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AGE-RELATED IMPAIRMENTS IN MEMORY AND IN CREB AND pCREB EXPRESSION IN HIPPOCAMPUS AND AMYGDALA FOLLOWING INHIBITORY AVOIDANCE TRAINING

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Abstract

This experiment examined whether age-related changes in CREB and pCREB contribute to the rapid forgetting seen in aged animals. Young (3-month-old) and aged (24-month-old) Fischer-344 rats received inhibitory avoidance training with a low (0.2 mA, 0.4 sec) or moderate (0.5 mA, 0.5 sec) footshock; memory was measured 7 days later. Other rats were euthanized 30 minutes after training, and CREB and pCREB expression levels were examined in the hippocampus, amygdala, and piriform cortex using immunohistochemistry. CREB levels decreased with age in the hippocampus and amygdala. After training with either shock level, young rats exhibited good memory and increases in pCREB levels in the hippocampus and amygdala. Aged rats exhibited good memory for the moderate but not the low shock but did not show increases in pCREB levels after either shock intensity. These results suggest that decreases in total CREB and in pCREB activation in the hippocampus and amygdala may contribute to rapid forgetting in aged rats. After moderate footshock, the stable memory in old rats together with absence of CREB activation suggests either that CREB was phosphorylated in a spatiotemporal pattern other than analyzed here or that the stronger training conditions engaged alternate mechanisms that promote long-lasting memory.

Keywords

Memory; CREB; hippocampus; amygdala; inhibitory avoidance

1. INTRODUCTION

As seen in humans, rats and mice exhibit age-related impairments in learning and memory on many tasks. Often, the impairments are characterized in terms of rapid forgetting, in which aged rodents perform similarly to young adult rodents on memory tests soon after training, but have poor memory at later times as compared to young rodents (Barnes, 1991;

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Foster, 1999; Gold, 2005, 2001; Korol, 2002; Winocur, 1988). There are many examples of accelerated forgetting in aged rodents, with specific time courses that differ by task. In particular, memory for inhibitory avoidance training remains stable in young adult rats for weeks after training but decays within hours to days in old rats. Importantly, the age-related difference in forgetting rate is apparent despite similar or lower foot shock perceptual thresholds in old rats (Foster and Kumar, 2007; Frye et al., 2010; Gold et al., 1981; Morris et al., 2010). Rapid forgetting is also evident in a variety of other tasks, including the water maze (Burke et al., 2008; Gage et al., 1984; Mabry et al., 1996; Rapp et al., 1987), reward reduction (Salinas and Gold, 2005), social transmission of food preference (Countryman and Gold, 2007), visual discriminated avoidance (Gold et al., 1981), Barnes circular maze (Barnes and McNaughton, 1985), spatial reversal (Zornetzer et al., 1982), spontaneous alternation (Da Silva Costa-Aze et al., 2011; McNay and Gold, 2001; Stone et al., 1997), odor-reward association (Roman et al., 1996), and eye-blink classical conditioning (e.g. Solomon et al., 1995; Woodruff-Pak et al., 2007). The breadth of examples of rapid forgetting suggests that this is a key characteristic of age-related changes in memory.

Rapid forgetting seen during aging is analogous to similar findings seen after many treatments that interfere with cell and molecular processes associated with the formation of new memories. Rapidly decaying memory is seen after administration of protein synthesis inhibitors, ERK/MAPK inhibitors, and inhibitors of transcription factors, such as cAMP response element-binding protein (CREB), and is also seen in several knockout and transgenic mice with alterations aimed at these and other molecular targets (e.g. Alberini, 2009; Apergis-Schoute et al., 2005; Costa-Mattioli and Sonenberg, 2008; Goelet et al., 1986; Guzowski et al., 2000; Houpt and Berlin, 1999; Izquierdo et al., 2006; Kandel, 2001; McGaugh, 2000; Taubenfeld et al., 2001; Trifilieff et al., 2006). The parallels between the rapid forgetting in these experiments and those seen in aged rodents suggest that similar cellular mechanisms may be involved.

Of particular relevance to the experiments reported here, considerable evidence suggests that CREB is a transcription factor important to the formation of durable memories, perhaps converting rapidly decaying memories and short-term potentiation to more permanent forms (Benito and Barco, 2010; Carlezon et al., 2005; Colombo et al., 2003; Josselyn, 2010; Silva et al., 1998; Taubenfeld et al., 1999; Yin and Tully, 1996). For example, interfering with CREB function via mutations or inhibitors generally disrupts memory assessed at long but not short times after training (Bourtchuladze et al., 1994; Brightwell et al., 2008, 2005; Frankland et al., 2004; Guzowski and McGaugh, 1997; Josselyn et al., 2004; Yin et al., 1994), and these findings are analogous to the rapid forgetting seen in aged rodents. Several studies have examined CREB functions in aged rodents (cf. Lund et al., 2004). One consistent finding is that aged rodents are impaired in training-related activation of phosphorylated CREB (pCREB) in the hippocampus (Countryman and Gold, 2007; Kudo et al., 2005; Monti et al., 2005; Porte et al., 2008; Xu et al., 2010). Several studies of chronically administered treatments have identified substances that can attenuate age-related memory impairments while also enhancing CREB phosphorylation (Assunção et al., 2010; Li et al., 2009; Trofimiuk et al., 2010; Xu et al., 2010). Mouravlev et al. (2006) demonstrated that somatic gene transfer of CREB protein into the hippocampus of young adult rats prevented later formation of age-associated memory impairments. Also, Brightwell et al. (2004) found that aged rats with poor spatial memory have lower hippocampal CREB levels than do those with good spatial memory. However, prior aging studies have apparently not examined whether altering training conditions to promote stable memory formation in aged rodents would reverse deficits in CREB activation.

The present report tested the hypothesis that age-related differences in the expression and activation of CREB and pCREB may contribute to the rapid forgetting that is characteristic

of aged rodents. Therefore, we used an inhibitory avoidance task in which the rate of forgetting is accelerated in aged rats. In addition, increases in the aversive component of training result in better maintenance of memory, providing a comparison of conditions in which memory is rapidly or slowly forgotten. Thus, in the experiments reported here, rats were trained with either a low intensity foot shock, which led to age-related rapid forgetting, or a moderate intensity foot shock, which led to stable memory formation in both young and old rats. In past studies of aging and memory, CREB and pCREB expression were assessed only in the hippocampus. In the present experiments, CREB and pCREB expression levels were assessed with immunohistochemistry in brain sections collected 30 min after training from the amygdala and piriform cortex, as well as from hippocampal dentate gyrus, area CA3, and area CA1 (see Figure 1).

