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## Predictable Chronic Mild Stress Improves Mood, Hippocampal Neurogenesis and Memory

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### Abstract

Maintenance of neurogenesis in the adult hippocampus is important for functions such as mood and memory. As exposure to unpredictable chronic stress (UCS) results in decreased hippocampal neurogenesis, enhanced depressive- and anxiety-like behaviors and memory dysfunction, it is believed that declined hippocampal neurogenesis mainly underlies the behavioral and cognitive abnormalities after UCS. However, the effects of predictable chronic mild stress (PCMS) such as the routine stress experienced in day-to-day life on functions such as mood, memory, hippocampal neurogenesis are unknown. Using forced swim and elevated plus maze tests in a prototype of adult rats, we demonstrate that PCMS (comprising 5 minutes of daily restraint stress for 28 days) decreases depressive- and anxiety-like behaviors for prolonged periods. Moreover, we illustrate that decreased depression and anxiety scores after PCMS are associated with ~1.8 fold increase in the production and growth of new neurons in the hippocampus. Additionally, we found that PCMS leads to enhanced memory function in water maze as well as novel object recognition tests. Collectively, these findings reveal that PCMS is beneficial to the adult brain function, which is exemplified by an increased hippocampal neurogenesis and an improved mood and cognitive function.

### Keywords

anxiety, adult neurogenesis; dentate neurogenesis; depression; elevated plus maze test; forced swim test; hippocampal plasticity; neural stem cells; stress and neurogenesis

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#### Conflict of Interest

The authors declare no conflict of interest in relation to the work described in this manuscript.

#### Supplemental Information

Supplemental information is available at Molecular Psychiatry's website.

## Introduction

The hippocampus, important for functions such as mood and memory, is one of the few brain regions where addition of new neurons occurs throughout life.<sup>1-5</sup> It is now well established that neural stem cells (NSCs) that reside in the subgranular zone (SGZ) of the dentate gyrus proliferate and give rise to new granule cells, which migrate into the granule cell layer and integrate into the hippocampal circuitry with specific afferent and efferent connectivity.<sup>6-8</sup> While multiple earlier studies have suggested a role for hippocampal neurogenesis in several cognitive functions,<sup>9-15</sup> there has been no consensus regarding this issue because of contrasting findings in other studies.<sup>16-18</sup> However, recent studies including investigations that utilized selective neurogenesis ablation techniques strengthen the purported role of hippocampal neurogenesis in functions such as mood,<sup>19-21</sup> learning and memory.<sup>22-25</sup> Thus, maintaining higher levels of hippocampal neurogenesis in adulthood appears to be beneficial for improving mood and cognitive functions.

Indeed, studies have demonstrated reduced depressive-like behavior and/or improved cognitive function with increased levels of hippocampal neurogenesis following chronic antidepressant treatment,<sup>13,26</sup> exposure to enriched environment<sup>27</sup> or physical exercise.<sup>6,14</sup> Likewise, conditions that suppress hippocampal neurogenesis such as unpredictable chronic stress (UCS) or greatly increased levels of stress hormones have been linked to amplified depressive-like behavior and/or cognitive dysfunction.<sup>19,26,28-32</sup> Thus, studies support a link between the stress-induced depression & cognitive dysfunction and lower levels of hippocampal neurogenesis. Pertaining to stress, it is apparent that a large number of individuals in the general population display different levels of chronic stress in their day-to-day lives.<sup>33</sup> Yet, in only a fraction of individuals, chronic stress leads to mental illnesses such as depression and anxiety, suggesting that the type, intensity and duration of stress play roles in the development of stress-related mental illnesses. For example, multiple studies clearly report that UCS leads to increases in depressive- and anxiety-like behaviors and decline in hippocampal neurogenesis, even when the UCS is milder in intensity.<sup>31,32,34,35</sup> It has been demonstrated that UCS reduces the proliferation of hippocampal NSCs and the psychosocial stress reduces the survival of newly born neurons in the hippocampus, which likely contribute to hippocampal atrophy typically seen after chronic stress.<sup>18,34-35</sup> However, the effects of predictable chronic mild stress (PCMS) on mood, learning, memory and hippocampal neurogenesis are mostly unknown. Because the majority of population experiences a type of chronic stress that is predictable and relatively milder in nature, it is of great interest to examine the effects of PCMS on depression- and anxiety-like behaviors, cognitive function and hippocampal neurogenesis.

We tested the hypothesis that PCMS enhances functions such as mood, learning and memory in adults via stimulation of NSCs and increased neurogenesis in the hippocampus. We exposed a group of adult rats to 5 minutes of daily restraint stress for 28 days (a model of PCMS) and investigated the stress-mediated effects on depressive- and anxiety-like behaviors using the forced swim test (FST) and the elevated plus maze (EPM) test. Additional group of age-matched adult rats that were handled similarly but not exposed to restraint stress (controls) were also examined with FST and EPM test for comparison. We next examined the extent of hippocampal neurogenesis to determine links between the level

of depression and anxiety with the extent of production of newly born cells and neurons. We also compared the dendritic growth of newly born neurons between the two groups to ascertain the effects of PCMS on growth of newly born neurons. As our results showed an association between enhanced hippocampal neurogenesis and declined depressive- and anxiety-like behavior in rats subjected to PCMS, we extended this study to examine the effects of PCMS-mediated increased neurogenesis on learning and memory function. Through investigation of additional groups of rats at 1.5-2.0 months after PCMS or handling regimen using water maze test (WMT), novel object recognition test (NORT), FST and EPM test, we demonstrate that exposure to PCMS considerably improves memory function and PCMS mediated improved mood persists for prolonged periods.

## Materials and methods

The experimental design comprising animal groups, different behavioral procedures, timing of tissue harvest and processing, and quantitative morphometric measurements are illustrated in Figure 1 of the supplemental document.

