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Inhibition of phosphodiesterase 2 reverses impaired cognition and neuronal remodeling caused by chronic stress

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Abstract

Chronic stress and neuronal vulnerability have recently been recognized as factors contributing to cognitive disorders. One way to modify neuronal vulnerability is through mediation of phosphodiesterase 2 (PDE2), an enzyme that exerts its action on cognitive processes via the control of intracellular second messengers, cGMP and, to a lesser extent, cAMP. This study explored the effects of a PDE2 inhibitor, Bay 60-7550, on stress-induced learning and memory dysfunction in terms of its ramification on behavioral, morphological and molecular changes. Bay 60-7550 reversed stress-induced cognitive impairment in the Morris water maze (MWM), novel object recognition and location tasks (ORT/OLT), effects prevented by treatment with 7-NI, a selective inhibitor of neuronal nitric oxide synthase (nNOS); MK801, a glutamate receptor (NMDAR) inhibitor; myr-AIP, a CaMKII inhibitor; and KT5823, a PKG inhibitor. Bay 60-7550 also ameliorated stress-induced structural remodeling in the CA1 of the hippocampus, leading to increases in dendritic branching, length, and spine density. However, the neuroplasticity initiated by Bay 60-7550 was not seen in the presence of 7-NI, MK801, myr-AIP or KT5823. PDE2 inhibition reduced stress-induced ERK activation and attenuated stress-induced decreases in

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transcription factors (e.g., Elk-1, TORC1, and pCREB) and plasticity-related proteins (e.g, Egr-1 and BDNF). Pre-treatment with inhibitors of NMDA, CaMKII, nNOS, PKG (or PKA), blocked the effects of Bay 60-7550 on cGMP or cAMP signaling. These findings indicate that the effect of PDE2 inhibition on stress-induced memory impairment is potentially mediated via modulation of neuroplasticity-related, NMDAR-CaMKII-cGMP/cAMP signaling.

Keywords

Stress; PDE2; Bay 60-7550; Cognition; Neuroplasticity

1. Introduction

The major factors involved in age-related neurodegenerative diseases, such as progressive decline in cognitive functions and the loss of synaptic contacts, remain poorly understood. There is significant evidence indicating that chronic stress and neuronal vulnerability are interrelated events contributing to age-related pathologies, such as Alzheimer's disease (Xu et al, 2009). In response to sustained stress, the brain undergoes a complex array of cellular and molecular changes that lead to maladaptive remodeling which presents itself in ways such as learning and memory impairment (McEwen BS, 2000; Mitra and Sapolsky, 2008). Presently, a plethora of different molecular "culprits" have been linked to architectural changes of neurons during chronic stress. NMDA receptor (NMDAR)-mediated Ca^{2+} influx is a major cellular mechanism for synaptic plasticity and learning and memory and may affect cyclic AMP (cAMP)/cyclic GMP (cGMP) formation through Ca^{2+}-calmodulin-dependent adenylyl cyclase (AC) and neuronal nitric oxide synthase (nNOS) related guanylyl cyclase (GC) (Huang et al, 1994; Chen et al, 2010). Cyclic AMP and cGMP are two likely candidates contributing to intracellular signaling transduction and gene transcription in the process of learning and memory (Burns et al, 1996; Suvarna and O'Donnell, 2002). The dysregulation of cAMP/protein kinase A (PKA) and cGMP/protein kinase G (PKG) signaling in states of prolonged stress reduces neuronal firing and rapidly affects cognitive function through disruption of neuroplasticity-associated proteins and their upstream transcriptional regulators (Birnbaum et al, 2004; Hains et al, 2009; Bodhinathan et al, 2010).

The main therapeutic option when regulating cAMP or cGMP is through intervention of phosphodiesterase (PDEs), which are a superfamily of enzymes that regulate cAMP and cGMP by hydrolysis. The discovery of phosphodiesterases inhibitors (PDEIs) brings hope for revealing the processes underlying stress-induced cognitive deficits and uncovering strategies for the treatment of age-related disorders, such as Alzheimer's disease. There are at least eleven distinct PDEs, and their inhibitors have been shown to be involved in intracellular signaling pathways associated with neurodegenerative disorders or their treatment (Xu et al, 2011). One of particular interest within the central nervous system is PDE2, primarily due to the high expression in the limbic nervous system and the adrenal cortex (Van Staveren et al, 2003), areas associated with cognitive functions and hypothalamus-pituitary-adrenal (HPA) axis regulation. Expression in these key areas helps promote the idea that PDE2 plays a role in the pathogenesis of stress-related disorders,

including learning and memory impairment (Reneerkens et al, 2009). However, it is not clear whether PDE2 inhibitors contribute to architectural changes in the hippocampus or how they affect the processes of learning and memory by regulation of series of molecular events when animals are subjected to unpredictable stress.

The present study was conducted to determine whether the PDE2 inhibitor Bay 60-7550 could ameliorate the structural remodeling of hippocampal neurons and related memory changes observed following chronic stress. The findings will help provide evidence that PDE2 inhibitors can modulate neuroplasticity and cognitive function by linking NMDAR-CaMKII-cGMP/cAMP signaling to downstream synaptic proteins expression.

2. Materials and Methods

2.1. Animals

Male ICR mice (Harlan, Indianapolis, IN) weighing between 22 and 25 g (3 months old) at the start of the experiment were obtained from the Animal Center of West Virginia University. Mice were housed five per cage under standard colony conditions, with a 12-h light/12-h dark cycle and access to food and water ad libitum. All experiments were carried out according to the ‘NIH Guide for the Care and Use of Laboratory Animals’ (NIH Publications No. 80-23, revised 1996). Experimental procedures were approved by the Animal Care and Use Committee of West Virginia University Health Sciences Center.

2.2. Drugs and treatments

Bay 60-7550 (2- [(3, 4- dimethoxyphenyl)methyl]- 7- [(1R)- 1- hydroxyethyl]- 4- phenylbutyl]- 5-methyl- imidazo[5, 1- f][1, 2, 4]triazin- 4(1H)- one) and KT5823 (2, 3, 9, 10, 11, 12- hexahydro- 10R- methoxy- 2, 9- dimethyl- 1- oxo- 9S, 12R- epoxy- 1H- diindolo[1, 2, 3- fg:3’, 2’, 1’- kl]pyrrolo[3, 4-i][1, 6]benzodiazocine- 10- carboxylic acid, methyl ester) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Myristoylated autocamtide-2-related inhibitory peptide (myr-AIP), MK801 and H89 were purchased from Calbiochem (San Diego, CA, USA). 7-Nitroindazole (7-NI) and Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) were purchased from Sigma Aldrich.

Bay 60-7550 was dissolved in 0.5% dimethyl sulfoxide (DMSO) and was administered via the intraperitoneal route (i.p.). myr-AIP, MK801, KT5823 and H89 were dissolved in artificial cerebrospinal fluid. Bay 60-7550 (1 and 3 mg/kg) or vehicle was given 30 min before stress procedures once per day for 14 days. MK801 (10μM), myr-AIP (20 μM), 7-NI (20 mg/kg), L-NAME (20 mg/kg), KT5823 (20 μM) and H89 (5 μM) were administered 30 min before treatment with Bay 60-7550. Animals were given bilateral microinjections of 2 μl MK801 and myr-AIP (1 μl/side) into the CA1 of the hippocampus. All the behavioral tests were performed 24 h after last drug treatment.

2.3. Surgery for brain cannula implantation

Animals were anesthetized intraperitoneally with a ketamine/xylazine mixture (100 mg/kg and 10 mg/kg, i.p., respectively) and placed in a stereotaxic frame (Stoelting Instruments, USA) with flat-skull position. Two holes are drilled on the skull based on the coordinates for

hippocampal CA1 (AP −1.7 mm from bregma, ML ±0.8 mm from midline, DV −2.0 mm from dura) (Franklin and Paxinos, 1997) before a guide cannula (30-gauge) was inserted in each hole and fixed in place. The cannula was anchored to the skull with dental cement, and then stainless steel stylets were inserted into the guide cannula to maintain patency prior to microinjections and to prevent occlusion. All surgery was performed under aseptic conditions. The mice were allowed to recover for 7–10 days.

2.4. Chronic unpredictable stress

Chronic unpredictable stress paradigm comprises exposure of mice to two different stressors twice daily for 14 consecutive days according to the procedure described earlier with minor modification (Willner et al, 1992; Ortiz et al, 1996; Perrotti et al, 2004). The order of stressors used was as follows: Day 1, shaker stress (high speed, 45 min), cold water swim (12 °C, 5 min); Day 2, restraint stress (1 h), tail pinch (1 min); Day 3, food/water deprivation (6 h), 6-h social isolation (the mice were placed individually in different cage in another housing room and were returned to their home cage 6 h later); Day 4, cold water swim (12 °C, 5 min), lights on overnight; Day 5, cage tilting (6 h), shaker stress (high speed, 1 h); Day 6, tail pinch (1 min), food/water deprivation (6 h); Day 7, cold room (4 °C, 15 min), 6-h social isolation; Day 8, shaker stress (high speed, 1h), restraint (1 h); Day 9, switching cages (6 h); lights on overnight; Day 10, Cage tilting (6 h), cold water swim (12 °C, 5 min); Day 11, 6-h social isolation, tail pinch (1 min); Day 12, Humid sawdust (6 h), food/water deprivation (6 h); Day 13, cold room (4 °C, 15 min), switching cages (6 h); Day 14, Lights on overnight, cage tilting (6 h). This protocol has been shown to cause significant effects on a number of cellular, biochemical, and neurochemical parameters characteristic of depressive/anxiogenic behaviors (Willner et al, 1992; Ortiz et al, 1996; Perrotti et al, 2004). Control groups were also handled everyday and kept in their home cages for the 14-day period. Three sets of mice were used for experiments including Morris water maze, novel object recognition and novel object location tests, each of which had ten groups of mice.

2.5. Morris water maze

The apparatus consisted of a circular, plastic pool (95 cm diameter ×25 cm high) located in a well-illuminated room with external cues visible from the inside of the pool, which was filled with opaque water (21±1°C). A hidden circular platform (8.5 cm diameter ×15.5 cm high) was submerged 1 cm under the water in one of four quadrants. The acquisition trials (training to escape to the hidden platform) were carried out for six blocks consisting of three (60 s) trials separated by 20 min inter-block intervals during which the platform remained in the same location relative to the distal cues in the room. On each trial, the mice were placed in the water at different start locations (E, S, W and N), which equally spaced from each other and were offset from the goal location by 45°. One hour following the sixth block, the hidden platform was removed and the mice were scored during a 60 s probe trial for latency to reach, and crossings over, the previous platform location (memory recall). Another probe trial was run 24 h after training to assess consolidation and retrieval of memory (Nicholas et al, 2006).

2.6. Object recognition test (ORT) and object location test (OLT)

The object recognition test was performed as described previously (De Lima et al, 2005; Li et al, 2011). All mice were given a habituation trial in which they could freely explore the apparatus for 5 min. And then the training session was conducted by placing an individual mouse in the center of the apparatus facing the wall. Mice were presented with a pair of two identical objects (Lego blocks) located in two diagonal corners and allowed to explore for 3 min (Sample session, T0). One hour (T1) or twenty-four hours (T2) later, mice were put back to the center of the same box except that one of the familiar objects was replaced with a novel one. The cumulative time spent exploring the familiar and novel objects was recorded manually using a computer during a 3 min period. For the object location test, instead of replacing a familiar object with novel object, one of the familiar objects was moved to a novel location (Ennaceur et al, 1997). The novel position of the object could be either 20 cm toward the front or 20 cm toward the back of the arena for both objects. Exploration was defined as actively touching or facing (within 2 cm toward) the object. Sitting on the object was not considered exploratory behavior. Objects were thoroughly cleaned with 70% ethanol after each individual trial to prevent a build-up of olfactory cues.