2. MATERIALS AND METHODS

2.1. Subjects

Young adult (3 to 4 mo.) and old (24 to 25 mo.) male Fischer-344 rats (Taconic Farms, Germantown, NY) were individually housed in translucent cages with a 12-h light/dark cycle (lights on at 07:00 h) and *ad libitum* access to food and water. Animal pain and discomfort were minimized, and all experiments were conducted in accordance with animal care guidelines established by the National Institute of Health and the University of Illinois, which is fully accredited (AAALAC).

2.2. Inhibitory Avoidance Training

Rats were handled on 5 consecutive days for 5 min each day prior to behavioral training. All training and testing took place between 1200 and 1600 h. The inhibitory avoidance apparatus was a trough-shaped alleyway (91 cm long, 22.9 cm wide at the top, 7.6 cm wide at the bottom, and 15.2 cm deep) divided into lit (31 cm) and dark (60 cm) compartments by a sliding door that could be lowered through the floor. Each rat was placed in the lit chamber facing the door. When the rat turned completely around, the door was lowered to a height approximately 2 cm above the floor. When the rat again turned toward the door, a timer was started to record the latency to enter the dark chamber. Upon entering the dark chamber, the rat received a brief foot shock (either 0.2 mA, 0.4 sec, or 0.5 mA, 0.5 sec) and the door was closed to prevent reentry into the lit chamber. The rat was then returned to the holding cage. For the initial behavioral characterization, retention latencies (max of 600 sec) were tested 7 days after training using the same procedure. To measure CREB and pCREB levels, rats were euthanized 30 min after training and immunostaining procedures were used at a later time. Groups for the CREB and pCREB measures also included a cage control group, in which rats were left in their home cage until euthanasia, and a no shock group, in which rats were placed in the training apparatus and allowed to cross from the lit to dark compartment, but did not receive a foot shock. Ns = 4 for the initial behavioral characterization. For the immunohistochemistry studies, N = 7 for the young cage control group and Ns =4 for all other groups.

2.3. Perfusion and Brain Slicing

Rats were deeply anesthetized with an overdose i.p. injection of sodium pentobarbital (Sigma-Aldrich, St. Louis, MO) and then perfused intracardially with 80 ml of 0.1 M phosphate-buffered saline followed by 80 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Rats were decapitated and the brains were removed and placed into 4% paraformaldehyde in 0.1 M PB for ~72 hrs. The brains were transferred to 20% glycerol in 0.1 M PBS for ~48 hrs. Frozen sections (40 μM) were collected at −30° C with a Leica 1800 cryostat (Leica Microsystems, Wetzlar, Germany). Slices through the dorsal hippocampus were collected and stored in a cryopreservative solution (250 mM 40 KD

polyvinylpyrrolidone, 880 mM sucrose, 30% v/v ethylene glycol, 50 mM sodium phosphate) at −20° C.

2.4. Immunohistochemistry

All steps took place at room temperature. All reactions were performed in duplicate, using alternating slices for CREB and pCREB staining. Slices were washed three times for 10 min each time in 0.05 M PBS initially and between all subsequent steps. Slices were first incubated in blocking solution (1% H_2O_2, 1% normal goat serum, 0.02% triton x-100, 0.05 M PBS) for 10 min. They were transferred to a pre-incubation solution (2% NGS, 0.4% triton x-100, 0.05 M PBS) for 20 min and then incubated overnight in a solution (1% NGS, 0.4% triton x-100, 0.05 M PBS) containing a rabbit primary antibody for CREB or Ser-133 phosphorylated CREB (Millipore, Billerica, MA) diluted 1:4000. The next day, the slices were placed for 1 hr in a solution (1% NGS, 0.2% triton x-100, 0.05 M PBS) containing a goat anti-rabbit biotinylated secondary antibody (Santa Cruz, Santa Cruz, CA). They were next incubated for 30 min with ABC reagent (Vector, Burlingame, CA) in 0.05 M PBS, followed by incubation with DAB substrate (Vector) for 4 min. Slices were mounted onto slides and allowed to dry overnight. The next morning, slices were dehydrated with a graded ethanol series of washes, then coverslipped using DPX mountant (Sigma-Aldrich).

2.5. Image Acquisition and Analysis

Sections were imaged using a Leica DM 6000B/CTR6000 light microscope and a Leica DFC350 FX video camera, which was interfaced to a PC computer. This system was used in conjunction with Image-Pro software (Media Cybernetics, Inc., Bethesda, MD) for image acquisition and for correction of unevenness in illumination across images. Image J software (NIH, Bethesda, MA) was used to quantify the optical density of CREB and pCREB staining in subregions of the hippocampus, amygdala, and piriform cortex. Figure 1 shows the specific regions that were analyzed. A statistical thresholding method in Image J was used to ensure that only specifically labeled cells were being measured. For each image, the optical density of a nearby region with no or little specific staining was calculated and used for background subtraction.

2.6. Statistical Analyses

All analyses were performed using Statview software. The optical densities of CREB and pCREB immunostaining were analyzed using two-way ANOVAs with post hoc Fisher PLSD tests where appropriate. Behavioral results were analyzed using a non-parametric Kruskal-Wallis one-way analysis of variance, followed by Mann-Whitney tests for individual comparisons.

3. RESULTS

3.1. Behavioral performance on the inhibitory avoidance task

Figure 2 shows training latencies (Left) and 7-day retention latencies (Right) of young and old rats trained on an inhibitory avoidance task. There were no significant age-related differences in training latencies. Young rats had maximum median retention latencies of 600 sec following a 0.2 mA, 0.4 sec or 0.5 mA, 0.5 sec foot shock. The old rats had a maximum median retention latency following the moderate intensity foot shock, but a median retention latency of only 66.9 sec with the lower intensity foot shock. Nonparametric analysis of variance revealed a significant group effect ($H = 8.7$, $p < .05$). Post-hoc analyses showed that old rats receiving the low intensity foot shock had significantly lower retention latencies compared to each of the other three groups (ps < .05).

3.2. CREB and pCREB immunostaining following training

Differences in immunostaining of pCREB, total CREB, as well as pCREB:CREB ratios, were examined in young and old rats 30 min after inhibitory avoidance training with a low (0.2 mA, 0.4 sec) or moderate (0.5 mA, 0.5 sec) foot shock. These groups were compared to cage controls and to no shock controls, which were trained on inhibitory avoidance without the foot shock. These results are summarized in Table 1.