### Animals and induction of PCMS

Three-months old Sprague-Dawley rats obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA) were used. All experiments performed in this study were approved by the animal studies subcommittee of the Durham VA Medical Center. For induction of PCMS, a rat restrainer made up of Plexiglas with small circular holes on sides for ventilation and a sliding door to facilitate the restraint during the stress procedure was used (Fig. 1). The animal was first guided to enter the restrainer and was allowed to stay for 5 minutes with no mobility (Fig. 1). At the end of 5 minutes, the rat was withdrawn gently from the restrainer and placed back into its home cage. This stress paradigm continued for 28 days and all rats were stressed at the same time every day (i.e. between 3-5 PM) to maintain the predictability of the timing of stress over the experimental duration. To deduce the effects of handling on behavior and cognitive function, animals assigned for the control group underwent similar handling every day for 28 days except the restraint stress procedure. The handling of each rat in the control group comprised removal from its cage, placing near the restraint stress apparatus for 5 minutes, and placing back in its cage. The handled group is termed as the control group throughout the manuscript.

### Analyses of depressive-like behavior using Forced swim test (FST)

One day after the completion of 28-day PCMS or handling regimen, depressive-like behavior in animals belonging to both groups (n=6/group) were ascertained using the FST. This test is widely used for measurement of depression-like behavior in rodents,<sup>36</sup> and the description of the procedure is detailed in the supplement document. Time spent in immobility for the trial duration was calculated for every rat and utilized as an index of depressive-like behavior. Data from PCMS and control groups were compared using the two-tailed unpaired Student's t-test.

### **Characterization of anxiety-like behavior using elevated plus maze (EPM) test**

Anxiety-like behavior in animals belonging to both groups (n=6/group) were ascertained using the EPM test. The EPM has been pharmacologically validated as a test for anxiety-like behavior in rats, with a decrease in anxiety-related EPM indices occurring in response to anxiolytic agents.<sup>37</sup> The description of the procedure employed is detailed in the supplement document. The number of open arm entries and duration of time spent in open arms for the duration of the test were used for calculation of the extent of anxiety-like behavior in each rat. Data from PCMS and control groups were compared using the two-tailed unpaired Student's t-test.

### **5'-bromo-2'-deoxyuridine (BrdU) injections and tissue processing**

To label the newly born cells that are generated over a 24-hr period in the neurogenic (SGZ-GCL) region of the hippocampus, each rat belonging to both groups received four intraperitoneal injections of BrdU (one injection every 6 hr over 18 hrs at a dose of 50 mg/Kg b.w.5) on the 3<sup>rd</sup> day after 28-day PCMS or handling regimen. At 6 hrs after the last BrdU injection, rats in both groups underwent intracardiac perfusion with 4% paraformaldehyde. The brains were dissected out and collected in 4% paraformaldehyde.

### **Analyses of neurodegeneration and neuroinflammation after PCMS**

The brains were cryoprotected and 30 $\mu$ m-thick cryostat sections were cut coronally through the entire hippocampus and collected serially. Serial sections (every 15<sup>th</sup>) through the entire hippocampus were selected in each animal belonging to different groups and processed for Fluoro-Jade B histochemical staining, as described in our earlier report.<sup>38</sup> Additional series (every 15<sup>th</sup>) of sections were immunohistochemically processed for visualization of surviving neurons, reactive astrocytes, and activated microglial cells using mouse monoclonal antibodies against neuron specific nuclear antigen (NeuN, a marker of mature neurons; Chemicon), nestin (a marker of reactive astrocytes; Hybridoma Bank), ED-1 (a marker of activated microglial cells; SeroTech) and the avidin-biotin complex (ABC) method.<sup>39-40</sup>

### **BrdU and doublecortin (DCX) immunohistochemistry**

Serial sections (every 15<sup>th</sup>) through the entire hippocampus were selected in each animal belonging to different groups and processed for BrdU immunostaining using a monoclonal antibody to BrdU (1:500; BD Biosciences) and the ABC method, as described in our earlier reports.<sup>4,41</sup> Another series (every 15<sup>th</sup>) of sections from each animal were processed for doublecortin (DCX) immunostaining using a goat polyclonal antibody to DCX (1: 250; Santa Cruz Biotechnology) using the ABC method, as detailed in our earlier study.<sup>4</sup>

### **Quantification of the numbers of newly born cells (BrdU) and neurons (DCX) in the hippocampus**

In order to determine the production of new cells per day and the status of hippocampal neurogenesis, stereological quantifications of BrdU<sup>+</sup> cells and DCX<sup>+</sup> cells in the SGZ-GCL were performed using serial sections (every 15<sup>th</sup>) immunostained for BrdU or DCX and the

optical fractionator method. The details pertaining to this procedure are described in the supplement document.

### **Analyses of the neuronal fate-choice decision of newly born cells**

We utilized the BrdU-DCX dual immunofluorescence and confocal microscopic analyses for identifying newly born cells that differentiated into neurons. As DCX expression occurs within 3 hrs after birth in newly born cells that are committed to neuronal lineage and DCX is an excellent immature marker of newly generated neurons,<sup>4,41-42</sup> this method facilitated the quantification of net neurogenesis (i.e. the fraction of newly born cells that differentiate into neurons). The detailed methodology is described in the supplement document.

### **Analysis of the dendritic growth of DCX<sup>+</sup> newly born neurons**

In order to compare the dendritic growth of newly born neurons between the PCMS and control groups, we quantified the dendritic growth of relatively mature neurons among DCX<sup>+</sup> + newly born neurons using the NeuroLucida neuron tracing system (Microbrightfield).<sup>4-5,41</sup> Relatively mature neurons within the DCX<sup>+</sup> neuronal population are defined as described in our earlier studies.<sup>4,41</sup> The methodology is also briefly described in the supplement document.