The values E0, E1 and E2 are measures of the total exploration time of both objects during T0, T1 and T2, respectively. E0 is the measure of the time spent exploring both identical objects (A1 and A2) in T0 (E0=A1+A2). E1 and E2 are the measures of the time spent exploring both the familiar (A) and relocated/novel object (Bl/Bo) in T1 (E1=A+Bl for OLT and E1=A+Bo for ORT) and T2 (E2=A+Bl for OLT and E2=A+Bo for ORT), respectively. The value discrimination index (DI) was considered as a relative measure of discrimination between the relocated, or novel, and the familiar objects (DI=B-A/E1 or DI=B-A/E2 for T1 and T2) (Rutten et al, 2009).

2.7. Rapid Golgi staining and quantitative analysis

Whole brains were quickly removed and processed according to the directions of a rapid Golgi staining kit (FD NeuroTechnologies, Ellicott city, MD). Serial sections were cut (100 μm) through the entire hippocampus (1.72mm to −6.72 mm from the bregma, Paxinos and Watson, 2005) on a freezing microtome. The staining procedure followed previously established methods that successfully stain hippocampal pyramidal cells (Titus et al, 2007; McLaughlin et al, 2007; Xu et al, 2009). For Golgi analysis, cells were chosen based on the following criteria: the cell body and dendrites were fully impregnated, the cell was relatively isolated from surrounding neurons, and the cell was located in the CA1 region of the hippocampus.

For morphological quantification of hippocampal neurons, 5 pyramidal neurons from each animal (10 mice/group, 50 neurons/group) were analyzed from area CA1 of the dorsal hippocampus (Vyas et al, 2002). A camera lucida drawing tube attached to an Olympus microscope BX51 (Olympus, Tokyo, Japan) was used to trace selected neurons for subsequent computerized image analysis. Using the center of the soma as reference point, branch points, and dendritic lengths were measured as a function of radial distance from the soma by adding up all values in each successive concentric segment (Xu et al, 2009; Shankaranarayana Rao et al, 2001; Vyas et al, 2002). The branch points and dendritic length

were quantified from 50 mm to 400 mm distances from the soma. To calculate the spine density, the exact length of the dendritic segment was calculated, and the number of spines along the length was counted (to yield spines/10 μm).

2.8. Immuno-blot analysis

Mice were decapitated and their brains were rapidly removed and stored at −70°C. The hippocampi were dissected out on a cold plate (−16 °C) (Franklin and Paxinos, 1997). The tissue samples were lysed with RIPA lysis buffer (Upstate Chemicon, Temecula, CA) containing protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL) and centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was assayed for total protein concentrations using BCA assay kit (Thermo Scientific, Rockford, IL). Samples were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and the separated proteins were transferred onto polyvinylidene difluoride membranes. Blots were then incubated in blocking buffer (phosphate buffered saline containing 0.1% sodium azide) for 2 h at room temperature, washed in tris-buffered saline with 0.1% Tween 20 (TBST), and incubated with the appropriate primary antibodies over night at 4 °C (anti-pERK1/2, 1:1000; anti-ERK1/2, 1:1000; anti-CREB, 1:1000; anti-p-CREB, 1:1000; anti-TORC1, 1:1000; anti-c-fos, 1:1000; anti-Egr-1, 1:1000; anti-pElk, 1:1000; anti-Elk, 1:1000; anti-BDNF, 1:1000; anti-Arc, 1:1000; and anti-β-actin, 1:1000). After washing, the blots were incubated with the secondary antibodies (IRDye 800CW Gt Anti-Mouse lgG (H+L) or IRDye 700CW Gt anti-Rabbit lgG (H+L), 1:10000) for 1h at room temperature. The detection quantification of specific bands was carried out using a fluorescence scanner (Odyssey Infrared Imaging System, LI-COR Biotechnology, South San Francisco, CA) at 700 nm and 800 nm wavelengths.

2.9. Statistical analysis

All data were presented as mean ± standard error of the means (SEM). The data from behavioral tests and western blot were subjected to Student's *t*-test or one-way ANOVA followed by a Dunnett's test to determine whether the means differed significantly from the vehicle control. A significance value of $p < 0.05$ was used for the statistical tests.

3. Results

3.1. Bay 60-7550 reverses chronic stress-induced impaired spatial learning and memory in Morris water maze

Although all mice reliably learned to locate the platform throughout six blocks of acquisition training, the groups significantly differed in their latency to reach the platform during the six training blocks. Post-hoc analyses showed that stressed mice took longer to reach the platform from block 2 to block 6 compared to non-stressed mice ($p < 0.01$; Fig. 1A). This impairment was not present in the stressed mice treated with Bay 60-7550 (3 mg/kg, i.p.); the latencies to reach the platform for Bay 60-7550-treated mice were significantly shorter than the latencies of the vehicle-treated stressed group starting from the second block ($p < 0.01$). Interestingly, the effects of Bay 60-7550 on acquisition were attenuated by pre-treatment with NMDA antagonist MK801 (10 μM, 30 min), specific CaMKII inhibitor myr-AIP (20 μM, 30 min), nNOS inhibitor 7-NI (20 mg/kg, 30 min; it inhibits both nNOS and

eNOS in higher dose), and PKG inhibitor KT5821 (20 μM, 30 min) ($p < 0.01$; Fig. 1B–D). Conversely, the eNOS inhibitor L-NAME at dose of 20 mg/kg did not reverse the amelioration conferred by Bay 60-7550 although a higher dose of L-NAME inhibits both eNOS and nNOS based on the previous study (Idigo et al, 2012). Similarly, PKA inhibitor H89 (5 μM, 30 min) did not completely reverse the effect of Bay 60-7550.

One hour after training, the platform was removed and mice were tested on a probe trial. Stressed mice exhibited significantly longer latencies to reach the platform position and fewer number of crossing over the platform position compared to non-stressed mice ($p < 0.001$ and $p < 0.01$). Bay 60-7550-treated mice (3 mg/kg) took significantly less time to reach the platform position, and made more crossings over the platform, than stressed mice [$F(2, 27) = 7.410$, $p < 0.01$; $F(2, 27) = 3.266$, $p < 0.05$, respectively] (Fig. 2A and 2B). These effects of Bay 60-7550 were blocked by pre-treatment with MK801, myr-AIP, 7-NI, and KT5821, whereas the eNOS inhibitor L-NAME and PKA inhibitor H89 only partially prevented the effects of Bay 60-7550 on short time memory (1 h) (Fig. 2A and 2B).

Memory retention for the platform location on the probe trial 24 h later was worse for stressed mice, as indicated by longer latencies ($p < 0.001$; Fig. 2C) and fewer platform crossings ($p < 0.01$; Fig. 2D). Bay 60-7550 (3 mg/kg, i.p.) ameliorated the detrimental effects of stress on platform latency and crossings [$F(2, 27) = 6.025$, $p < 0.01$; $F(2, 27) = 2.864$, $p < 0.05$, respectively] (Fig. 2C and 2D). Bay 60-7550's effects were partially prevented by pre-treatment with MK801, as evidenced by longer latencies to the platform position ($p < 0.05$) and a trend towards fewer platform crossing ($p > 0.05$). In contrast, myr-AIP, 7-NI, KT5823, L-NAME and H89 did not block the effects of Bay 60-7550 on long-term memory (24 h).

Swim speed in the 1 h or 24 h probe trials did not differ following chronic stress or drug treatment (data not shown), suggesting that observed differences in performance were not the result of differences in overall activity levels. Notably, MK801, myr-AIP, L-NAME, 7-NI, KT5823 and H89 by themselves did not cause any significant effects on learning and memory, indicating that inhibition of these receptors and kinases specifically reversed the amelioration of Bay 60-7550's effects on the processes of cognition (Supplementary figure 1 and Supplementary figure 2).

3.2. Bay 60-7550 reversed impaired episodic-like memory tasks in the object recognition test (ORT) and the object location test (OLT)

During the sample session (T0), all groups exhibited a comparable amount of time exploring the two identical objects during the familiarization phases, indicating that these objects elicited similar interest in the animals (data not shown). As shown in Fig. 3A and 3B, chronic stress induced significant memory impairment, as evidenced by a lower discrimination index (DI) during T1 (1h) and T2 (24h) when compared to vehicle-treated control mice (ps <0.01) in ORT. One-way ANOVA analyses revealed Bay 60-7550 ameliorated the stress-induced decrease in DI at both 1h and 24h [$F(2, 27) = 9.39$, $p < 0.05$ and $p < 0.01$; $F(2, 27) = 8.095$, $p < 0.05$ and $p < 0.01$, respectively]. MK801, myr-AIP and KT5823 blocked the effect of Bay 60-7550 at 1h [$F(5, 54) = 9.197$, $p < 0.001$; $p < 0.01$ and $p < 0.05$, respectively] and 24h [$F(5, 54) = 10.68$, $p < 0.001$; $p < 0.01$ and $p < 0.05$, respectively],

whereas 7-NI and H89 blocked the effect of Bay 60-7550 on 24 h memory [F (5, 54)=10.68, p <0.01 and p <0.05, respectively], but not 1 h memory.

In the OLT, total time of exploration towards the two identical objects did not differ significantly among any of the groups (data not shown). During both the 1h and 24h test sessions, the stressed mice exhibited an impaired preference for the novel location over the familiar location, as shown by a significant decrease in DI (ps <0.01) (Fig. 3C and 3D). Bay 60-7550 (3 mg/kg) administration significantly increased exploration of the object in the novel location compared with vehicle-treated stress group 1h [F (2, 27) =8.06, p < 0.01] and 24h after training [F (2, 27) =7.68, p < 0.01]. MK801, myr-AIP, 7-NI and KT5823 blocked the effect of Bay 60-7550 for 1h memory (p < 0.05; p <0.01; p <0.01 and p <0.01). myr-AIP and 7-NI still blocked the effect of Bay 60-7550 on 24 h memory, while MK801 and KT5823 did not prevent Bay 60-7550's effect for long-term memory (24 h) (p >0.05). L-NAME and H89 partially inhibited the effect of Bay 60-7550 1h and 24h after training, but the differences were not significant.

Similar to the MWM test, MK801, myr-AIP, L-NAME, 7-NI, KT5823 and H89 by themselves did not have any significant effect on cognition in either the ORT or OLT (Supplementary figure 3).

3.3 Bay 60-7550 reversed hippocampal dendritic hypotrophy under conditions of chronic stress

The morphological analysis is based on a total of 500 Golgi-impregnated neurons from 100 animals. The examples of hippocampal CA1 pyramidal neurons from control animals at different magnifications are shown in Fig. 4A–C, and representative neurons from each experimental group can be found in Fig. 4D–M.

To determine changes in morphology of CA1 neurons, the number of branch points and dendritic length were evaluated by Sholl analysis. The analysis showed that the number of dendritic apical branching points from chronically stressed mice decreased significantly as compared to neurons in the control, non-stressed group (Fig. 5). This can be found comparing the segments from 150 μm (p < 0.001), 200 μm (p < 0.001) and 250 μm (p < 0.001). The apical dendritic length in the stressed vehicle group was also significantly shorter at 200 μm (p < 0.001) and 250 μm (p < 0.001) as compared to the non-stressed control group (Fig. 6). The atrophy of the basal dendrites, again comparing the non-stressed control with stressed group, was as pronounced as that observed in the apical dendrites; the basal branch points were significantly decreased at 100 μm (p < 0.001) and 150 μm (p < 0.001) in the chronically stressed mice (Fig. 7). The basal dendritic lengths were decreased by 37%, 24% and 34% at the segments measured at 100 μm (p < 0.001), 150 μm (p <0.001), and 200 μm (p < 0.001), respectively, in the stressed versus non-stressed group (Fig. 8).

