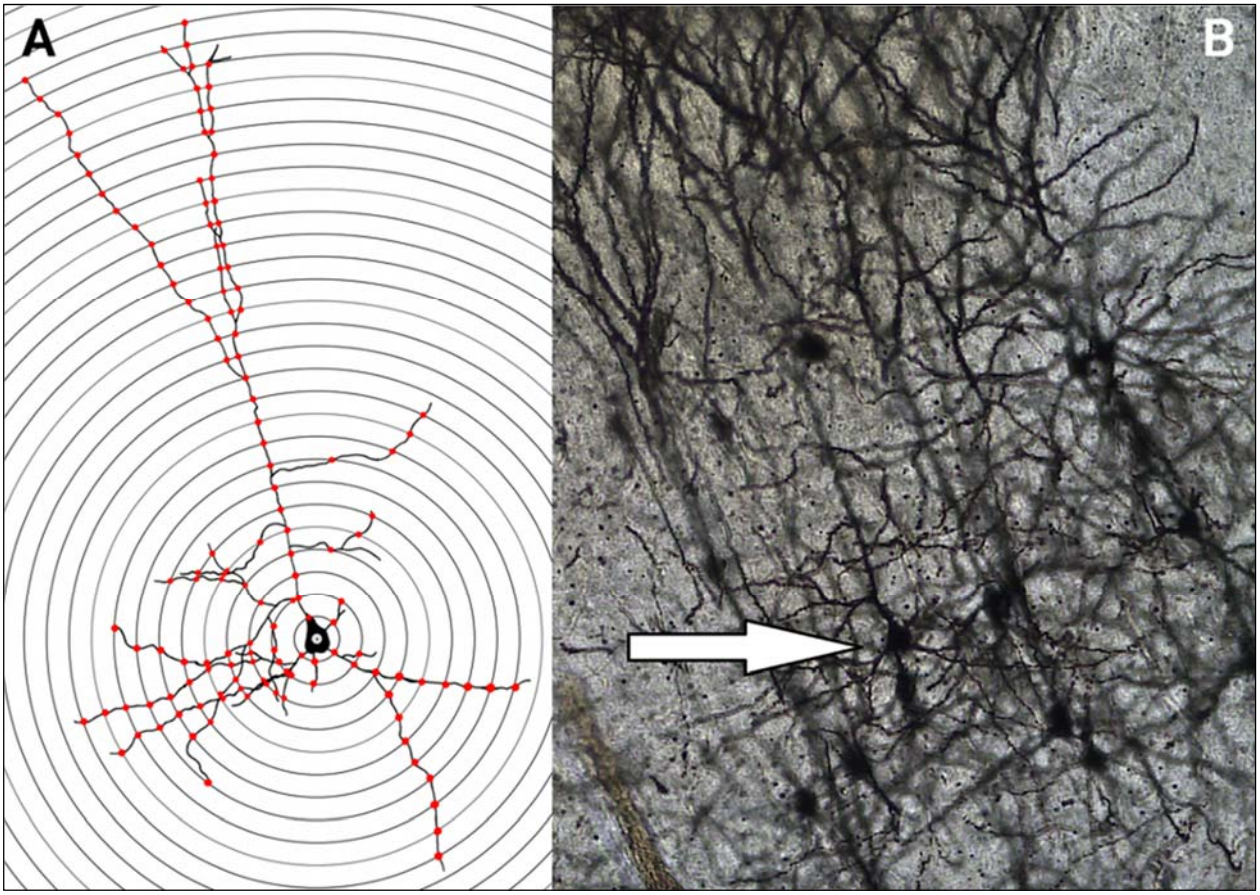

Figure 2. Secondary motor cortex (M2) pyramidal neuron. (A) A drawing of one of the neurons with an overlay of the rings used for Sholl ring analysis (not to scale). Intersections of dendrites with each of the rings are marked in red. (B) A photomicrograph of the neuron in (A) as it appeared in the tissue at 200x magnification.