### **Characterization of learning and memory function using the water maze test (WMT)**

Because rats subjected to PCMS exhibited decreased depressive- and anxiety-like behaviors with enhanced hippocampal neurogenesis, we wondered whether PCMS-mediated increased neurogenesis also improves learning and memory function. Therefore, using additional groups of rats subjected to PCMS or handling (n=6/group), we examined hippocampal-dependent spatial memory function using the Morris WMT,<sup>43</sup> which is a closest parallel to the episodic memory in humans. The WMT was performed at 1.5 months after the completion of PCMS or handling regimen. The delay between PCMS or handling and the WMT in this study is based on the earlier finding that newly generated neurons typically take about 6 weeks to get incorporated into the spatial memory circuits.<sup>15</sup> The procedure employed for WMT is detailed in the supplement document.

### **Analyses of memory function using novel object recognition test (NORT)**

As a second measure of memory function, each rat belonging to both groups were examined with NORT<sup>25</sup>. The protocol used for NORT is described in the supplement document. Memory analysis with NORT is based on the natural tendency of rodents to investigate a novel object instead of a familiar one. The choice to explore the novel object reflects the use of learning and (recognition) memory processes.<sup>25</sup>

### **Analyses of depressive- and anxiety-like behavior at extended time points after PCMS**

In order to determine whether PCMS mediated decreases in depressive and anxiety-like behaviors last for prolonged periods after PCMS, animals used for WMT and NORT above were also tested for FST and EPM test as described earlier. These analyses characterized the long-term outcome of PCMS on depression and anxiety.

## Results

### PCMS promotes antidepressant-like effects

In the first FST performed one day after the conclusion of PCMS or handling regimen, the overall depressive-like behavior in rats subjected to PCMS was 51% less than that observed in control rats ( $p < 0.01$ ; Fig. 1[A]). Thus, exposure to PCMS considerably reduces depressive-like behavior, which is an indication of improved mood function or antidepressant-like effect mediated by PCMS. Analyses of additional groups of rats at ~2.0 months after the stress regimen also revealed better mood in rats exposed to PCMS, in comparison to age-matched control rats ( $p < 0.05$ ; Fig. 1 [B]), suggesting that antidepressant like effects of PCMS persists for prolonged periods.

### PCMS reduces anxiety-like behavior

In the first EPM test performed two days after the conclusion of PCMS or handling regimen, rats subjected to PCMS displayed nearly 3 fold increase in the number of entries into the open arm in comparison to control rats ( $p < 0.05$ ; Fig. 1 [C]). Furthermore, comparison of the total time spent in the open arm between the two groups revealed that PCMS treated rats spent 4.3 folds greater amount of time in the open arm ( $p < 0.05$ ; Fig. 1 [D]). Thus, exposure to PCMS reduces anxiety-like behavior, which is also an indication of improved mood function. Investigation of additional groups of rats at ~2.0 months after the stress regimen also revealed reduced anxiety in rats exposed to PCMS in comparison to age-matched control rats (Fig. 1 [E, F]), as PCMS treated rats spent 6.3 folds greater amount of time in the open arm ( $p < 0.05$ ; Fig. 1 [F]). Thus, the anxiolytic effects of PCMS are enduring.

### PCMS does not cause neurodegeneration or neuroinflammation

We analyzed separate sets of serial sections through the hippocampus with Fluoro-Jade B staining and immunostaining for NeuN, nestin and ED-1. These analyses revealed no signs of neurodegeneration or neuroinflammation in PCMS treated rats (see Figs. 2 and 3 of the Supplemental document).

### PCMS enhances the production of new cells in the SGZ-GCL of the hippocampus

Visualization of BrdU+ cells with BrdU immunostaining revealed an increased production of newly born cells in the SGZ-GCL of rats that underwent PCMS, in comparison to control rats (Fig. 2 [A1-B2]). While newly born cells appeared as clusters in both groups, the PCMS group exhibited an obvious increase in both density and size of clusters (Fig. 2 [A2, B2]). Quantification of the total number of BrdU+ cells in the SGZ-GCL confirmed the increased production of newly born cells per day in rats subjected to PCMS. Overall, the production of new cells per day was increased by 1.5 folds in the PCMS group in comparison to the control group ( $p < 0.01$ , Fig. 2 [C1]). Thus, PCMS considerably enhances the overall production of new cells per day, likely by increased proliferation of NSCs.



### **PCMS does not alter the neuronal fate-choice decision of newly born cells in the hippocampus**

Quantification of the neuronal differentiation of newly born cells in the SGZ-GCL of PCMS and control groups via BrdU and DCX dual immunofluorescence and Z-section analyses in a confocal microscope (Fig. 2 [D1-D4]) revealed no changes in neuronal fate-choice decision of newly born cells in rats that underwent PCMS. The average fraction of newly born cells that differentiate into DCX+ neurons is 66% ( $66.3 \pm 2.1\%$ ,  $n=6$ ) in the control group and 70% ( $70 \pm 7.1\%$ ) in the PCMS group ( $p>0.05$ ; Fig. 2 [E1]). Thus, PCMS does not modify the extent of neuronal fate-choice decision of newly born cells in the neurogenic region of the hippocampus.

### **PCMS greatly increases the production of new neurons per day in the hippocampus**

By using percentages of neuronal differentiation among newly born cells and the overall generation of new cells per day (i.e. BrdU+ cell counts), we calculated the production of new neurons per day in both control and PCMS groups to ascertain net hippocampal neurogenesis. This revealed an increased production of new neurons per day in the PCMS group, in comparison to the control group. The overall production of new neurons per day in the PCMS group is 1.6 fold greater than the control group ( $p<0.01$ ; Fig. 2 [F1]). As neuronal differentiation of newly born cells were comparable between the PCMS and control groups, increased numbers of newly born neurons in the PCMS group mainly reflects increased proliferation of NSCs with exposure to PCMS.