The morphological changes within the CA1 neurons seen during chronic unpredictable stress were not apparent in mice treated with Bay 60-7550 (3 mg/kg; Fig. 5A). The number of apical dendritic branch points was increased at the dose of 3 mg/kg Bay 60-7550 at a radial distance of 150 μm [F (2, 17)=3.768, p < 0.05], 200 μm [F (2, 17)=6.958, p < 0.01] and 250 μm [F (2, 17)=22.99, p < 0.001] from the soma. However, MK801, myr-AIP, 7-NI,

and KT5823 blocked the effect of Bay 60-7550 (3mg/kg) on the number of apical dendritic branch points at the radial distance of 150 μm ($p < 0.001$), 200 μm (ps < 0.001), and 250 μm ($p < 0.001$) from the soma (Fig. 5B, 5C and 5D). The length of the apical dendrites increased from 20% to 40% at segments 200 and 250 μm with the higher dose of Bay 60-7550 (3 mg/kg) (Fig. 6A). Similar to what were observed for apical branch points, MK801, myr-AIP, 7-NI and KT5823 also blocked the effects of Bay 60-7550 on apical dendritic length ($p < 0.001$; Fig. 6B, 6C and 6D)

Bay 60-7550 at 3 mg/kg also increased the number of basal branch points at the radial distance of 100 μm [$F (2, 17)=8.539$, $p <0.01$] and 150 μm [$F (2, 17)=5.9$, $p < 0.05$] (Fig. 7A). MK801, myr-AIP, 7-NI and KT5823 blocked the effects of Bay 60-7550 on the number of the basal branch points at the radial distance of 100 μm ($p <0.001$) and 150 μm ($p <0.001$). L-NAME and H89, however, only partially blocked the effects of Bay 60-7550 (Fig. 7B, 7C and 7D). In addition, the basal dendritic length was increased at segments 100, 150 and 200 μm when Bay 60-7550 was administered at 3mg/kg (Fig. 8A) [$F (2, 17)=11.24$, $p < 0.01$ at 100 μm; $F (2, 17)=9.19$, $p < 0.01$ at 150 μm; $F (2, 17)=5.895$, $p < 0.01$ at 200 μm]. MK801, myr-AIP, 7-NI and KT5823 reversed Bay 60-7550's effects from segments 100, 150 to 200 μm ($p < 0.001$), but H89 only blocked the effects of Bay 60-7550 at 200 μm from the soma. Overall, this data shows that with increasing doses of Bay 60-7550 there are more dendritic branching points and increased dendritic length under conditions of chronic unpredictable stress. However, these effects were reversed by pre-treatment with different inhibitors, such as MK801, myr-AIP, 7-NI, KT5823 or H89.

Another interesting finding in this study was that atrophy of the dendritic spine density was apparent after chronic stress ($p <0.01$) (Fig. 9A). Increased spine density was observed after treatment with high dose of Bay 60-7550 (3 mg/kg) [$F (2, 17)=8.45$, $p <0.01$]. The synaptogenesis initiated by Bay 60-7550 was not seen in the presence of MK801, myr-AIP, 7-NI, KT5823 ($p <0.01$; $p <0.05$; $p <0.05$ and $p <0.01$, respectively). L-NAME and H89 partially attenuated the effect of Bay 60-7550, but the differences were not significant. Similar to the behavioral data, MK801, myr-AIP, L-NAME, 7-NI, KT5823 and H89 by themselves did not affect neuronal morphology (Supplementary figure 4 and Supplementary figure 5).

3.4 Immuno-blot results

3.4.1 Bay 60-7550 activated neuronal ERK1/2 signaling—Extracellular regulated protein kinases (ERKs) are widely expressed intracellular signaling molecules that are involved in functions including the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells. Analysis of Immuno-blot data showed that ERK1/2 phosphorylation (pERK1/2) was increased, i.e. the ratio of pERK1/2/ERK1/2 was significantly increased, after chronic stress (Fig. 9B, $p <0.01$). This increase of pERK1/2/ERK1/2 was reversed by treatment with Bay 60-7550 [$F (2, 27) =5.331$, $p <0.01$]. However, MK801, myr-AIP, 7-NI, and KT5823 blocked Bay 60-7550's effects on ERK phosphorylation [$F (5, 54)=5.764$, $p <0.01$; $p <0.05$; $p <0.05$ and $p <0.01$, respectively], suggesting an involvement of NMDA receptor, CaMKII and PKG in the PDE2 related ERK activation. The eNOS inhibitor L-NAME and PKA inhibitor H89 did not induce statistical differences on ERK

phosphorylation. These inhibitors themselves did not affect the ERK1/2 phosphorylation (Supplementary figure 6A).

3.4.2 Bay 60-7550 contributed to nuclear accumulation of the CREB phosphorylation (pCREB), co-activator TORC1 and Elk-1 phosphorylation (pElk-1)—ERK are known to activate many transcriptional proteins, such as CREB, TORC1 and Elk-1. This study investigated whether PDE2 is involved in phosphorylation of CREB at Ser-133, a key event in ERK relevant transcription (Ortega et al, 2011). The amount of pCREB between the stressed group and control (non-stressed) group was found to be significantly different, i.e. chronic stress decreased pCREB. However, this decrease in the pCREB was reversed following treatment with Bay 60-7550 (3 mg/kg) [F (2, 27)=16.56, p <0.001]. MK801, myr-AIP, 7-NI, KT5823, and H89 blocked the effect of Bay 60-7550 on pCREB [F (5, 54)=8.200, p <0.001; p <0.01; p <0.05; p <0.01 and p <0.01, respectively]. L-NAME partially prevented Bay 60-7550's effect on pCREB, but the effect did not reach statistical significance (Fig. 10A).

TORC translocation into the nucleus is an essential step in CREB-mediated transcription. The present results showed that TORC was decreased in chronically stressed mice (p <0.01). The stress-induced down-regulation of TORC proteins was reversed by pretreatment with 3mg/kg Bay 60-7550 [F (2, 27)=18.12, p <0.001]. As with pCREB, the effect of Bay 60-7550 on TORC was blocked by MK801, myr-AIP, 7-NI, KT5823, and H89 [F (5, 54)=9.534, p <0.001; p <0.01; p <0.01; p <0.001 and p <0.001) but not L-NAME (p > 0.05) (Fig. 10B).

To investigate the existence of a link between phosphorylation of Elk-1 (pElk-1) and the ERK signaling, we examined phosphorylation of Elk-1 on Ser-383, the major residue enabling ternary complex formation and transactivation (Gille et al, 1995) (Fig. 10C). Chronic stress induced a decrease in the ratio of pElk-1/Elk-1 (p <0.01), and pretreatment with PDE2 inhibitor Bay 60-7550 (3 mg/kg) reversed this effect [F (2, 27)=18.14, p <0.001]. Bay 60-7550's effect was prevented by MK801, myr-AIP, 7-NI, KT5823, and H89 [F (5, 54)=12.49, p <0.01; p <0.01; p <0.001; p <0.001 and p <0.05, respectively], but not L-NAME (p > 0.05). These treatments alone did not induce significant changes in CREB, TORC1 or Elk-1 under conditions of chronic stress (Supplementary figure 6).

3.4.3 Bay 60-7550 activated Egr-1 and the brain derived neurotrophic factor (BDNF) expression—The present study investigated hippocampal immediate early genes (IEG) expression, i.e. c-fos, Egr-1 and Arc, as indirect measures of neural activation and plasticity in the brain after treatment with Bay 60-7550 during chronic stress (Fig. 11A, B and C). Neither chronic stress nor Bay 60-7550 administration altered c-fos expression (Fig. 11A). Chronic stress significantly decreased Egr-1 expression, an effect reversed by treatment with 3 mg/kg of Bay 60-7550 [F (2, 27)=9.930, p <0.01; Fig. 11B). MK801, myr-AIP, 7-NI, and KT5823 inhibited Bay 60-7550's effect on Egr-1 [F (5, 54)=5.235, p<0.01; p <0.01; p <0.01 and p <0.01]. Though chronic stress decreased Arc expression, neither 1 mg/kg nor 3 mg/kg of Bay 60-7550 attenuated this effect (Fig. 11C).

Given that the primary role of BDNF is to promote the functional architecture of neurons in the brain (Cho et al, 2013), the present study investigated BDNF expression in the hippocampus in the presence of Bay 60-7550 after chronic stress. As shown in Fig. 11D, BDNF levels were decreased in the hippocampus of stressed mice when compared to the non-stressed controls (p <0.01), but down-regulated BDNF levels were significantly increased following pretreatment with Bay 60-7550 [F (2, 27) =17.6, p <0.01]. Moreover, MK801, myr-AIP, 7-NI, KT5823 and H89, but not L-NAME (p > 0.05), reversed Bay 60-7550's effect [F (5, 54)=11,21, p <0.01; p <0.01; p <0.01; p <0.01 and p <0.05]. These treatments alone did not induce any significant effects on immediate early genes or BDNF expression (Supplementary figure 7).

4. Discussion

Stressful events are positively correlated to aging and age-related disorders by behavioral, neuroendocrinological and clinical studies, suggesting that some of the neurobiological abnormalities found in stressed animals parallel those found in Alzheimer's disease (AD) patients (Chen et al, 2010; Palumbo et al, 2007). The present study found that chronic stress impairs hippocampus-dependent cognitive function and neuroplasticity in mice. The subsequent studies support the ideology that chronic stress affects NMDAR-dependent nNOS and CaMKII production at hippocampal synapses, thereby resulting in enhanced neuronal PDE2 activity. This enhanced PDE2 activity accelerates the metabolism of cAMP/cGMP to adenosine and guanosine, resulting in overall impairment of learning and memory processes. Selective PDE2 inhibitor Bay 60-7550 ameliorated cognitive deficits and dendritic structural alterations in the hippocampus by enhancing cAMP/PKA and/or cGMP/PKG formation, leading to downstream increases in transcriptional factors and synaptic-related protein expression. This is consistent with previous studies suggesting PDE enzymes are targeted to particular cellular processes, which make them valuable as potential targets for treatment of age-related cognitive disorders (Suvarna and O'Donnell, 2002; Rutten et al, 2009; Sierksma et al, 2013), although the underlying mechanism of action remains to be elucidated.