Statistical methods

Statistical analyses were conducted using Prism GraphPad (version 6.07) software. Two-way repeated measures analyses of variance (rmANOVAs) were used to evaluate dendritic complexity, with ring included as the repeated measure. These tests were run with apical and basal dendrites analyzed both together and separately. In all analyses, statistical significance was set at $p < 0.05$. The final sample size was n = 8 CON rats, 6-7 neurons/rat and n = 10 CVS rats, 5–8 neurons/rat, with a total of 112 neurons analyzed across both groups. Three neurons were excluded from analyses due to disagreement between experimenters regarding the connectedness of several branches.

Results

The average number of total dendritic intersections (Figure 2A) did not significantly differ between CON (M = 105.63, SD = 21.81) and CVS rats (M = 108.90, SD = 13.71; Figure 3B). There was no main effect of stress on dendritic complexity in the M2, $F_{1, 16} = 0.15$, p = 0.70, and no significant interaction between ring and stress, $F_{23, 368} = 0.64$, p = 0.90 (Figure 3A). Similarly, there was no difference in the total number of apical intersections (CON: M = 61.75, SD = 15.68; CVS: M = 65.30, SD = 17.57), $F_{1, 16}$ = 0.29, p = 0.60, between the control and CVS groups. The analysis of basal intersections also showed no differences (CON: M = 48.50, SD = 11.78; CVS: M = 47.36, SD = 8.67), $F_{1, 16}$ = 0.27, p = 0.61. In addition, there was no significant