### **PCMS enhances the overall status of hippocampal neurogenesis as measured by numbers of DCX+ newly born neurons**

We ascertained whether the overall status of hippocampal neurogenesis is enhanced with exposure to PCMS via DCX immunostaining (Fig. 3 [A1-B2]) and stereological quantification of all DCX+ neurons in the SGZ-GCL of control rats and rats subjected to PCMS. Earlier studies have revealed that cells expressing DCX in the SGZ-GCL of rats are new neurons that were born mostly during the two weeks prior to euthanasia.<sup>41</sup> Thus, DCX immunostaining reveals a mixed population of 1-14 day old newly born neurons and hence provides a good measure of the ongoing status of hippocampal neurogenesis. Morphologically, a vast majority of DCX+ cells in both groups exhibited vertically orientated apical dendrites projecting into the dentate GCL and the molecular layer (Fig. 3 [A1-B2]). Quantification of DCX+ cells in the SGZ-GCL revealed that exposure to PCMS increases the overall addition of new neurons to the SGZ-GCL by 1.8 folds ( $p<0.0001$ , Fig. 3 [C1]). Thus, the increase in hippocampal neurogenesis observed in the PCMS group is consistent in terms of both net neurogenesis per day and the status of neurogenesis measured via quantification of all DCX+ neurons.

### **PCMS considerably enhances the dendritic growth of newly born neurons in the hippocampus**

Morphometric analyses revealed that, relatively mature DCX+ neurons in rats exposed to PCMS exhibit more complex dendritic trees with increases in the total dendritic length and numbers of dendritic nodes and endings, in comparison to their counterparts in the control

group (Fig. 4 [A]). The overall increase in the PCMS group is 1.8 fold for the total dendritic length ( $p < 0.001$ ; Fig. 4 [B]), 1.9 fold for the total number of dendritic nodes ( $p < 0.001$ ; Fig. 4 [C]) and dendritic endings ( $p < 0.001$ ; Fig. 4 [D]). The concentric circle analyses of Sholl also revealed a similar trend (see Fig. 4 of the supplemental document for details).

### **PCMS does not alter learning but improves memory retention in the water maze task**

Rats in both PCMS and control groups quickly learned to locate the position of the submerged platform using spatial cues. This was evidenced by decreases in the latency to reach the platform over the 7 training sessions. By 7th learning session, animals in both PCMS and control groups displayed an ability to locate the platform within 8-12 seconds after their release into the pool. Regression analyses revealed that both learning curves and  $r^2$  values are identical between the two groups (Fig. 5 [A]). Comparison of values of the first and last learning sessions revealed that the latency to reach the platform decreases over the training period by 86% in the PCMS group ( $p < 0.0001$ ) and 80% in the control group ( $p < 0.001$ ; Fig. 5 [B]). Thus, PCMS does not seem to alter the hippocampal-dependent spatial learning ability for the age group tested in this study. However, analyses of memory retrieval function revealed considerably improved memory function in rats that underwent PCMS. This was evidenced by enhanced scores for all four parameters of memory retention in the probe test that was conducted at 24 hrs after the last (7<sup>th</sup>) training session. First, in comparison to rats in the control group ( $n=6$ ), rats in the PCMS group ( $n=6$ ) exhibited considerably reduced latency to reach the platform area after their release into the pool ( $4.9 \pm 0.8$  seconds [control group] versus  $2.3 \pm 0.6$  seconds [PCMS group]),  $p < 0.05$ ; Fig. 5 [C]). Second, rats in the PCMS group spent more time in the platform area than control rats ( $4.7 \pm 0.5$  seconds [control group] versus  $8.1 \pm 0.8$  seconds [PCMS group]),  $p < 0.01$ ; Fig. 5 [D]). Third, rats that underwent PCMS displayed greater numbers of platform area crossings than rats in the control group ( $3.3 \pm 0.5$  [control group] versus  $5.3 \pm 0.4$  [PCMS group]),  $p < 0.05$ ; Fig. 5 [E]). Fourth, rats in the PCMS group exhibited increased dwell time in the platform quadrant (i.e. the quadrant where platform was placed during learning sessions) than rats in the control group ( $15.5 \pm 1.9$  seconds [control group] versus  $21.3 \pm 1.5$  seconds [PCMS group]),  $p < 0.05$ ; Fig. 5 [F]). Thus, exposure of rats to PCMS considerably increases memory retention ability.

### **PCMS improves novel object recognition ability**

We used the amount of time spent with the novel object and the discrimination index for the novel object as measures of memory function in NORT. The preference of the rat to delve into the novel object indicates the use of learning and (recognition) memory processes<sup>25</sup>. This test is particularly ideal for examining neurogenesis related memory function, as an earlier study using NORT in animals that underwent ablation of hippocampal neurogenesis and animals having normal neurogenesis has demonstrated that hippocampal neurogenesis plays a key role in the novel object recognition.<sup>25</sup> In comparison to control rats, rats that underwent PCMS spent similar time with the familiar object ( $p > 0.05$ ; Fig. 5 [G]) but spent 2.4 folds greater time with the novel object ( $p < 0.05$ ; Fig. 5 [H]). Furthermore, in rats treated with PCMS, the total exploration time was 2.0 folds greater than control rats ( $p < 0.06$ ; Fig. 5 [I]) and the discrimination index for the novel object was 17% greater than control rats



( $p < 0.05$ ; Fig. 5 [J]). Thus, NORT analyses clearly showed improved recognition memory in PCMS treated rats.