PDEs are the only known enzymes that hydrolyze cAMP and cGMP, and thus, are integral to the regulation of cyclic nucleotide signaling and cellular communication during chronic stress (Kleppisch, 2009). Among eleven PDE inhibitors, at least seven of them, such as inhibitors of PDE 1, 2, 4, 5, 9, 10, and 11 may be implicated in behavioral changes related to emotional and cognitive disorders (Blokland et al, 2006; Xu et al, 2011). However, current PDE4 and PDE5 inhibitors are not candidates for the treatment of memory disorders because of their severe side effects, such as emesis for PDE4 and visual impairments for PDE5, at the prolonged high doses necessary for therapeutic benefit (Robichaud et al., 2002; Azzouni and Samra, 2011). The widespread presence of PDE2 in the HPA axis and adrenal cortex makes increasing the levels of cAMP and cGMP with a single, lower dose more plausible. This lower dose, single candidate approach might influence stress-related events, such as depression and cognitive disorders, all while avoiding the issues present with both cAMP and cGMP inhibitors usage. Our previous studies provided a link between PDEs and cognition (Li et al, 2011; Zhang et al, 2005). Other studies also reported that PDE2 inhibition reverses pharmacologically induced memory deficits (van Donkelaa et al, 2008;

Reneerkens et al, 2013), but there has been lack of evidence for the role of PDE2 in memory processes during chronic stress. In the first set of experiments in this study, mice were subjected to chronic unpredictable stress, which attempts to mimic the psychosocial stress that humans encounter in daily life, and increases PDE2 expression predominantly (Xu et al, 2013). The stress paradigm results in MWM deficits, as mice exposed to stress have longer latencies to reach the platform during the training blocks, indicating the impaired acquisition. In probe trials, the latencies to reach the previous platform location, and the number of crossings over the previous platform were longer than those of control group, suggesting poorer memory recall and retrieval. However, the stressed mice that received PDE2 inhibitor Bay 60-7550 learned faster in the training sessions, and Bay treatment restored memory performance to that of non-stressed controls during the probe trials. Interestingly, pre-treatment with NMDA inhibitor MK801 prevented the effect of Bay 60-7550 both on the short-term (1 h) and long-term memory (24 h). On the other hand, the nNOS inhibitor 7-NI, CaMKII specific inhibitor myr-AIP and PKG inhibitor KT5823 blocked the effects of Bay 60-7550 only on short-term learning and memory processes (Fig. 2). The eNOS inhibitor L-NAME and PKA inhibitor H89 only showed a nonsignificant trend towards prevention of Bay 60-7550's effect on the memory. These results support the notion of critical roles of the NMDAR-NO pathway on Bay 60-7550 induced cGMP formation in short-term memory processes during chronic stress (Rodefer et al, 2012). Indeed, PDE2 may metabolize both cyclic nucleotides in a tissue-dependent manner; it has slightly higher affinity for cGMP than cAMP in the hippocampus (Garcia-Osta et al, 2012). The fact that myr-AIP and 7-NI only reversed Bay 60-7550's effect on short-term memory suggests that CaMKII-nNOS signaling in the hippocampus mainly works by modulating cGMP levels (Deshmukh et al, 2009).

Since the first sign of cognitive decline in patients suffering from age-related degenerative disorders, such as AD, is episodic memory deficits (Dere et al, 2005), spatial memory was next confirmed using object recognition (ORT) and object location tests (OLT), both of which may represent different aspects of episodic-like memory, that is, the aspect of "what" and "where", respectively (Clayton et al, 2001). Usually unstressed mice spend more time exploring the novel (what) and relocated (where) objects in the ORT and OLT, respectively, which reflect an implicit form of episodic memory (Morris, 2001). In the present study, stressed mice showed impairment of short-term (1h) and long-term (24h) memory consolidation and retrieval processes in both of ORT and OLT. Bay 60-7550 exerted memory-enhancing effects; stressed mice treated with Bay 60-7550 spent more time exploring the replaced novel objects and relocated familiar objects 1h and 24h after treatment. These memory enhancements may involve PDE2 inhibitor improving NMDA- and CaMKII-related neuronal NO production and cGMP or cAMP formation, as evidenced by MK801, myr-AIP, 7-NI preventing the effects of Bay 60-7550 on memory consolidation in both ORT and OLT. Additionally pre-treatment with KT5823, a PKG inhibitor, differentially modulated memory performance induced by Bay 60-7550 at both of 1h and 24h memory retention in ORT; it prevented the amelioration of Bay 60-7550 on the short term (early phase) memory processes but its effect on long term memory was weak in OLT. Considering that early-phase cGMP/PKG signaling requires late-phase cAMP/PKA signaling in memory formation (Bollen et al., 2014). It is plausible that both of cGMP and

cAMP signaling are involved in the cognitive enhancement of Bay 60-7550 in the ORT and OLT, although the magnitude of this effect on cAMP was markedly less than cGMP. The nNOS inhibitor 7-NI blocked the improvement on ORT and OLT induced by Bay 60-7550; while the eNOS inhibitor L-NAME only partially reduced the effect of Bay 60-7550. To our knowledge, there is presently no published data concerning the antagonistic action of KT5823 and H89 on Bay 60-7550-induced memory enhancing for locations in which objects were explored. This is the first time to determine the specific contributions of KT5823 and H89 to, and interactions with, episodic-like memory encoding, consolidation, and retrieval in the presence of Bay 60-7550 in stressed mice.

The behavioral changes after treatment with Bay 60-7550 are in line with the sequence of morphological and molecular changes taking place in the hippocampus. It's well known that chronic stress adversely affects hippocampal neuronal metabolism and cell survival, as well as dendritic morphology and basic functioning (McEwen et al, 2000; Nicholas et al, 2006). The CA1 neuronal response to stress is less well characterized compared to that of CA3, but these neurons are sensitive to stress (Gourley et al, 2013). Restoration of neuronal plasticity, reflected by an increased dendritic branches and neuronal sprouting in the CA1, were found in the presence of Bay 60-7550. However, these effects were blocked by pre-treatment with MK801, myr-AIP, 7-NI, and KT5821, demonstrating a role of NMDAR-CaMKII-nNOS-cGMP pathway on dendritic remodeling during stress. Pre-treatment with H89 also partly prevented these Bay 60-7550's effects on changes of neuronal structure in the CA1, indicating the involvement of cAMP/PKA in the effect of Bay 60-7550. It seems that stimulation of cGMP or cAMP by Bay 60-7550 is closely associated with the restructuring on dendritic complexity and spine density of hippocampal neurons, thereby stimulating excitatory working memory networks (Frank et al, 2004).

Considering that persistent changes in neuronal function and structure require multiple signaling cascades that convey synaptic information to the cell nucleus for new gene expression (Flavell and Greenberg, 2008; Gallo and Iadecola, 2011), we investigated the extracellular signal-regulated kinase 1 and 2 (ERK1/2) cascade that is a major link between synaptic stimuli and new protein synthesis (Thomas and Huganir, 2004). An increase of ERK1/2 phosphorylation (pERK1/2) was found after chronic stress, but it was reversed by treatment with Bay 60-7550. Phospho-ERK1/2 may mediate cellular stress by interacting with nuclear targets involved in neuronal survival and plasticity, which includes cAMP responsive element-binding protein (CREB) and other transcription factors, such as TORC1 and Elk-1 (Finkbeiner et al, 1997; Martinez-Finley et al, 2011; Kleppisch and Feil, 2009). Some transcription factors drive the expression of key plasticity-related genes, such as c-fos and Egr-1 (Flavell and Greenberg, 2008). The results presented in this study show that Bay 60-7550 significantly reversed the decreased pElk-1, pCREB and its coactivator, TORC1, levels induced by stress. However, these effects were blocked by pre-treatment with MK801, myr-AIP, 7-NI, KT5823, and H89. H89 not only blocked Bay 60-7550's effect on these three transcription factors, it also blocked Bay 60-7550's effects on downstream brain-derived neurotrophic factor (BDNF) expression. These findings, however, are inconsistent with the memory performance that suggested H89 only partially blocked the memory-enhancing potential of Bay 60-7550, possibly because behavioral changes are not

significantly sensitive to small changes in these transcription factors and BDNF expression in the brain.

Stress hormones may affect neuronal sprouting and growth through its deleterious effects on the function and expression of plasticity-related proteins and neurotrophins, such as c-fos, Egr-1, Arc and BDNF (Flavell and Greenberg, 2008; Reddy et al, 2005). Exposure to stress is detrimental in that it serves to decrease BDNF expression in the hippocampus. However, not all of the plasticity-related proteins have identical responses to all stress stimuli. For example, Egr-1 in the hippocampus appears to be more responsive to memory processes during stress, whereas c-fos and Arc are more responsive in the hypothalamus to acute stress (Girotti et al, 2007; Wisden et al, 1990). The present study found that chronic stress reduced Egr-1, Arc and BDNF expression, and Bay 60-7550 only reversed stress-induced decreases in Egr-1 and BDNF expression. These effects were blocked by pre-treatment with MK801, myr-AIP, 7-NI, KT5823, and H89, suggesting Bay 60-755 is acting mainly through its action on NMDAR-CaMKII-nNOS-cGMP or cAMP to protect against stress-induced plasticity-related proteins and BDNF abnormalities. Furthermore, it is suggested that a postsynaptic CaMKII-NOS-cGMP/PKG-CREB and/or CaMKII-cAMP/PKA-CREB pathway, as well as presynaptic cGMP-PKG signaling are implicated in the cognitive performance (Impey et al., 1996; Lu et al., 1999; Reneerkens et al., 2009; Lee et al., 2011). Considering that the few existing studies investigating the localization of PDE2, the fact that PDE2 inhibitor Bay 60-7550 influences the levels of the second messengers cGMP and cAMP (with preference to cGMP) via CaMKII-nNOS-cGMP/PKG and/or CaMKII-cAMP/PKA dependent signaling indicates the effects of PDE2 inhibitor may be involved in both pre- and post-synaptic mechanisms.

In conclusion, Bay 60-7550 is able to reverse chronic stress-induced impairment of cognitive performance and structural architecture of hippocampal neurons through modulation of the NMDA receptor-CaMKII-cGMP/cAMP pathway, which are in line with the observations that Bay 60-7550 regulates the ERK signaling, transcription factors, and plasticity-related proteins (Fig. 12). The increases in cGMP and/or cAMP levels seem to be tightly regulated by PDE2 activity and it may be the main reason for the neuroprotective properties of Bay 60-7550 against chronic stress. The fact that inhibition of NMDA, CaMKII, nNOS, PKG or PKA by different antagonists prevents the effects of Bay 60-7550 confirms the crucial roles of this pathway in regulation of Bay 60-7550's effects on behaviors, neuronal remodeling and plasticity-related protein expression during chronic stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The authors declare that our manuscript is based on original research and has not been previously published or submitted elsewhere. The experimental procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the West Virginia University Institutional Animal Care and Use of Laboratory Animals (IACUC) guidelines.

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Highlights

1. Inhibition of PDE2 reverses stress-induced cognitive disorders.
2. Inhibition of PDE2 ameliorates stress-induced neural remodeling.
3. The underlying mechanism involves in NMDAR-CaMKII-nNOS-cAMP/cGMP signaling.