3.2.1. Hippocampus: Dentate Gyrus—Figure 3 (A and B) shows pCREB immunostaining in the dentate gyrus region of the hippocampus. Activation of CREB by training was evident in young but not aged rats. A two-way ANOVA revealed a main effect of training on pCREB staining ($F_{(3,27)} = 7.30$, $p < .01$). In young rats, training with a 0.2 mA foot shock significantly enhanced pCREB staining compared to cage controls ($p < .01$). Training with a 0.5 mA foot shock enhanced pCREB staining compared to cage control, no shock, and 0.2 mA groups (ps < .03). In old rats, there were no significant increases in pCREB staining in response to training at either foot shock intensity. There was a main effect of age on pCREB ($F_{(1,27)} = 18.17$, $p < .001$), indicating lower pCREB staining in old compared to young rats. Post-hoc tests were used to identify age-related differences within training groups (e.g. young cage controls vs. old cage controls). There were significant age-related differences between the young and old rats in the 0.2 mA and 0.5 mA shock groups (ps < .05). In addition to the training and age effects, there was a significant age by training interaction ($F_{(3,27)} = 3.58$, $p < .05$).

There was an overall main effect of age ($F_{(1,27)} = 4.36$, $p < .05$) on CREB immunostaining, with lower staining observed in old rats (Figure 3C). There were no main effects of training on CREB staining ($F_{(3,27)} = 0.24$).

There was a main effect of training ($F_{(3,27)} = 4.76$, $p < .01$) but not age ($F_{(1,27)} = 1.38$) on pCREB:CREB ratios (Figure 3D). In young rats, training with a 0.2 mA shock increased pCREB:CREB ratios above those of cage control rats ($p < .03$). Training with a 0.5 mA shock increased pCREB:CREB ratios compared to both cage control and no shock groups (ps < .03). In old rats, training did not significantly alter pCREB:CREB ratios, regardless of the foot shock intensity.

3.2.2. Hippocampus: Area CA3—There was a main effect of age ($F_{(1,27)} = 6.45$, $p < .03$) but not training ($F_{(3,27)} = 2.40$) on pCREB immunostaining in area CA3 (Figure 4A and 4B). However, post-hoc analyses revealed no significant differences between groups. There was also a main effect of age ($F_{(1,27)} = 22.74$, $p < .0001$) but not training ($F_{(3,27)} = 0.06$) on CREB staining (Figure 4C). Post-hoc analyses revealed significantly lower CREB staining in old compared to young rats in the cage control, 0.2 mA shock, and 0.5 mA shock groups (ps < .05), but not in the no shock group. There was a main effect of age ($F_{(1,27)} = 4.87$, $p < .05$) on pCREB:CREB ratios (Figure 4D), suggesting higher ratios in old rats. There were no training effects on pCREB:CREB ratios ($F_{(3,27)} = 0.78$).

3.2.3. Hippocampus: Area CA1—In area CA1, the effects on pCREB, CREB, and pCREB:CREB ratios were very similar to those seen in the dentate gyrus, and are therefore not presented in detail here.

3.2.4. Basolateral Amygdala—Both training ($F_{(3,27)} = 5.19$, $p < .01$) and age ($F_{(1,27)} = 36.89$, $p < .0001$) significantly affected pCREB immunostaining in the basolateral amygdala (Figure 5A and 5B). In young rats, training with a 0.2 mA foot shock increased pCREB staining compared to cage controls ($p < .05$). Training with a 0.5 mA foot shock increased pCREB staining compared to both cage and no shock controls (ps < .01). In old rats, training

did not significantly alter pCREB staining, regardless of the shock intensity. Compared to cage controls, age-related deficits in pCREB activation were evident in the no shock, 0.2 mA shock, and 0.5 mA shock groups (ps < .05). There was also a significant age by training interaction ($F_{(3,27)} = 3.29$, $p < .05$).

There was a main effect of age ($F_{(1,27)} = 21.39$, $p < .0001$) but not training ($F_{(3,27)} = 0.20$) on CREB immunostaining (Figure 5C). Post-hoc analyses revealed significantly lower CREB staining in old rats in the cage control and 0.5 mA shock groups (ps < .05).

There was a main effect of training ($F_{(3,27)} = 3.39$, $p < .05$) but not age ($F_{(1,27)} = 2.36$) on pCREB:CREB ratios (Figure 5D). In young rats, the 0.2 mA shock group had significantly higher ratios compared to cage control rats ($p < .05$). The 0.5 mA shock group had significantly higher ratios compared to both the cage control and no shock groups (ps < .05). In old rats, training did not have a significant effect on pCREB:CREB ratios.

3.2.5. Lateral Amygdala—In the lateral amygdala, the effects on pCREB, CREB, and pCREB:CREB ratios were similar those seen in the basolateral amygdala, with the major exception being lack of a training effect on pCREB:CREB ratios in the lateral amygdala (see Table 1). Therefore, these results are not presented in detail here.

3.2.6. Piriform cortex—There were no main effects of age ($Fs_{(1,27)} < 3.67$) or training ($Fs_{(3,27)} < 0.07$) on pCREB, CREB, or pCREB:CREB ratios in the piriform cortex (Figure 6).

4. DISCUSSION

4.1. Age-Related Differences in Memory

Training with a low intensity foot shock produced stable 7-day memory in young rats, but led to poor 7-day memory in old rats. These results support prior work demonstrating rapid age-related forgetting following inhibitory avoidance training (Gold et al., 1981; Morris et al., 2010). In particular, Gold et al. (1981) trained rats with a low intensity foot shock and training-testing intervals ranging from 2 hours to six weeks. Young rats had stable memory for at least 3 weeks after training. Old rats had comparable or even better memory than young rats 2 hours after training. However, the old rats exhibited slight deficits after 1 day, and were significantly impaired after 7 days. Thus, old rats exhibited rapid forgetting for inhibitory avoidance, with memory intact for hours after training, but with memory impairments emerging during the days after training. Importantly, this operational definition of forgetting may reflect memories with variable time courses depending on the salience of the initiating events.

In contrast to the results with the low foot shock, training with a moderate intensity foot shock resulted in stable 7-day memory in both young and old rats. These results are similar to findings by Gold et al. (1981), and indicate that forgetting rates in old rats can be slowed by increasing the saliency of the training foot shock, perhaps with concomitant increases in neural modulators of memory associated with the higher training-related arousal levels. Together, the behavioral results presented here provide a good model with which to examine changes in CREB expression and activation that might contribute to rapid forgetting in aged rats.