## Discussion

This study provides novel evidence that PCMS is beneficial for several brain functions which include mood, memory and NSC plasticity. While the constructive effect of PCMS on mood was evidenced by noticeable decreases in depressive- and anxiety-like behaviors in FST and EPM test, the helpful effect of PCMS on cognitive function was exemplified by an improved hippocampal-dependent memory function in both WMT and NORT. Furthermore, enrichments in mood and memory after PCMS were associated with substantially increased hippocampal neurogenesis. In view of the role of hippocampal neurogenesis in cognitive functions, better mood and memory functions following PCMS appeared to be mediated via increased plasticity of hippocampal NSCs resulting in increased production of new neurons. As these findings pertaining to mood, memory and hippocampal neurogenesis are remarkably contrary to the effects observed in earlier studies employing unpredictable chronic stress (UCS) prototypes, our results underscore that chronic stress has the ability to facilitate many beneficial effects on brain function when it is both milder and predictable in nature.

### Links between chronic stress, depression, anxiety, memory, and hippocampal neurogenesis

Previous studies in several animal prototypes demonstrate that UCS leads to increased depressive- and anxiety-like behaviors<sup>44-45</sup> and learning and memory dysfunction.<sup>46</sup> While many changes in the hippocampus and the basolateral amygdala after UCS likely contribute to these dysfunctions, hippocampal neurogenesis has emerged as one of the focal issues underlying the stress-induced depression, anxiety and memory impairments.<sup>20-21,28,46-47</sup> Indeed, it has been shown that both acute and chronic forms of unpredictable stress reduces hippocampal neurogenesis.<sup>18,34-35,48</sup> Furthermore, studies reveal that decreased hippocampal neurogenesis, increased depressive & anxiety-like behaviors and memory dysfunction occur in tandem following exposure to UCS.<sup>49</sup> Additional studies demonstrate that chronic antidepressant treatment not only ameliorates impairments in mood and memory but also increases hippocampal neurogenesis.<sup>13,28,30,50-51</sup> Thus, stress mediated decrease in hippocampal neurogenesis appears to be one of the major reasons underling mood and memory dysfunction after UCS. Recent studies on selective ablation of hippocampal neurogenesis also support the role of hippocampal neurogenesis in reducing depression and anxiety,<sup>19-21,52-53</sup> and improving learning and memory function.<sup>22-25</sup> In contrast to findings in UCS prototypes, our results demonstrate reduced depressive- and anxiety-like behaviors at both early and extended time-points after PCMS. These were evidenced by decreases in the duration of immobilization in the FST and increased time spent in the open arm with the EPM test. Moreover, enhanced memory function was observed after PCMS in both WMT and NORT. This was illustrated by superior retention of the learned memory in WMT and an increased amount of time spent with the novel object in NORT. Interestingly, the overall decreases in depression and anxiety and improvements in memory function were associated with enhanced production and growth of new neurons in

the hippocampus. Thus, while our findings after PCMS are contradictory to what was reported in studies using UCS, the link between the amount of hippocampal neurogenesis and the extent of depressive-and anxiety-like behavior is conspicuously maintained as in previous studies. This association reinforces the earlier suggestion that increased hippocampal neurogenesis is beneficial for improving functions such as mood and memory.

### **Reasons underlying the beneficial effects of PCMS on mood and memory**

Maintenance of homeostasis in conditions where physical and social demands change frequently and/or unpredictably requires integration of neurobehavioral, neuroendocrine and autonomic responses.<sup>54</sup> Stimuli that severely perturb homeostasis (e.g. stress) induce recruitment of several neuronal pathways to adapt to the demand, culminating in the activation of hypothalamic-pituitary-adrenocortical (HPA) axis and an increased release of the stress hormone corticosterone.<sup>55</sup> Activation of HPA axis following positive forms of stress (e.g. predictable stress in day to day life) experienced by individuals is believed to be useful for preparing the brain and body for impending challenges and inducing pro-cognitive effects,<sup>55-56</sup> as this response is a natural reaction of the body involving enhanced heart rate and blood pressure, increased flow of glucose and oxygen to the muscles and brain, and moderate increase in the levels of corticosterone in the brain. On the other hand, negative forms of stress (e.g. UCS) resulting from inability to cope with life changes, unmanageable workload or difficult environment, can considerably increase the levels of corticosterone for protracted periods and cause the development of major depression, anxiety-like disorders and memory impairments.<sup>33,57-59</sup> Previous studies imply an “inverted U” relationship between stress and cognitive functions so that moderate increases in the levels of corticosterone (following mild stress) leads to pro-cognitive effects, while greater increases in corticosterone levels (following chronic stress) leads to detrimental effects on cognitive processing.<sup>55-56</sup>

Thus, the overall stress reaction in an organism can be advantageous for cognitive functions when it induces moderate increases in corticosterone levels and leads to adaptation and resistance. Conversely, the stress reaction can be severely damaging to the organism with considerable cognitive dysfunction when it facilitates much greater increases in corticosterone levels and leads to aberrant adaptation comprising neurodegeneration, neuroinflammation, increased depressive- and anxiety-like behaviors and memory dysfunction. From these perspectives, it is likely that PCMS prototype employed in this study induced only moderate increases in corticosterone levels for shorter durations over the 28-day exposure period. The following observations support the above possibility. First, PCMS paradigm used in our study did not induce neurodegeneration which is contrary to changes seen after UCS, severe restraint stress or greatly increased levels of corticosterone. Lack of neurodegeneration after PCMS was confirmed by the absence of Fluoro-Jade B positive cells and retention of the hippocampal cytoarchitecture that is comparable to the control hippocampus with NeuN immunostaining. Second, exposure to PCMS did not induce neuroinflammation typified by the rare occurrence of nestin+ reactive astrocytes and ED-1+ reactive microglial cells as in the control hippocampus. Third, PCMS regimen resulted in considerably increased production and enhanced dendritic growth of new neurons in the hippocampus. Fourth, exposure to PCMS significantly improved mood and memory

function, which is incongruent with the depression, anxiety and memory impairment observed after UCS, severe restraint stress or greatly increased levels of corticosterone. 19,26,28-32,44-46 Thus, PCMS regimen employed in this study likely elicited moderate increases in corticosterone levels which facilitated both adaptive and beneficial responses in the brain.