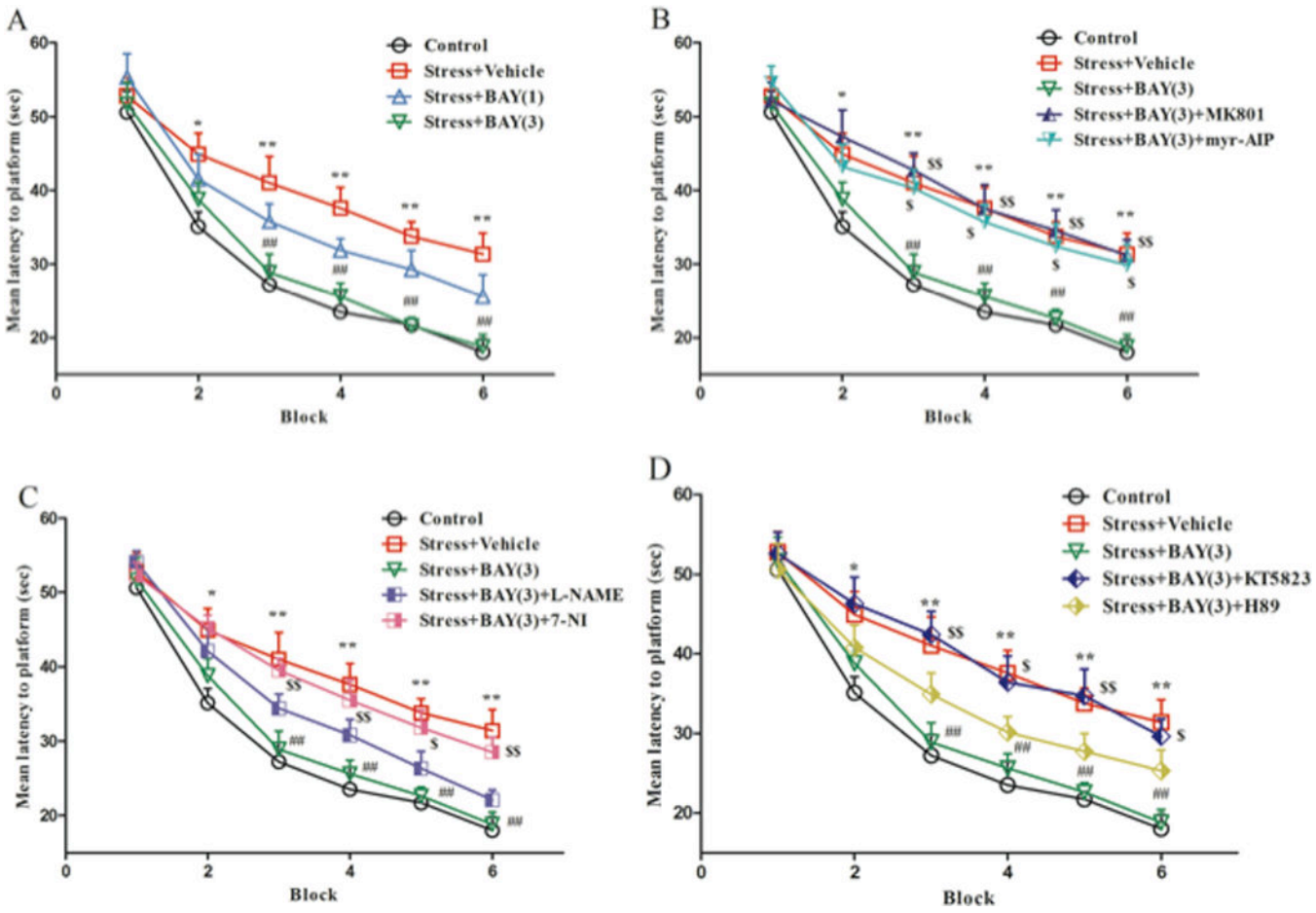

Fig. 1.

Learning curve in Morris water maze of control mice and stressed mice treated with vehicle, Bay 60-7550, MK801, myr-AIP, L-NAME, 7-NI, KT5823 and H89. Each point shows the average time taken for 10 mice. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. non-stressed control group. $^{\#\#}p < 0.01$, vs. vehicle-treated stressed group. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. BAY (3) (3 mg/kg Bay 60-7550) treated-group (n=10).

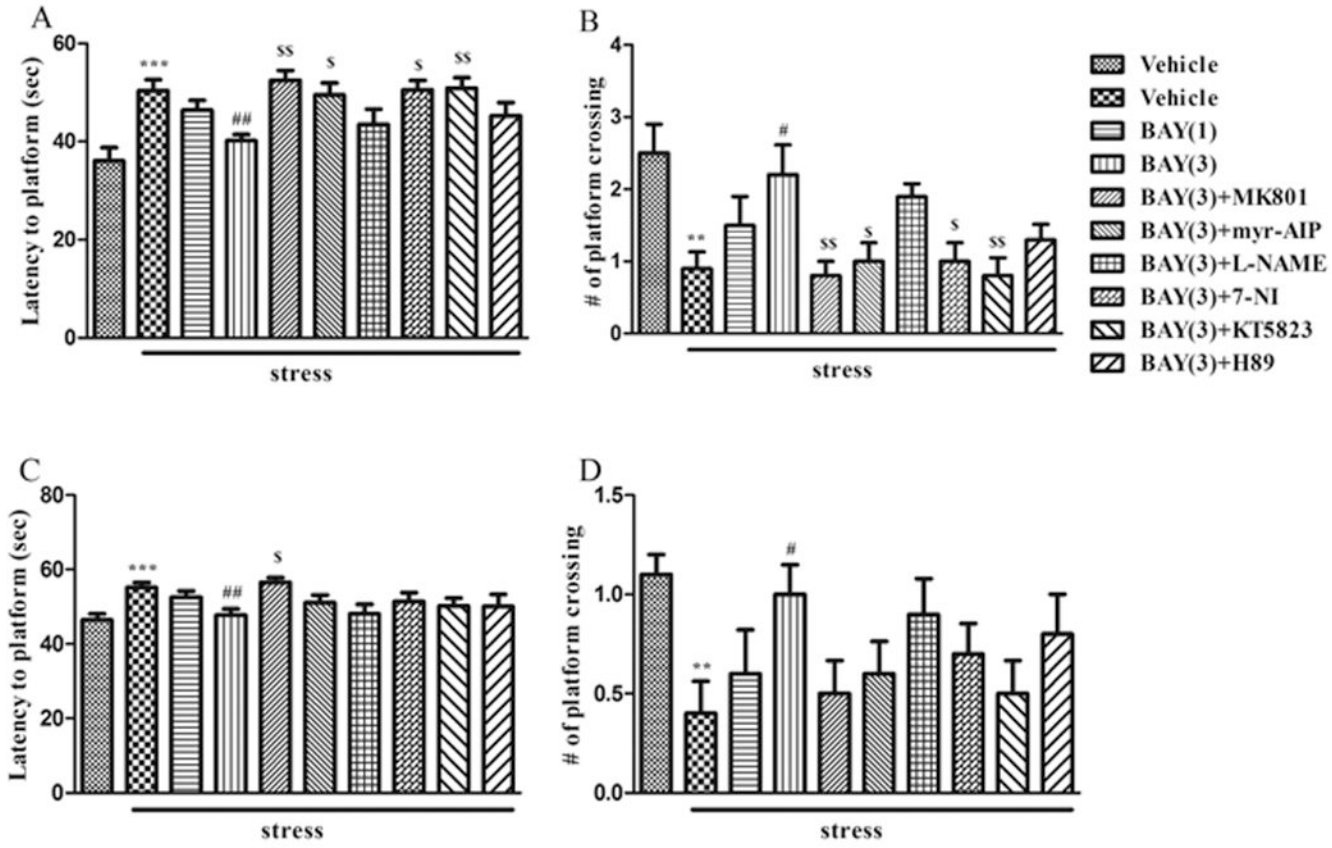

Fig. 2.

Chronic treatment with Bay 60-7550 (14 days) improves learning and memory behaviors on the Morris water maze task during the 1 h (A, B) or 24 h (C, D) test trials. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. non-stressed control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. vehicle-treated stressed group. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. BAY(3) treated-group (n=10).

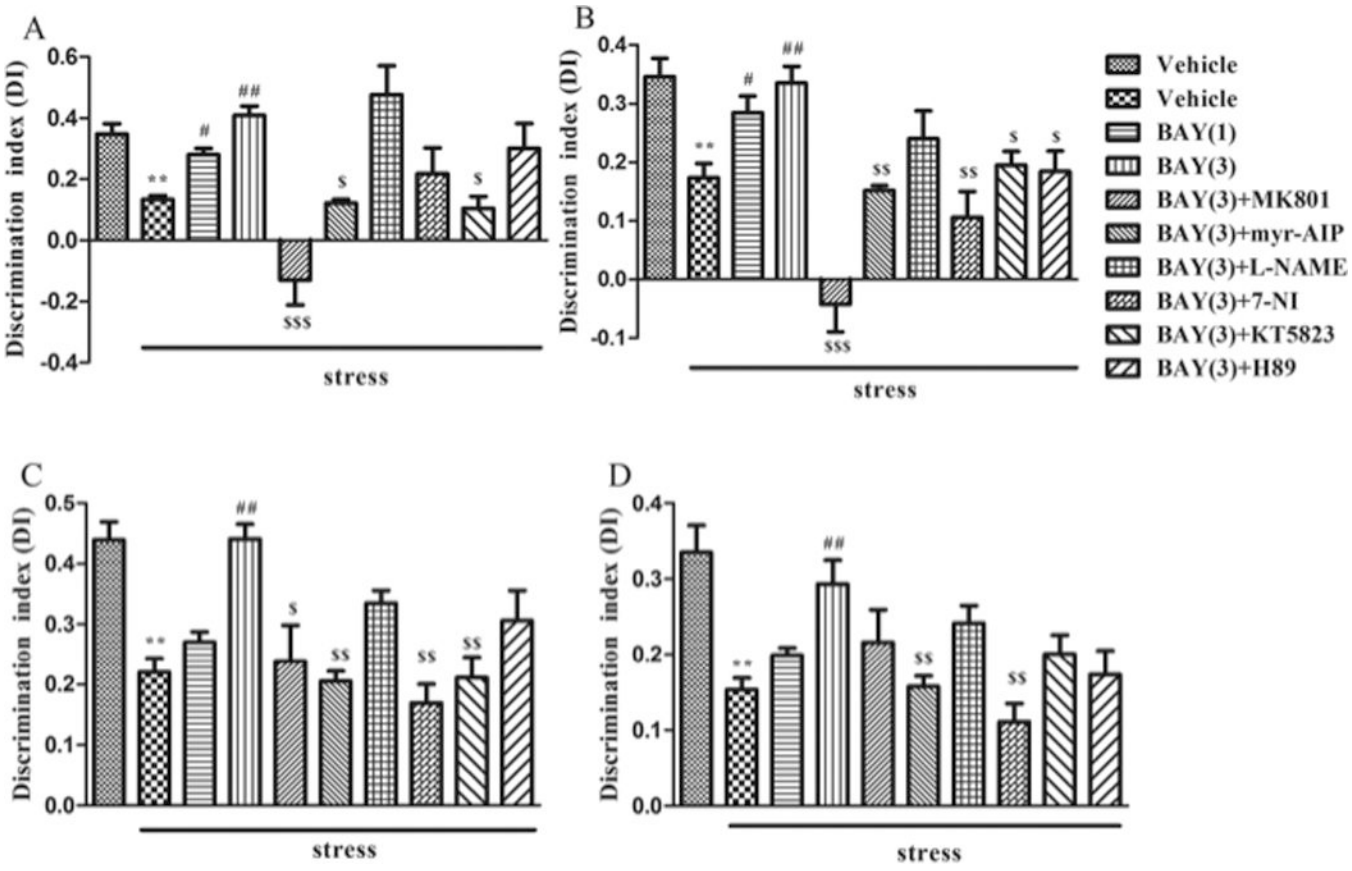

Fig. 3.

The effects of Bay 60-7550 on ORT (A, B) and OLT (C, D) memory performance between T1 and T2. Mean value of the discrimination index (DI) in the task with 1h (A, C) and 24h (B, D) retention delays. $^{**}p < 0.01$ vs. non-stressed control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. vehicle-treated stressed group. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ $^{\$\$\$}p < 0.001$ vs. BAY (3)-treated group (n=10).