4.2. Age-Related Differences in CREB and pCREB Expression at Baseline

At baseline, CREB levels were lower in aged vs. young adult rats in area CA3 and in the basolateral and lateral amygdala. Comparable changes were not evident in the dentate gyrus,

area CA1, or piriform cortex, although the latter brain region had high CREB expression levels as seen previously (Ferrer et al., 1996; Herdegen et al., 1993). The age-related reductions in hippocampal CREB observed here are consistent with past findings revealing decreased expression of hippocampal CREB in old Fischer-344 rats (Countryman and Gold, 2007). Other studies have shown age-related reductions in CREB levels in whole hippocampal homogenates from Long Evans (Brightwell et al., 2004) and Wistar rats (Trofimiuk et al., 2010). The age-related decreases in CREB expression in the amygdala have not, to our knowledge, been noted before. The decreased expression of CREB in the hippocampus and amygdala with age, as well as the decreased training-related activation of CREB described below, may contribute to age-related memory impairments. The memory impairments are analogous to those seen in CREB knockout mice and in rats and mice treated with CREB antisense oligonucleotides, RNA interference, or dominant negative mutations, any of which impairs the formation of long-lasting memory and reduces the durability of long-term potentiation (Bourtchuladze et al., 1994; Canal et al., 2008; Florian et al., 2006; Guzowski and McGaugh, 1997; Josselyn, 2010; Peters et al., 2009; Pittenger et al., 2002; Won and Silva, 2008).

Importantly, age-related changes in CREB, as well as changes in expression of pCREB described below, do not likely reflect differences in cell numbers or cell morphology between young and old rats. Studies utilizing stereological methods for quantification have found that neuron numbers are preserved in the hippocampus of old rats (Gallagher et al., 2003; Rapp and Gallagher, 1996; Rasmussen et al., 1996). Likewise, another study reported no significant volume or cell loss in the hippocampus or amygdala of aged mice (von Bohlen und Halbach and Unsicker, 2002).

The age-related decreases in CREB levels in several brain regions suggest that some estimates of age-related changes in behaviorally elicited increases in pCREB levels may reflect the loss of CREB *per se.* Several recent studies of changes during aging in pCREB levels in the hippocampus have measured only pCREB or have used total CREB levels to normalize for changes in pCREB (e.g. Assunção et al., 2010; Hattiangady et al., 2005; Li et al., 2009; Trofimiuk et al., 2010; Xing et al., 2010; Xu et al., 2010; Zhao et al., 2009). The present results suggest that quantifying total CREB levels is important to interpret correctly age- or activity-dependent changes in pCREB in old rats. Further, using CREB levels for normalization in aging studies may make it difficult to compare directly results across age groups, since total CREB levels may decline with age in a brain area-specific manner.

4.3. Age-Related Differences in pCREB Expression in Response to Training

CREB phosphorylation in the piriform cortex has been shown to be responsive to several stimuli (Alvarez-López et al., 2004; Dere et al., 2008; Estrada and Isokawa, 2009; Kim et al., 2006; Pandey et al., 2001a, b; Wang et al., 2007). However, this is the first study, to our knowledge, to examine CREB activation in the piriform cortex after inhibitory avoidance training, revealing no evidence for pCREB increases after training in either young or aged rats. The results contrast with those showing c-Fos expression and ERK activation in piriform cortex induced by active avoidance training in young adult rats (Radwanska et al., 2010).

A striking result was that there were substantial age-related differences in activation of CREB in response to training in the hippocampus and amygdala. In young adult rats, pCREB expression in the dentate gyrus, area CA1, and the lateral and basolateral amygdala increased 30 min after training with a low foot shock as compared to expression levels in young cage controls. Training with a moderate intensity foot shock further enhanced pCREB levels in the same brain regions. In parallel groups of young rats, both shock intensities produced high avoidance latencies in memory tests 7 days after training. These results are

consistent with the view that, in young adult rats, CREB activation may be an important component or marker of long-term memory processes occurring after training. The results obtained in hippocampal regions agree with a number of other studies showing increased hippocampal CREB phosphorylation following inhibitory avoidance training in young adult rats, which can be abolished by treatments that interfere with memory processes (Cammarota et al., 2000; Taubenfeld et al., 2001, 1999; Viola et al., 2000). The amygdala results also agree with several studies demonstrating enhanced CREB phosphorylation in the basolateral and/or lateral amygdala following fearful or stressful stimuli (Bilang-Bleuel et al., 2002; Hubbard et al., 2007; Ilin and Richter-Levin, 2009; Kogan and Richter-Levin, 2008; Lin et al., 2003; Saha and Datta, 2005; Shen et al., 2004; Stanciu et al., 2001).

In marked contrast to the results obtained with young adult rats, aged rats did not show significant increases in pCREB expression in any brain region after training with either the low or moderate shock intensity. In old rats, pCREB levels in the dentate gyrus, area CA1, and lateral and basolateral amygdala, i.e. those areas responsive to training in young rats, were similar to those of aged cage controls and were generally significantly lower than those in corresponding groups and samples of young rats. Viewed across all brain areas, age-related decreases in total CREB cannot entirely account for these impairments in CREB phosphorylation, since basal CREB levels in area CA1 and the dentate gyrus were similar between young and old rats. Rather, the findings suggest that age-related impairments upstream to CREB activation may be an important contributor to age-related impairments in memory. This interpretation is consistent with recent evidence that overexpression of CREB in the hippocampus improves memory in young but not middle-aged rats (East et al., 2011), perhaps due to an inability of middle aged rats to engage upstream activators of CREB phosphorylation when needed.

The present results are the first to identify substantial age-related impairments in CREB activation in the amygdala and hippocampus after inhibitory avoidance training. The failure to activate CREB in the amygdala may have significant consequences to modulation of memory in other neural systems, such as the hippocampus, and may contribute to the decreased pCREB after training in the hippocampus. Consistent with this possibility, intra-amygdala injections of β-adrenergic agonists and antagonists, drugs that enhance and impair memory respectively, increase and decrease expression of activity-regulated cytoskeletal protein (Arc) in the hippocampus in conjunction with modulation of memory (McIntyre et al., 2005). These findings suggest that age-related deficiencies in amygdala modulation of hippocampal functions may be important in mediating the rapid forgetting and rapid decay of neural plasticity seen in aged rodents. The present results also agree with a number of studies showing age-related impairments in CREB activation in a variety of hippocampal-dependent tasks (Countryman and Gold, 2007; Kudo et al., 2005; Monti et al., 2005; Porte et al., 2008; Xu et al., 2010). Recent studies also indicate that long-term administration of certain compounds, including procyandins (Xu et al., 2010), green tea (Assunção et al., 2010; Li et al., 2009), and St. John’s wort (Trofimiuk et al., 2010), can reverse age-related deficits in CREB activation in concert with memory enhancement. Thus, these findings are consistent with the view that a failure to activate CREB after training contributes to age-related memory impairments.