### **Potential reasons underlying increased hippocampal neurogenesis after PCMS**

Exposure to PCMS in this study induced ~1.8 fold increase in the production of new neurons in the hippocampus. As the extent of conversion of newly born cells into neurons was comparable between PCMS and control groups, enhanced neurogenesis after PCMS is a result of daily increases in the production of new cells via augmented proliferation of NSCs. Additionally, exposure to PCMS facilitated ~1.8 fold increase in the dendritic growth of newly born neurons. These changes are contrary to the reported decreases in hippocampal neurogenesis in models of UCS, subordination stress, foot shock stress, cold immobilization stress and predator odor stress.<sup>35,45,60-61</sup> The discrepancy between the current study and previous studies likely reflects the effects of different levels of corticosterone elicited by the chosen stress regimen. It is likely that PCMS induced moderate increases in corticosterone for shorter durations over the 28-day exposure period stimulated an increased proliferation of NSCs through multiple mechanisms. However, direct corticosterone effects on specific receptors in NSCs do not appear to be the major reason for increased proliferation of NSCs. This is because only a minority (~27%) of NSCs in the adult hippocampus expresses glucocorticoid receptors and mineralocorticoid receptors are not expressed in hippocampal NSCs.<sup>62</sup> Furthermore, administration of glucocorticoid receptor agonist dexamethasone actually suppresses cell proliferation.<sup>63</sup>

From the above view point, it is likely that moderately elevated corticosterone after PCMS enhanced hippocampal neurogenesis through indirect effects, which could involve the following. First, elevations in corticosterone following acute mild stress have been shown to enhance glutamatergic neurotransmission through increased expression of NMDA and AMPA receptors in the brain.<sup>55</sup> As increased neural activity promotes proliferation of NSCs,<sup>64-65</sup> increased hippocampal neurogenesis after PCMS may be due to increased excitatory neurotransmission in hippocampal circuits for brief periods over the 28-day PCMS regimen. This idea is consistent with the glucocorticoid-mediated improved memory observed in young humans, impaired memory seen in older humans with inhibition of cortisol synthesis<sup>66</sup> and biphasic effects of glucocorticoids on synaptic plasticity and hippocampal-dependent memory function<sup>67</sup>. Second, while severe stress reduces the levels of multiple neurotrophic factors, mild stress or lower doses of corticosterone or dexamethasone treatments have been shown to induce transient increases in levels of neurotrophic factors such as fibroblast growth factor-2, brain derived neurotrophic factor and insulin-like growth factor-1.<sup>68-69</sup> As all of these neurotrophic factors are capable of stimulating the proliferation of NSCs<sup>70-72</sup> and knockdown of some of these factors induces both reduced hippocampal neurogenesis and depression,<sup>21</sup> it is plausible that increased neurogenesis after PCMS is related to increased levels of these neurotrophic factors. Third, other neurogenesis stimulating, antidepressant, and memory enhancing factors such as serotonin, neuropeptide Y and phosphorylated cyclic AMP response element binding protein

might have also exhibited up-regulation with PCMS.73-75 Thus, activation of multiple beneficial factors after PCMS likely influenced increases in the hippocampal neurogenesis, mood and memory function. Studies on the levels of the above factors are needed in future to further understand the mechanisms underlying PCMS mediated beneficial effects on the adult brain.

## Supplementary Material

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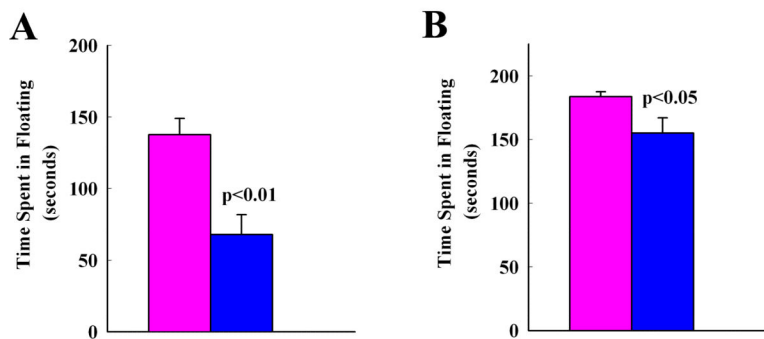
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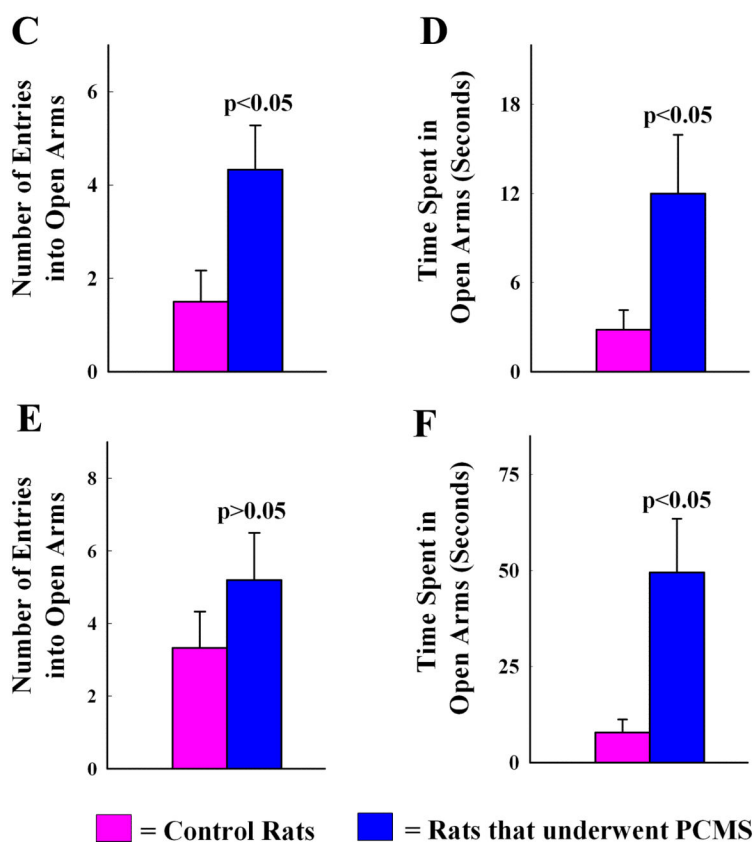
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### Analyses with Forced Swim Test (FST)