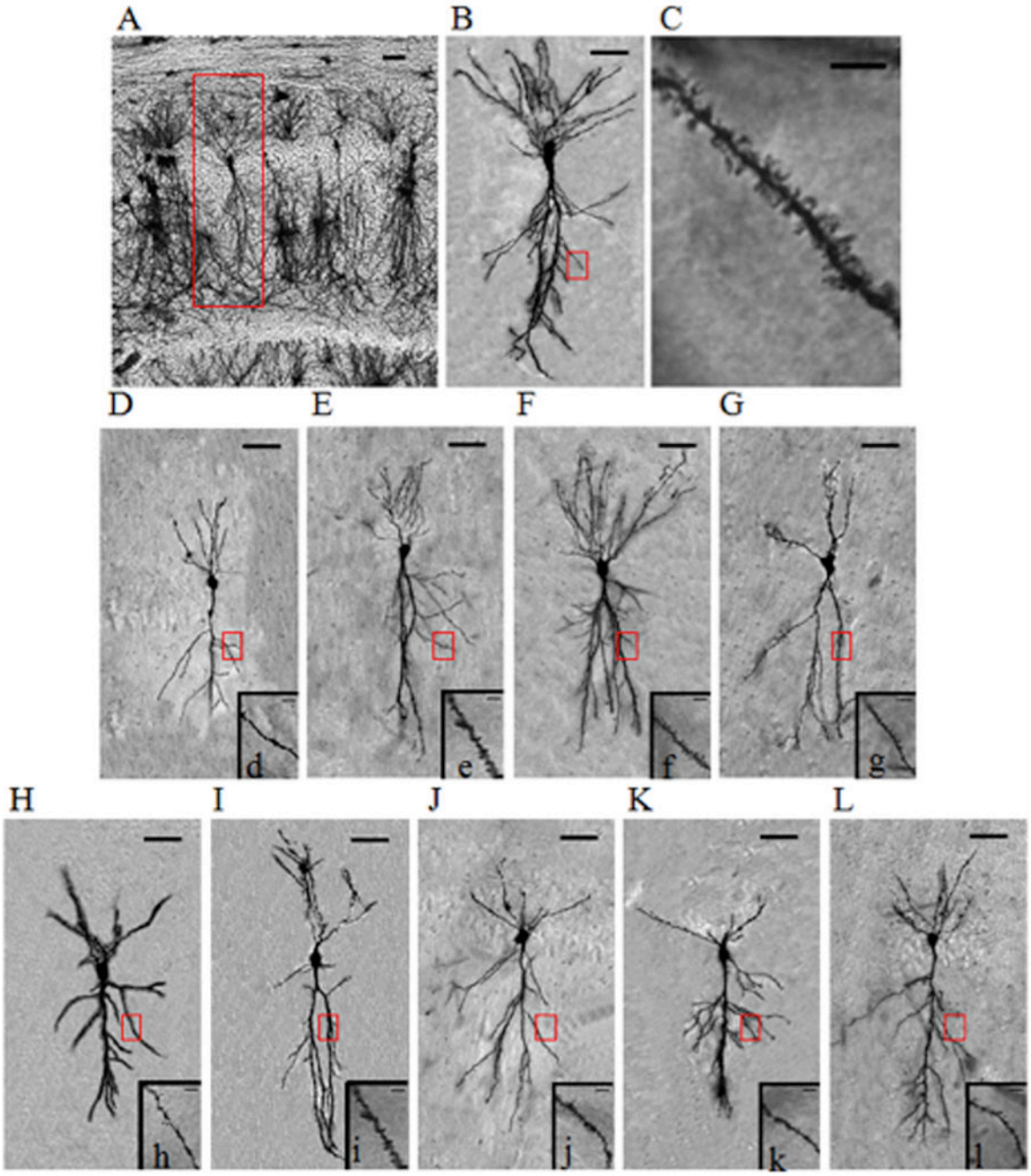

Fig. 4.

Photomicrographs of representative Golgi stained hippocampal CA1 pyramidal neurons from each group. A: The CA1 region in hippocampus of the mice. B: High magnification of the marked area in A. C: Spines on the dendrites. D: vehicle-treated stressed group. E: stress + BAY (1). F: stress + BAY (3). G: stress + BAY (3) + MK801 (10 μM). H: stress + BAY (3) + myr-AIP (20 μM). I: stress + BAY (3) + L-NAME (20 mg/kg). J: stress + BAY (3) +7-NI (20 mg/kg). K: stress + BAY (3) + KT5823 (20 μM). L: stress + BAY (3) + H89 (5 μM).

Magnification: 10× in A, 40× in B, D, E, F, G, H, I, J, K, L, M and 100× in C. Scale bars = 50 μm (B, D, E, F, G, H, I, J, K, L, M) and 5 μm (C, d, e, f, g, h, i, j, k, l, m) (n=10).

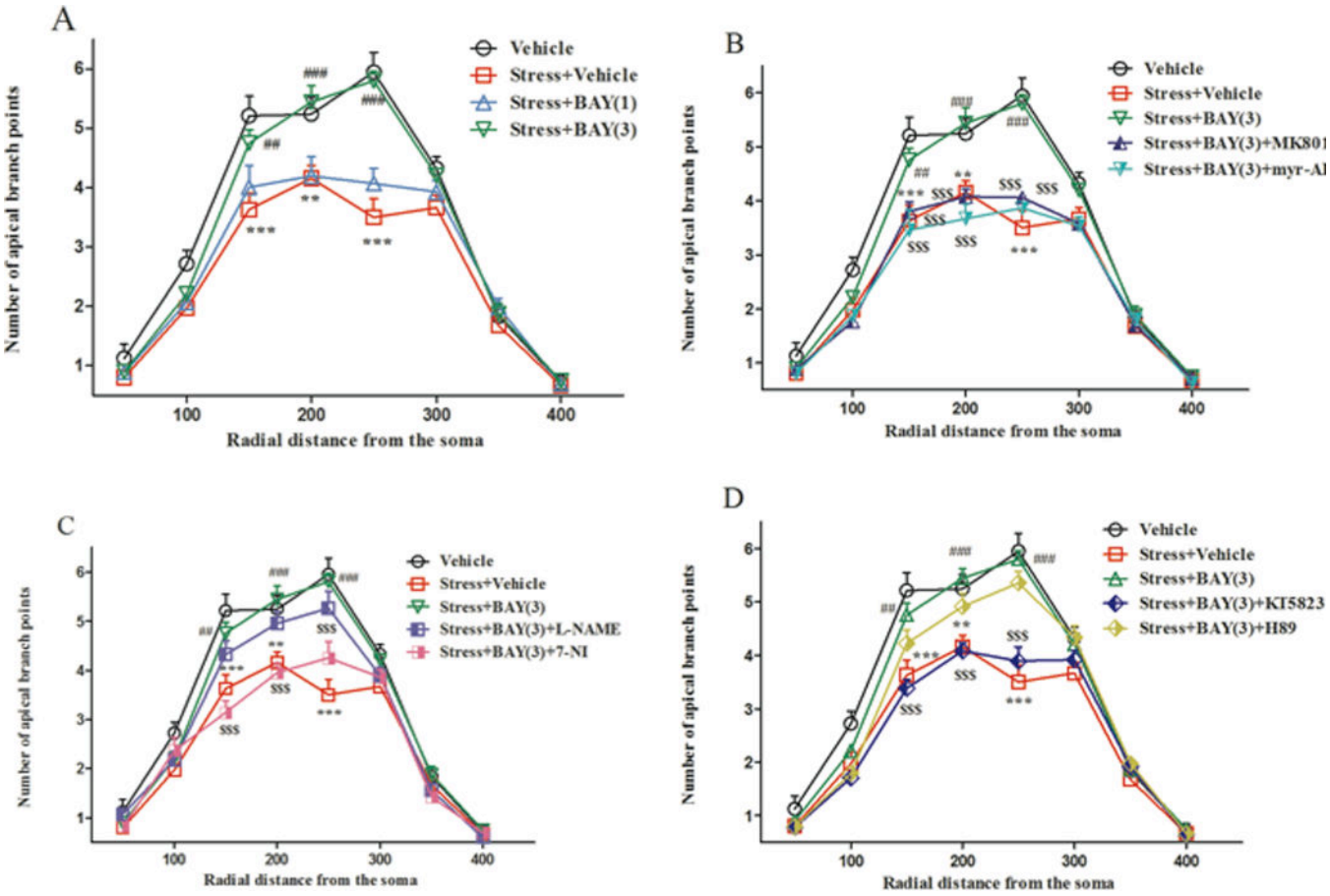

Fig. 5.

Subtle effects of stress and Bay 60-7550 on apical dendritic points. Analysis the number of branch points for 50-400 μm segments from the soma using Sholl analysis. Each point represents the mean ± SEM of 10 mice (50 neurons/ group). ** $p < 0.01$, *** $p < 0.001$ vs. non-stressed control group. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the vehicle-treated stressed group. $^{\$\$\$}p < 0.001$ vs. the Bay 60-7550-treated group.

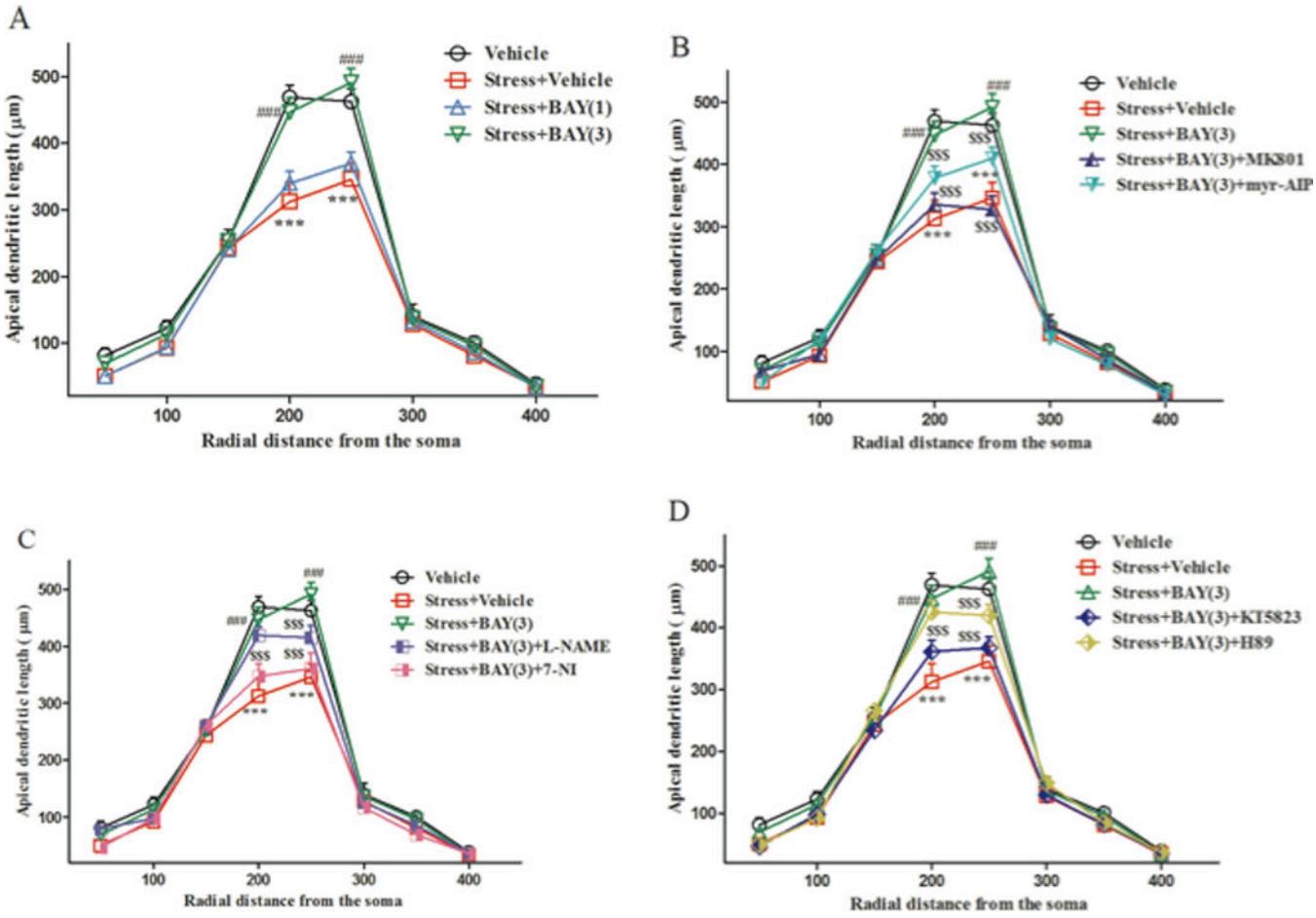

Fig. 6.

Subtle effects of stress and Bay 60-7550 on apical dendritic length. Analysis the dendritic length for 50-400 μm segments from the soma using Sholl analysis. Each point represents the mean ± SEM of 10 mice (50 neurons/ group). *** $p < 0.001$ vs. non-stressed control group. $^{###}p < 0.001$ vs. the vehicle-treated stressed group. $^{\$\$\$}p < 0.001$ vs. the Bay 60-7550-treated group.