The present experiments included a condition, moderate intensity foot shock used during training, which tested further the importance of CREB activation for long-lasting memory. On the basis of past findings (Gold et al., 1981), we expected the moderate shock condition to result in longer maintenance of memory for inhibitory avoidance training, a result confirmed here. We further expected that the moderate training shock would also result in greater CREB activation that would parallel the better memory scores. This result was clearly not evident: Raising the foot shock intensity reversed age-related impairments in 7-

day memory but did not increase CREB phosphorylation in the hippocampus or amygdala of old rats, as it did in young rats. Thus, CREB activation was dissociated from memory formation in old rats trained with the moderate foot shock intensity.

There are several possible explanations for the apparent dissociation between CREB activation and memory formation. In the present experiment, measurements of CREB activation were limited to a single time point, 30 min after training. Although some studies suggest there is rapid and long-lasting CREB activation following training (e.g. Taubenfeld et al., 2001, 1999), others have observed specific and even delayed time windows of CREB phosphorylation after training (Bernabeu et al., 1997; O'Connell et al., 2000). Therefore, an earlier or later time point might reveal training-related increases in pCREB levels that would be associated with the stable memory observed here after training with the moderate shock intensity. It is also possible that the quantitative histochemistry methods used here were not sufficiently sensitive to reveal increases after the moderate shock intensity. Given the greater increase in pCREB expression levels seen in young rats after the moderate shock intensity, this seems unlikely, though the possibility cannot be fully discounted. Although pCREB activation in young rats appeared more or less uniform across large numbers of cells, such widespread activation may not be necessary for memory formation. For instance, Han et al. (2009, 2007) found that altering CREB function in small numbers of sparsely distributed cells in the lateral amygdala can affect formation of auditory fear memory (Han et al., 2009, 2007; Zhou et al., 2009). Sparse distribution of critical neurons expressing CREB activation would likely be missed using the immunohistochemistry methods employed here. Utilization of a cell counting strategy or of more quantitative techniques, such as ELISAs or western blots, may be helpful in providing corroborative molecular evidence of the optical density measurements presented here.

Another possibility is that the dissociation between CREB and memory may indicate that CREB activation is not required for memory formation in old rats. This view is consistent with a number of studies showing stable formation of memory and long-term potentiation in CREB knockout and mutant mice (Balschun et al., 2003; Gass et al., 1998; Rammes et al., 2000). Although several studies do indicate deficits in long-term memory in CREB mutant mice (Bourtchuladze et al., 1994; Sekeres et al., 2010), these deficits can often be reversed by altering training procedures or by administering memory-enhancing agents (Frankland et al., 2004; Kogan et al., 1997; Canal et al., 2008). Further, the idea that CREB activation is not necessary for the formation of stable memories after the moderate shock is consistent with the possibility that the moderate shock level recruits additional molecular components of memory formation compared to those seen with the lower shock. In young rats, the behavioral measure of memory is maximal at both shock intensities, thereby obscuring the utility of additional molecular mechanisms. However, in aged rats, the stable memory after training with the moderate shock, coupled to the absence of CREB activation, may reflect the importance of these additional molecular mechanisms. In this regard, the study of stable memory in aged rats may be a condition favorable to investigation of these additional factors.

Highlights

- Old rats exhibit memory impairments following inhibitory avoidance training.
- Memory impairments are evident following a low but not moderate foot shock.
- Old rats have deficits in total CREB and in pCREB activation following training.
- pCREB deficits in old rats are present regardless of foot shock intensity.

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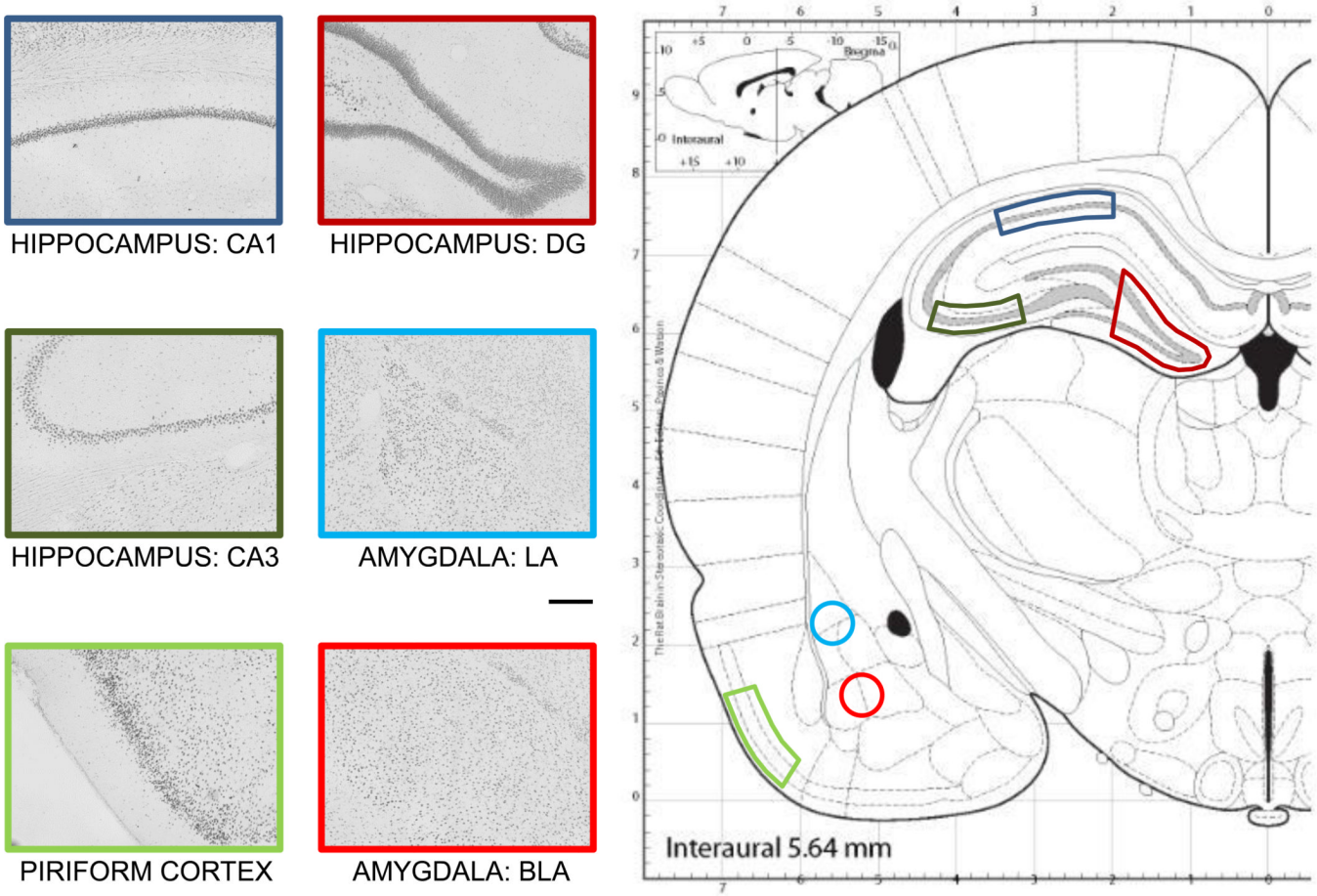

Figure 1.
Illustration of the areas targeted in this study, which were the dentate gyrus (DG), CA3, and CA1 of the hippocampus, the lateral (LA) and basolateral (BLA) nuclei of the amygdala, and the piriform cortex. Scale bar = 200 μm. Adapted with permission from Paxinos and Watson (2003).