### Analyses with Elevated Plus Maze (EPM) Test



**Figure 1.**

The bar charts A & B illustrate the results of forced swim test (FST) at one day (A) and 2 months (B) after the predictable chronic mild stress (PCMS) regimen. Note that, rats that underwent PCMS spend considerably reduced time in immobility (or floating), in comparison to the age-matched control rats that underwent handling alone at both early and extended time-points after PCMS. Reduced immobility time is an indication of reduced depressive-like behavior in the PCMS treated rats at both time-points. The bar charts C-F illustrate the results of elevated plus maze (EPM) test at 2 day (C, D) and 2 months (E, F)

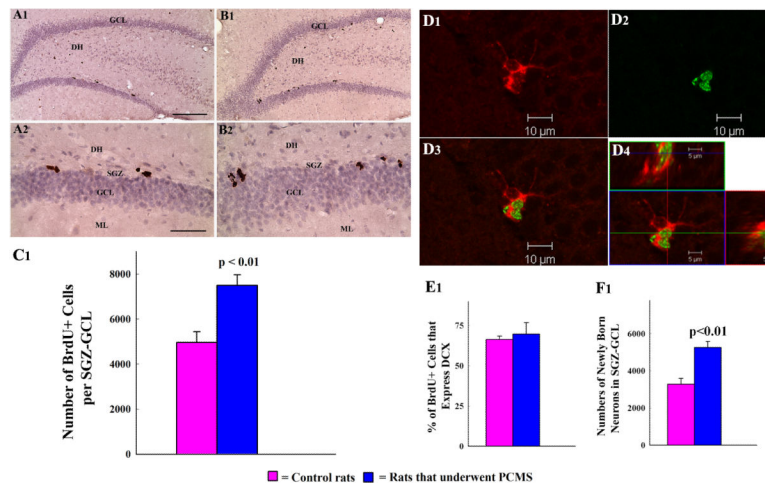
after the predictable chronic mild stress (PCMS) regimen. Note that, rats that underwent PCMS exhibit greater numbers of entries into the open arm and spend considerably greater time in the open arm, in comparison to the age-matched control rats that underwent handling alone at both early (C, D) and extended (E, F) time-points after PCMS. Reduced immobility time is an indication of reduced depressive-like behavior in PCMS treated rats at both time-points. The number of entries and time spent in open arms were used as indices of anxiety like behavior.

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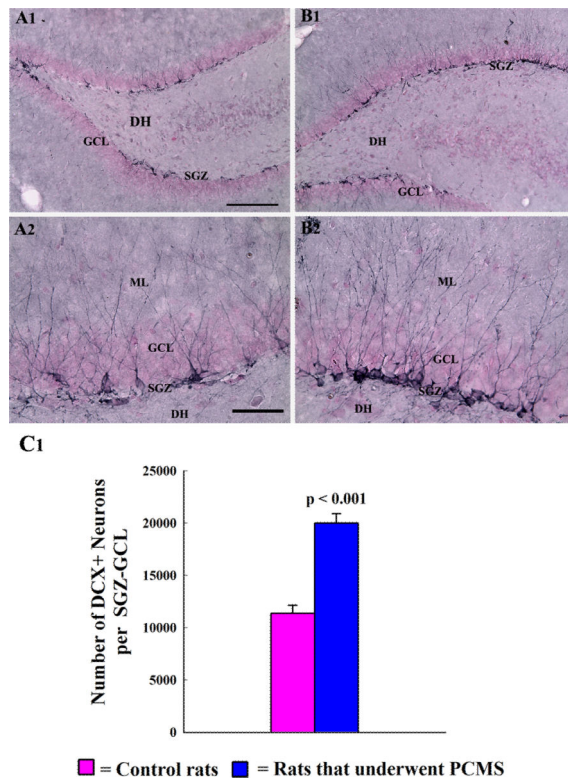
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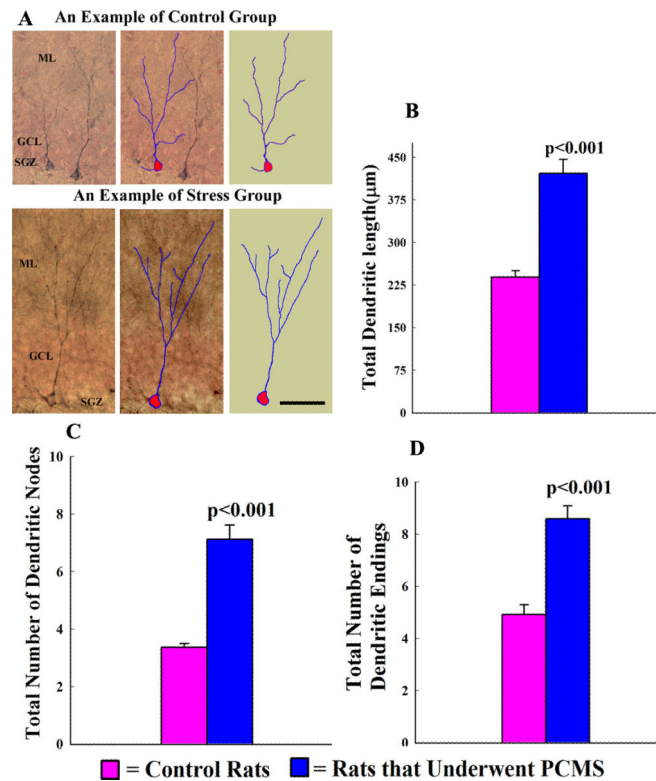
**Figure 2.**