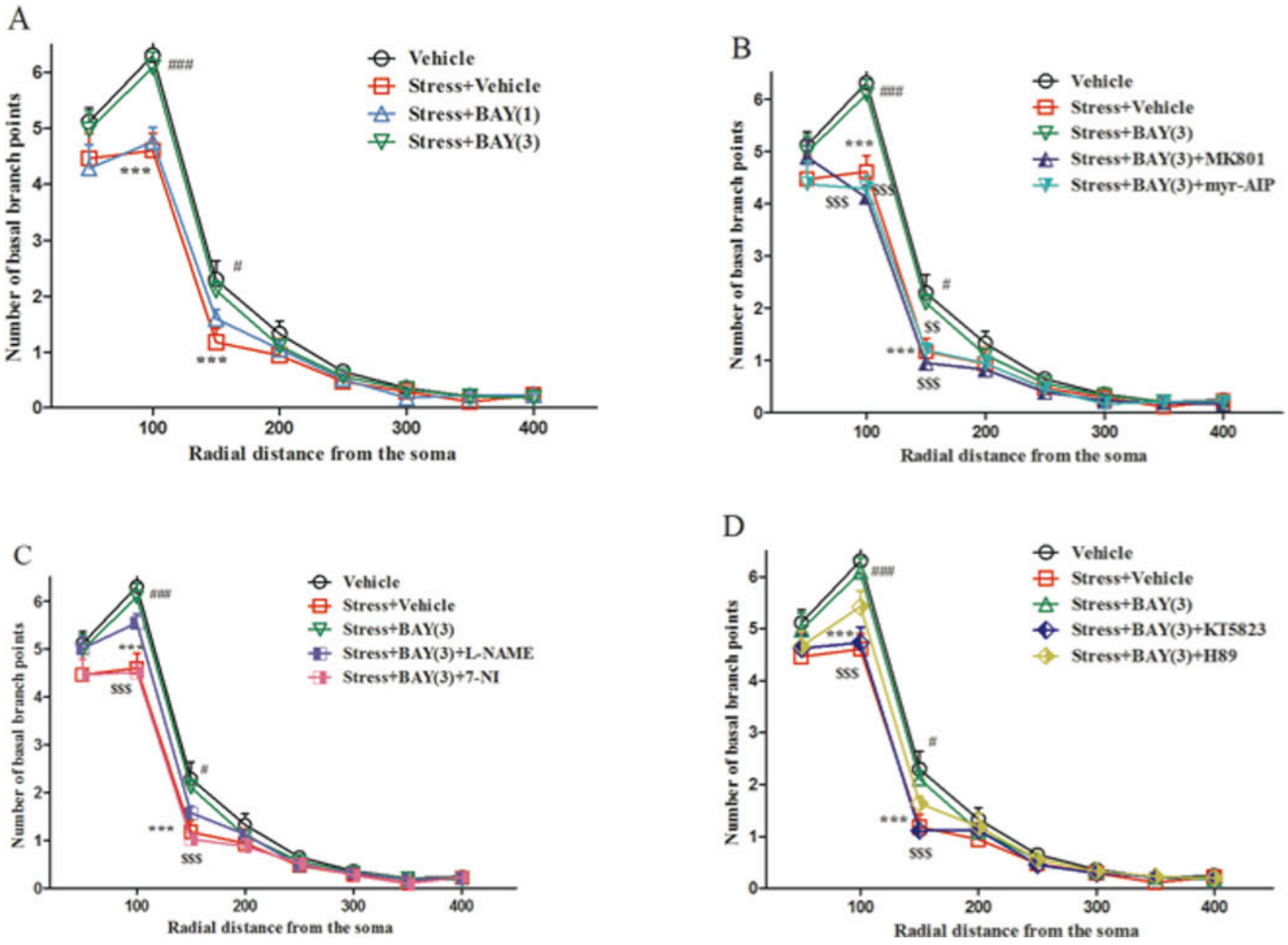

Fig. 7.

Subtle effects of stress and Bay 60-7550 on basal dendritic points. Analysis the number of branch points for 50-400 μm segments from the soma using Sholl analysis. Each point represents the mean ± SEM of 10 mice (50 neurons/ group). *** $p < 0.001$ vs. non-stressed control group. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. the vehicle-treated stressed group. $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. the Bay 60-7550-treated group.

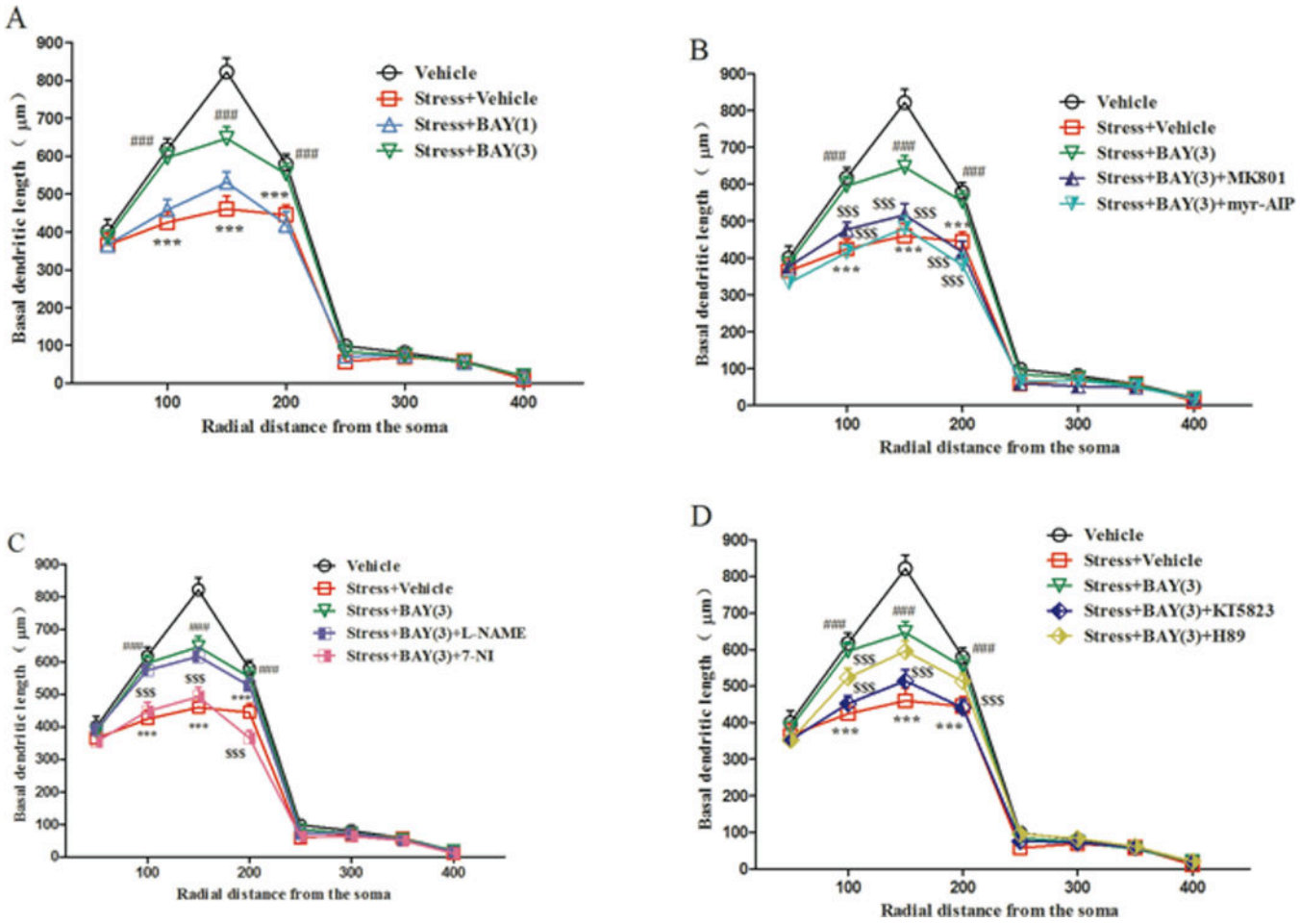

Fig. 8.

Subtle effects of stress and Bay 60-7550 on basal dendritic length. Analysis the dendritic length for 50-400 μm segments from the soma using Sholl analysis. Each point represents the mean ± SEM of 10 mice (50 neurons/ group). *** $p < 0.001$ vs. non-stressed control group. $^{###}p < 0.001$ vs. the vehicle-treated stressed group. $^{\$\$\$}p < 0.001$ vs. the Bay 60-7550-treated group.

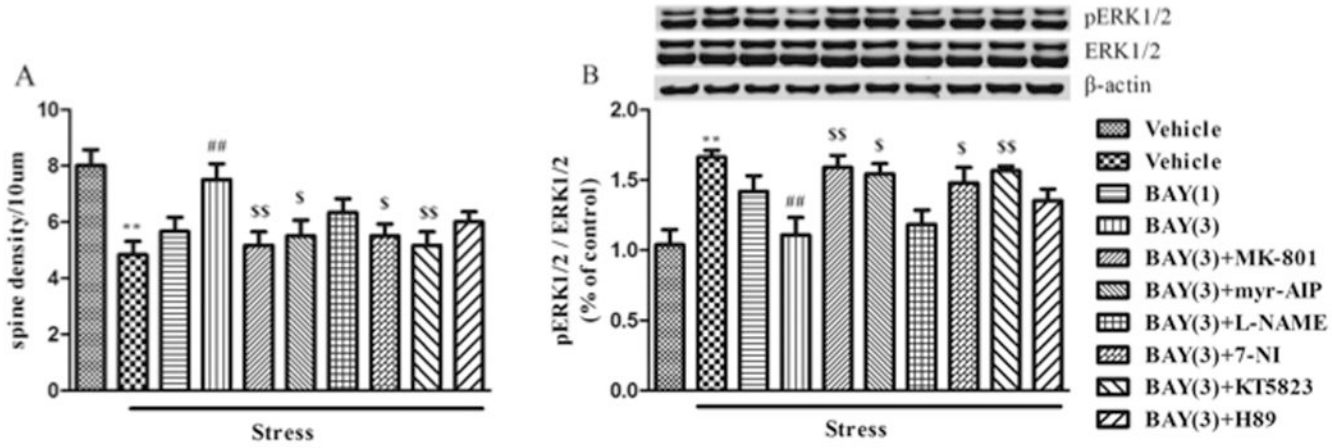

Fig. 9.

The effects of Bay 60-7550 on spine density (A) and the ratio of pERK1/2 and ERK1/2 (B) in chronically stressed mice. Results are expressed as mean ± SEM from 10 mice (for spine density) and 10 mice (for the ratio of pERK1/2 and ERK1/2). ** $p < 0.01$ vs. non-stressed control group; $^{\#\#}p < 0.01$ vs. vehicle-treated stressed group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. Bay 60-7550-treated group.