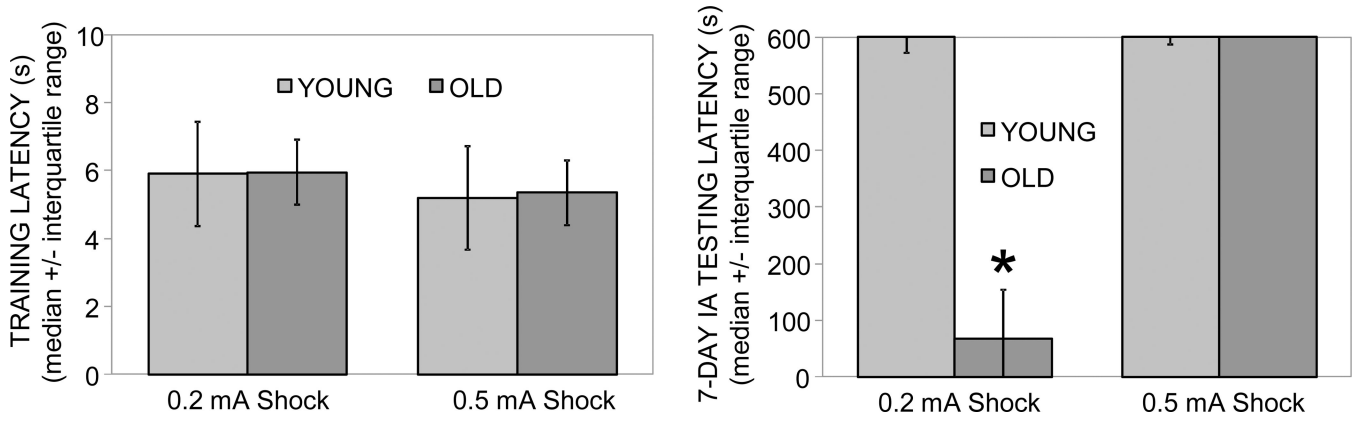

Figure 2.

Training and 7-day retention latencies in young and old rats trained on inhibitory avoidance with a 0.2 or 0.5 mA foot shock. (Left) There were no significant differences in training latencies across groups. (Right) Old rats had significantly lower 7-day retention latencies following the low intensity foot shock than did all other groups (*) ps < .05. Note that old rats exhibited maximal retention latencies after training with the higher foot shock intensity.

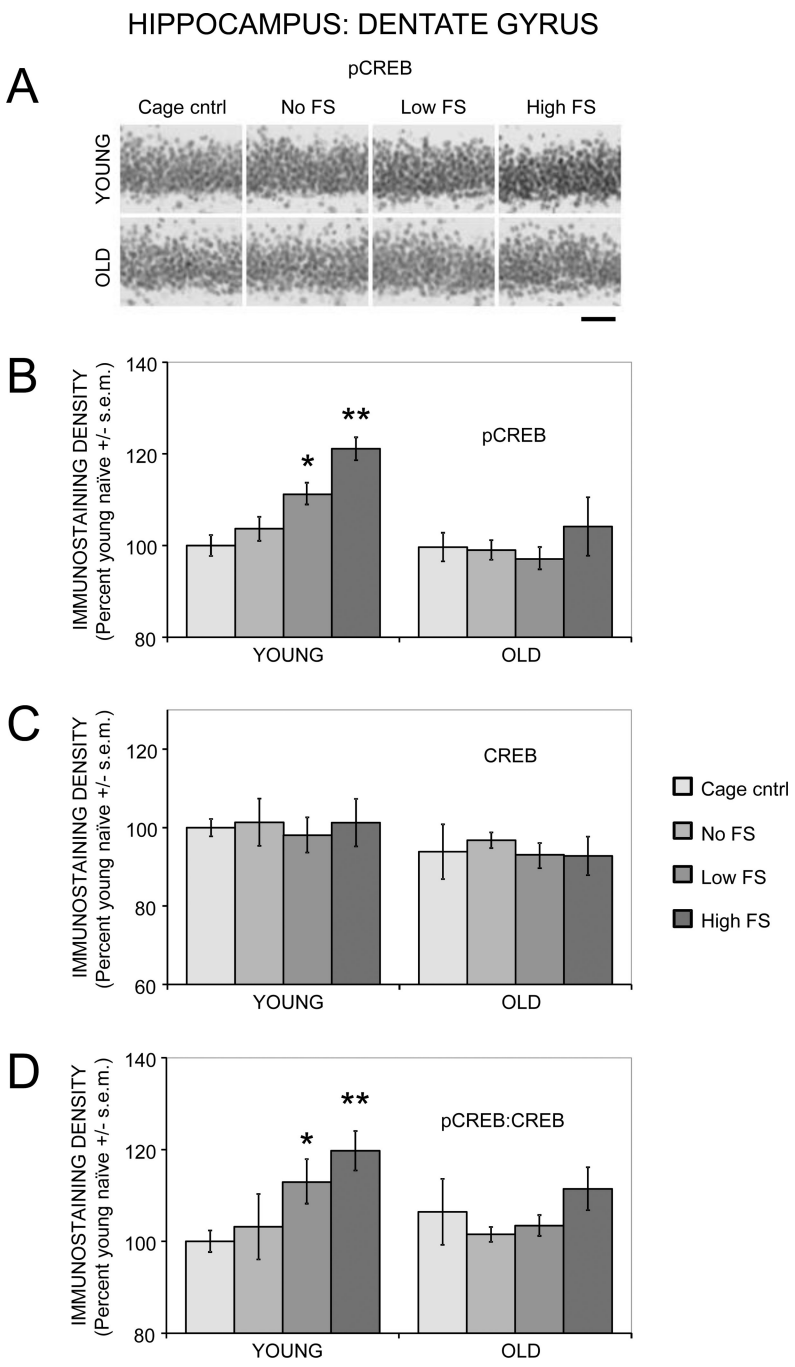

Figure 3.