Representative photomicrographs depicting the distribution of newly born cells (i.e. BrdU+ cells) in the subgranular zone-granule cell layer (SGZ-GCL) of hippocampi from an age-matched control rat (A1, A2) and a rat that underwent predictable chronic mild stress for 28 days (B1, B2). Scale bar, A1, B1 = 200 μm; A2, B2 = 50 μm; DH, dentate hilus. Figure C1 compares the total numbers of newly born cells generated per day in the SGZ-GCL between control rats (n=6) and rats that underwent PCMS (n=6). Values represent means and standard errors of the mean. Note that, PCMS group exhibits significantly greater numbers of newly born cells than the control group. Figures D1-D3 illustrate newly born cells (i.e. the BrdU+ cells shown in green color) expressing doublecortin (DCX, red color) in the subgranular zone-granule cell layer of a rat treated with PCMS, visualized through BrdU-DCX dual immunofluorescence and Z-section analyses using a laser confocal microscope. Figure D4 shows an orthogonal view of a newly born cell expressing BrdU and DCX. Figure E1 shows percentages of newly born cells that differentiate into DCX+ neurons in the control and PCMS treated groups. Note that, the neuronal fate choice decision is similar between the two groups. Figure F1 illustrates the net neurogenesis based on the total numbers of BrdU+ cells and percentages of BrdU+ cells expressing DCX. The net neurogenesis is much greater in the PCMS group, in comparison to the control group.



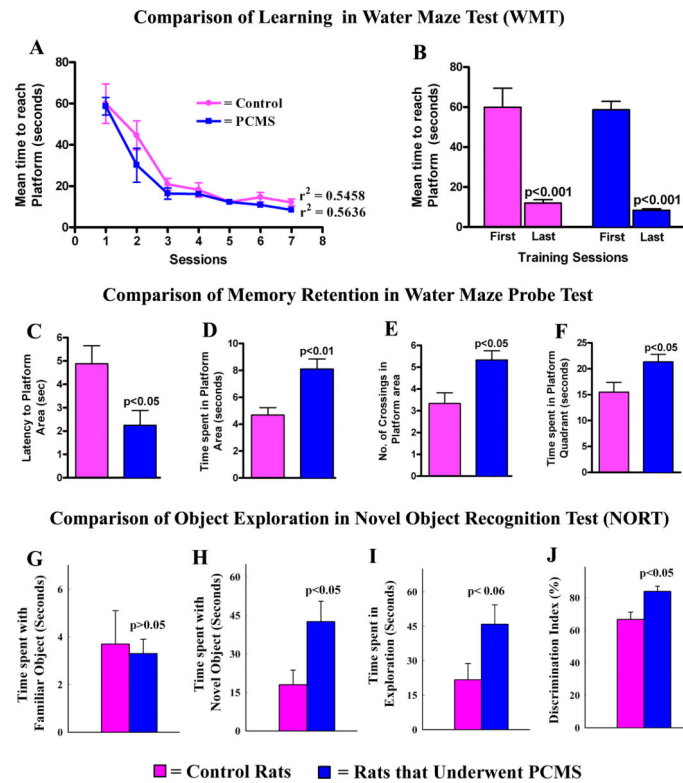
**Figure 3.** Distribution of newly born neurons expressing doublecortin (DCX) in the subgranular zone-granule cell layer (SGZ-GCL) of hippocampi from an age-matched control rat (A1, A2) and a rat that underwent predictable chronic mild stress for 28 days (B1, B2). Scale bar, A1, B1 = 200  $\mu$ m; A2, B2 = 50  $\mu$ m; SGZ-GCL to adult rats. DH, dentate hilus; ML, molecular layer. The bar chart in Figure C1 compares the total numbers of DCX+ neurons (depicting the overall status of neurogenesis) in the SGZ-GCL between control rats (n=6) and rats that underwent PCMS (n=6). Values represent means and standard errors of the mean. Note that, the PCMS group exhibits significantly greater numbers of newly born neurons than the control group.





**Figure 4.**

Figure A illustrates drawings of representative neurons from the control and PCMS groups using Neurolucida. Scale bar =  $50\mu\text{m}$ . ML, Molecular layer. The bar charts (B-D) compare the total dendritic length (B), numbers of dendritic nodes (C), and numbers of dendritic endings (D) of relatively mature DCX+ newly born neurons between control rats and rats treated with predictable chronic mild stress (PCMS). Note that, newly born neurons from the PCMS treated group exhibit considerably higher values for all parameters of the dendritic growth.

**Figure 5.**

Figures A-B illustrate data pertaining to learning in the water maze test (WMT) between control and PCMS treated groups. Note that, both groups exhibited excellent spatial learning ability, as evidenced by progressive decreases in the latency to reach the platform over 7 sessions and similar  $r^2$  values (A), and  $>80\%$  reduction in the latency to reach the platform between the first and last sessions of learning (B). Figures C-F compare the results of probe (memory retention) test performed at 24 hrs after the last learning session between control and PCMS treated groups. Note that all parameters of the memory retention (latency to reach the platform area, dwell time in platform area, platform area crossings and dwell time in the platform quadrant) in the PCMS treated group are superior to the control group. The bar charts G-I compare the control and PCMS treated groups for the time spent with: (i) the familiar object (G); (ii) the novel object (H); and (iii) both familiar and novel objects (i.e. total exploration time; I). The bar chart in J illustrates the discrimination index for the novel object in both groups. Note that, in rats treated with PCMS, both time spent with the novel object (H) and the discrimination index for the novel object (J) are significantly greater than in age-matched control rats, which are suggestive of a superior memory function in the PCMS treated rats.