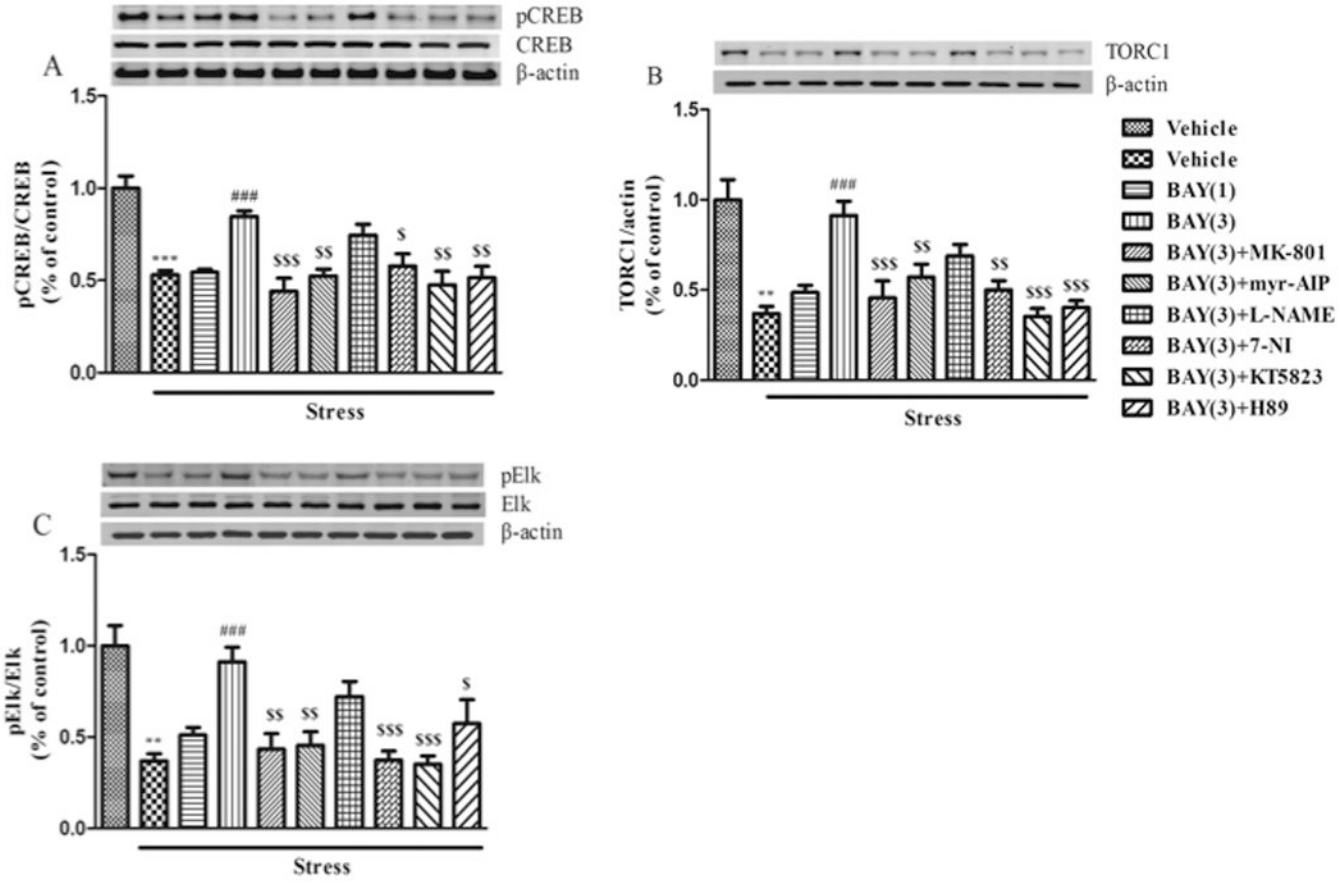

Fig. 10.

The effects of Bay 60-7550 on pCREB, TORC1 and pElk expression in chronically stressed mice. Results are expressed as mean ± SEM from 10 mice. ** $p < 0.01$, *** $p < 0.001$ vs. non-stressed control group. $^{###}p < 0.001$ vs. vehicle-treated stressed group. $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. Bay 60-7550-treated group.

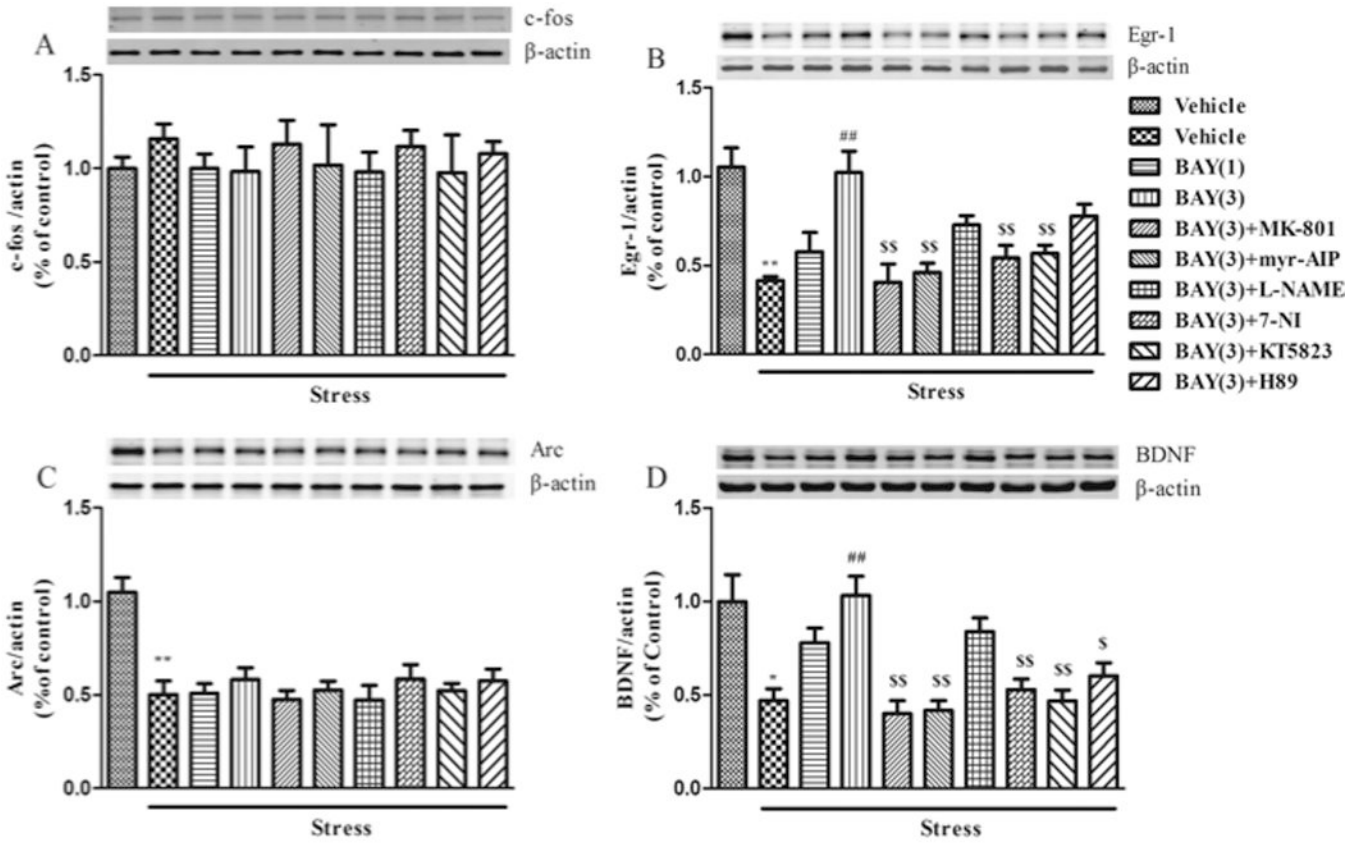

Fig. 11.

The effects of Bay 60-7550 on c-fos, Egr-1, Arc and BDNF expression in chronically stressed mice. Results are expressed as mean ± SEM from 10 mice. * $p< 0.05$, ** $p< 0.01$ vs. non-stressed control group; $^{\#\#}p< 0.01$ vs. vehicle-treated stressed group; $^{\$}p< 0.05$, $^{\$\$}p< 0.01$ vs. Bay 60-7550-treated group.

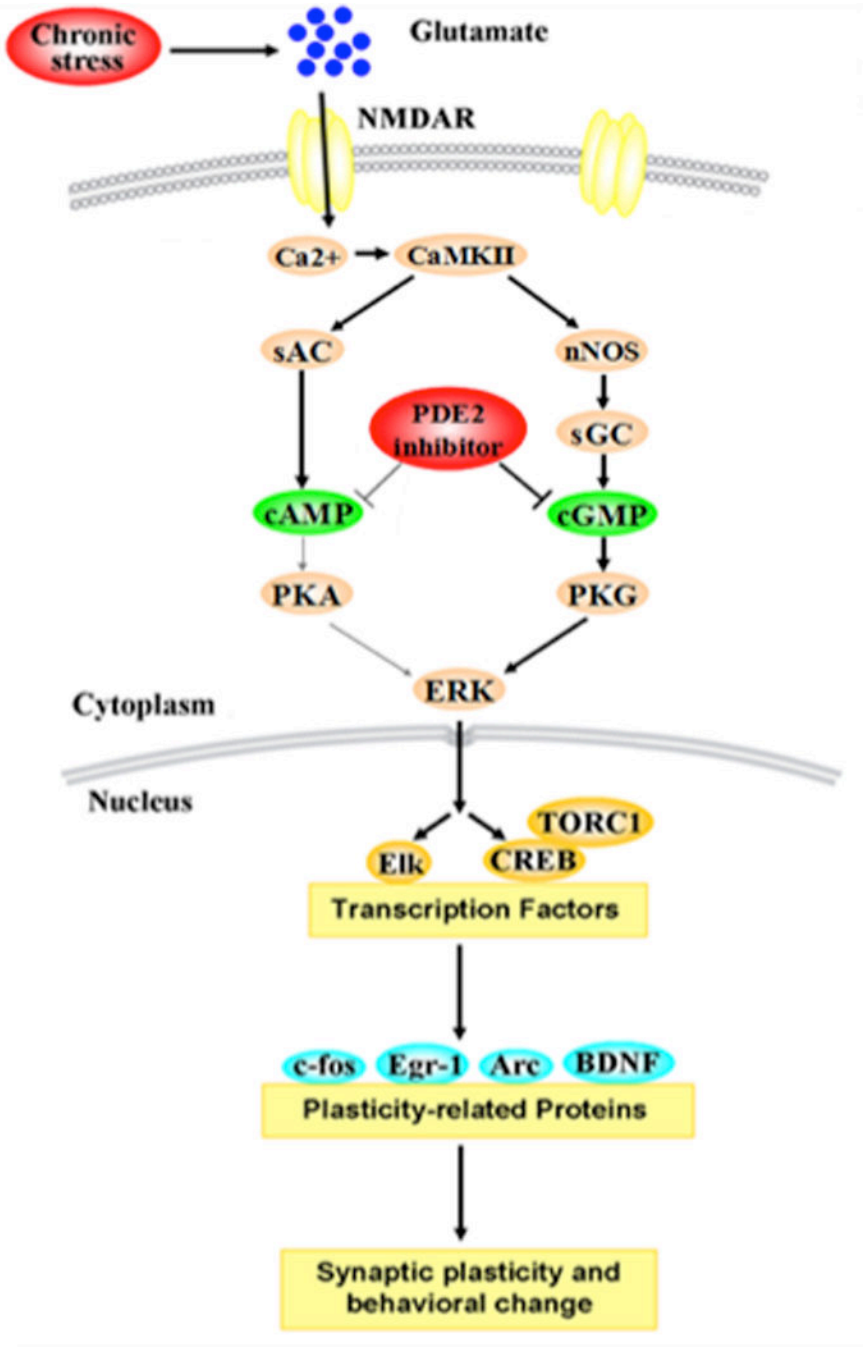

Fig. 12.

Representation of the involvement of PDE2 in NMDA-CaMKII-NO-cAMP/cGMP signaling. Ca^{2+} influx through NMDA receptor on the membrane activates nNOS and cAMP/cGMP, which are required for ERK activation and for the full expression of plasticity-related proteins, i.e. CREB, TORC1, Elk. This effect ignites the immediate early genes (IEG) and brain derived neurotrophic factor (BDNF) expression. And finally, all these changes regulate the synaptic plasticity and behaviors (n=10).