Age- and training-associated differences in pCREB and CREB immunoreactivity in the dentate gyrus of the hippocampus. (A) Representative photomicrographs of pCREB immunostaining. Scale bar = 50 μm. (B) Young rats had significantly enhanced pCREB levels following training with a low or moderate intensity foot shock, whereas old rats had training-related deficits in pCREB activation. (*) $p < .01$ vs. young cage controls. (**) $ps < .05$ vs. young cage control, no shock, and 0.2 mA. (C) Total CREB levels were similar between young and old rats and not altered by training. (D) Young rats had significantly higher ratios of pCREB:CREB after training with a low or moderate intensity foot shock,

whereas old rats had training-related deficits in pCREB:CREB ratios. (*) $p < .05$ vs. young cage controls. (**) $ps < .05$ vs. young cage controls and young no shock.

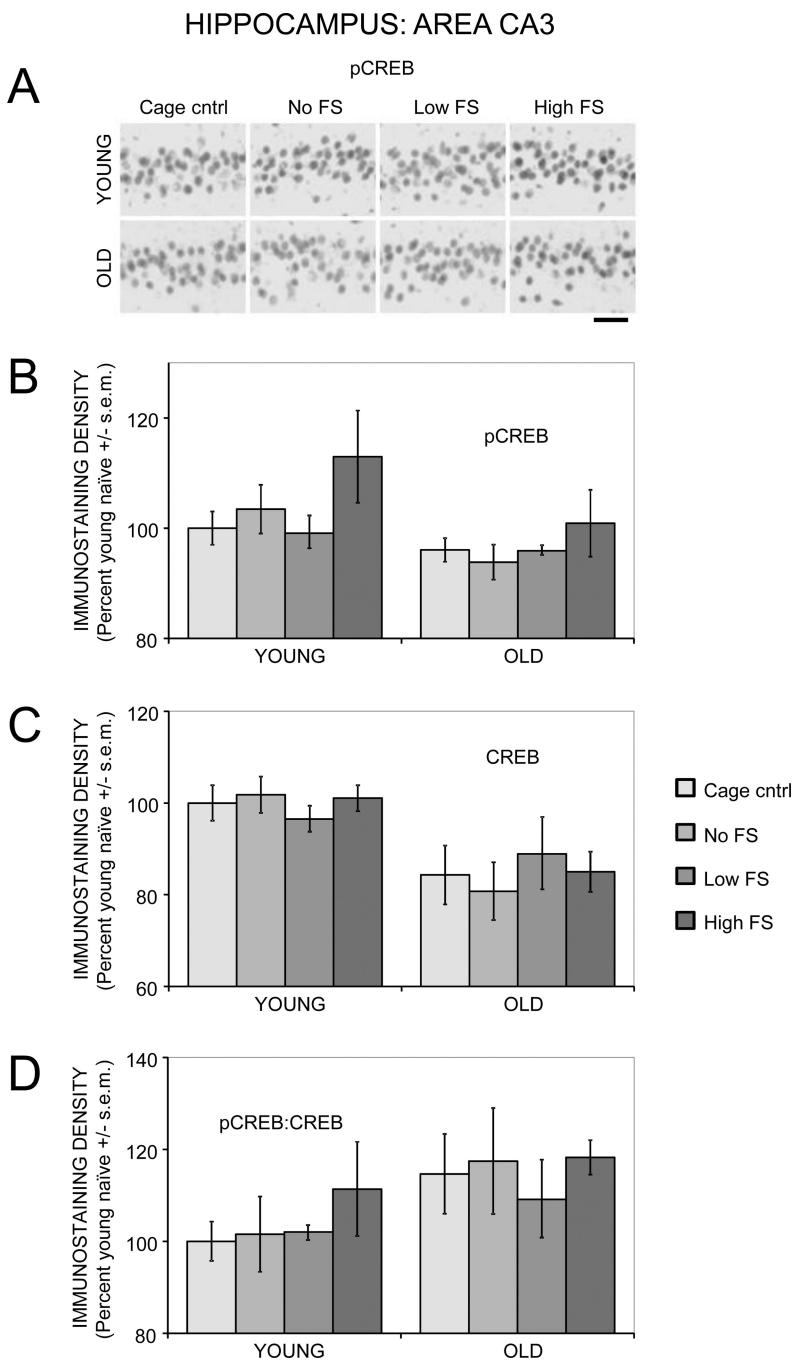

Figure 4.

Age- and training-associated differences in pCREB and CREB immunoreactivity in area CA3 of the hippocampus. (A) Representative photomicrographs of pCREB immunostaining. Scale bar = 50 μm. (B) pCREB levels were similar between young and old rats and not significantly altered by training. (C) There were significantly lower CREB levels in old compared to young rats. (D) There were no significant age- or training-related differences in pCREB:CREB ratios.