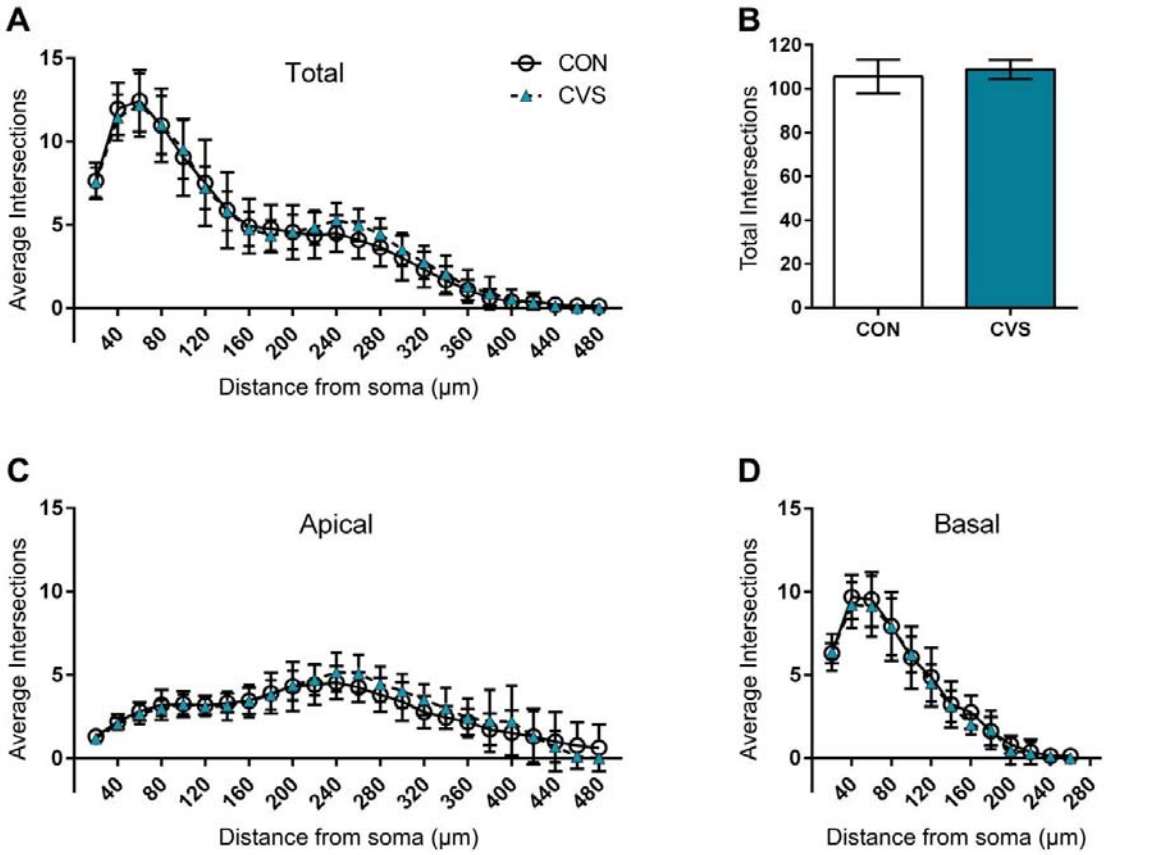

Figure 3. Dendritic complexity of secondary motor cortex (M2) pyramidal neurons. (A) Exposure to chronic variable stress (CVS) had no effect on whole-neuron dendritic complexity in this region. Ring-by-ring analyses were not conducted for these data because of the high level of variability. (B) No difference was found between the average number of dendrite-ring intersections in M2 neurons from CVS-exposed and control rats. (C & D) CVS exposure had no effect on the complexity of apical or basal dendrites measured separately. n = 5–8 neurons/rat, 8–10 rats/group.

interaction between ring and stress for either apical, $F_{23, 368} = 0.96$, $p = 0.51$ (Figure 3C), or basal dendrites, $F_{12, 192} = 0.34$, $p = 0.98$ (Figure 3D).

Discussion

Though we had predicted that CVS exposure would lead to decreased dendritic complexity of Layer 3 (L3) pyramidal neurons in the rat M2, our findings did not support this prediction. Furthermore, although prior research in other regions (Dias-Ferreira et al., 2009) has found a differential impact of chronic stress on the complexity of apical versus basal dendrites, we did not. This is consistent with Taylor et al. (2014) and Dias-Ferreira et al. (2009), both of whom found no chronic stress-induced morphological changes in the DMS, another region involved in goal-directed behavior.

The fact that no morphological effects of chronic stress were observed in either the DMS or the M2 regions of the same tissue could reasonably raise the concern that the duration of chronic stress used by Taylor et al. (2014) was not long enough to produce dendritic retraction. However, because Taylor et al. (2014) did find dendritic retraction in the NAc shell while examining this tissue, we believe that the lack of retraction we observed in the M2 is not due to insufficient stress exposure.

One interpretation of our findings is that the mechanisms facilitating flexible, goal-directed behaviors in the M2 and perhaps other cortical regions may not be as considerably impacted by chronic stress as more "automatic," subcortical regions like the amygdala and DLS (Taylor et al., 2014; Wilson et al., 2015). In other words, the increase in habitual action strategies seen in addiction studies may not be at the expense of goal-directed behavior mediated by the M2 (or DMS), specifically. Indeed, Schwabe et al. (2011) did argue that chronic stress shifts the *dominant* behavior pattern from goal-directed to habitual but maintained that both remain potential decision-making strategies.

In this study, we focused exclusively on pyramidal neurons in L3 of the M2, which contribute to local cortical circuits and have outputs to L5 neurons in this region; however, it is neurons in L5, not L3, that directly connect the M2 to the striatum through the corticostriatal loop (Kawaguchi, 2017). Therefore, one direction for further investigation would be to evaluate the dendritic complexity of neurons in L5 of the M2 to determine whether the direct connection of this layer to the striatum might affect its involvement in the action strategy shift. In addition, because previous studies have reported chronic stress-induced morphological changes in Layers 1 and 2 of cortical regions related to habitual behavior (e.g., the lateral OFC; Dias-Ferreira et al., 2009), these layers of the M2 may also be worth examining more closely in order to rule out stress-induced changes to the M2.

When considered with previous literature (Dias-Ferreira et al., 2009; Taylor et al., 2014), our findings suggest that chronic stress differentially impacts specific brain regions related to decision making strategies. Specifically, it suggests that stress facilitates the transition from goal-directed to more habitual strategies by favoring increased dendritic complexity in regions associated with habitual strategies while making less of an impact on regions associated with goal-directed strategies. Implications of these combined findings provide neurobiological evidence to support addiction prevention and recovery efforts that recognize the importance of proactive and adaptive management of stress, particularly in terms of preventing maladaptive habit formation.

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