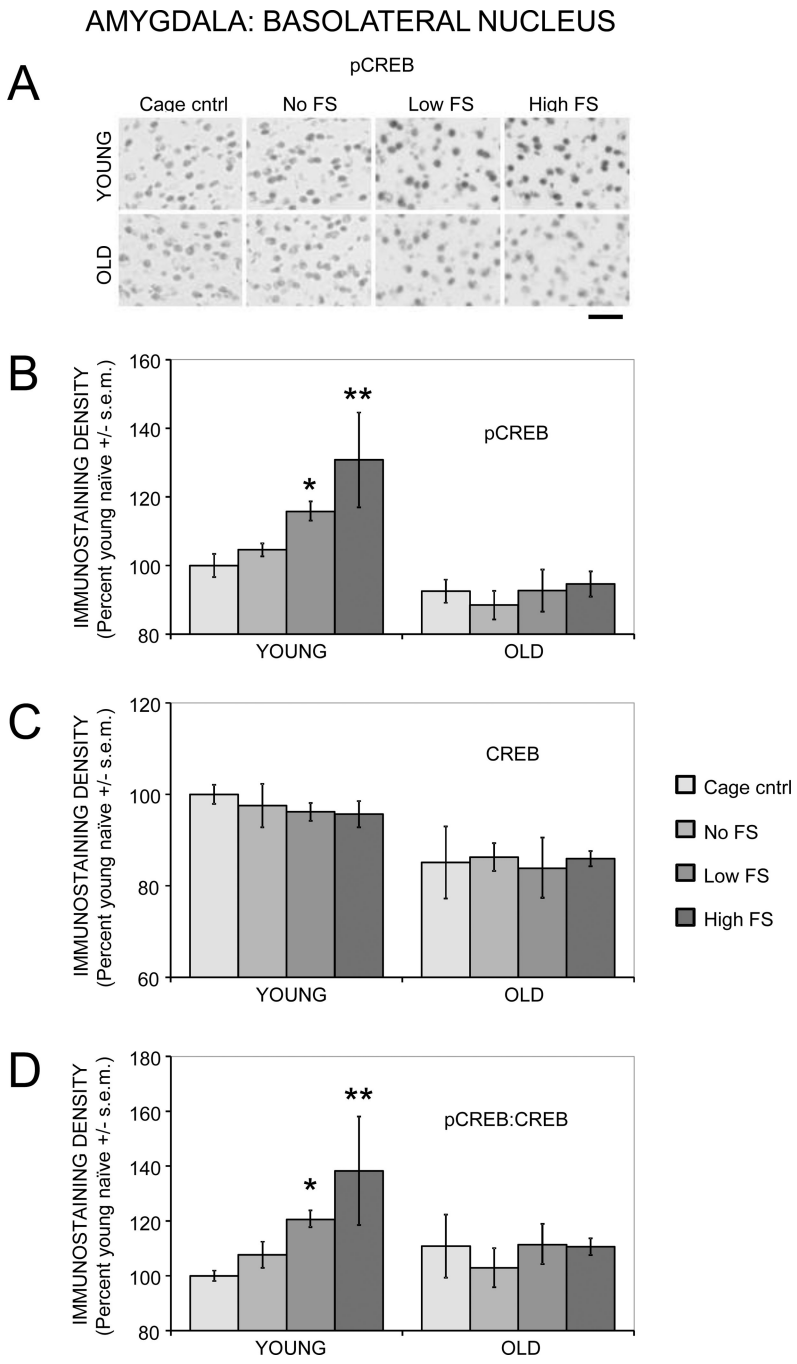

Figure 5.

Age- and training-associated differences in pCREB and CREB immunoreactivity in the basolateral amygdala. (A) Representative photomicrographs of pCREB immunostaining. Scale bar = 50 μm. (B) Young rats had significantly enhanced pCREB levels following training with a low or moderate intensity foot shock, whereas old rats had training-related deficits in pCREB activation. (*) $p < .05$ vs. young cage controls. (**) $ps < .01$ vs. young cage controls and no shock. (C) Total CREB levels were significantly lower in old compared to young rats. (D) Young rats had significantly higher ratios of pCREB:CREB after training with a low or moderate intensity foot shock, whereas old rats had training-related deficits in

pCREB:CREB ratios. (*) p < .05 vs. young cage controls. (**) ps < .05 vs. young cage controls and no shock.

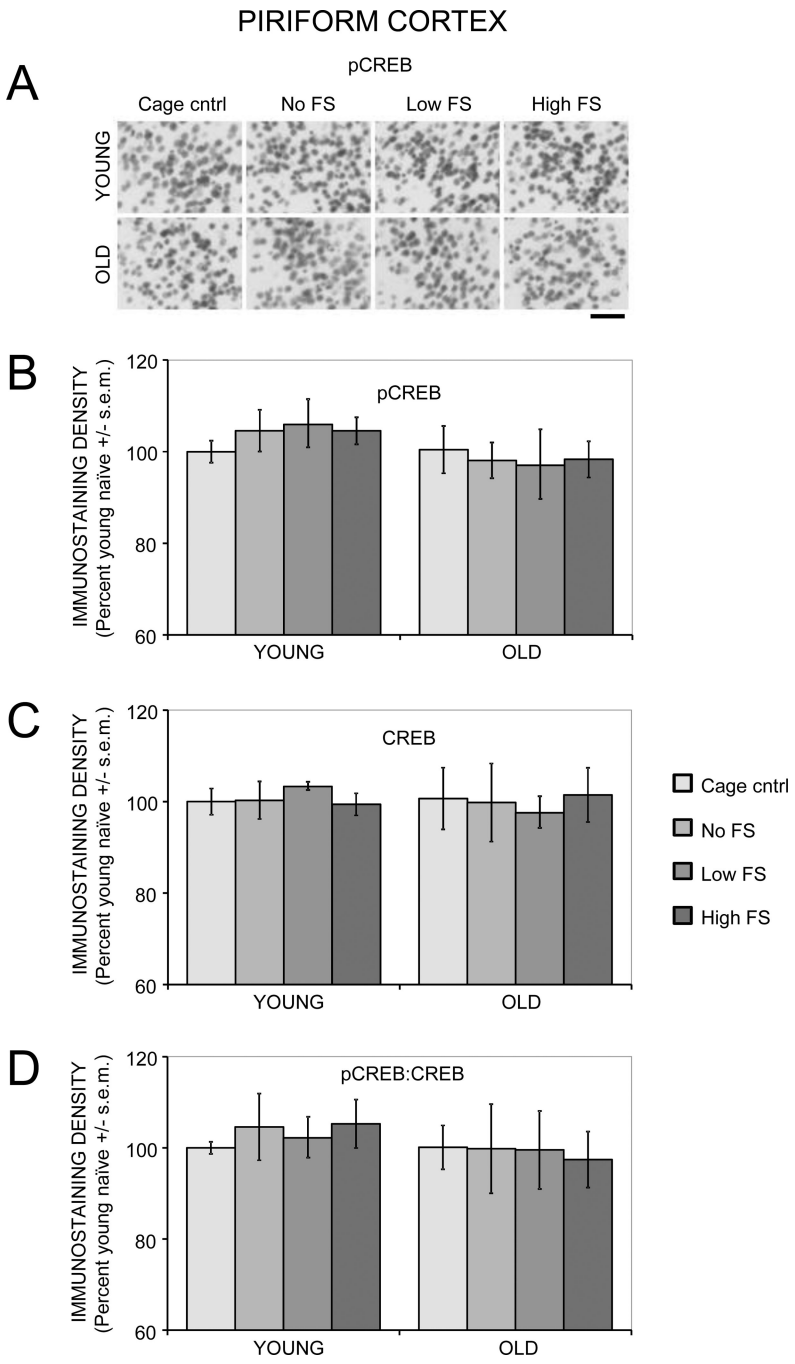

Figure 6.

Age- and training-associated differences in pCREB and CREB immunoreactivity in the piriform cortex. (A) Representative photomicrographs of pCREB immunostaining. Scale bar = 50 μm. There were no age- or training-related differences in pCREB (B), CREB (C), or ratio of pCREB:CREB (D).

Table 1

Summary of Immunohistochemistry Results

	TRAINING EFFECT		AGE EFFECT
	YOUNG RATS	OLD RATS	
pCREB			
DG	✓		✓
CA1	✓		✓
CA3			
BLA	✓		✓
LA	✓		✓
PIR			
CREB			
DG			
CA1			
CA3			✓
BLA			✓
LA			✓
PIR			
pCREB:CREB			
DG	✓		
CA1	✓		
CA3			
BLA	✓		
LA			
PIR			

✓ denotes significant main effect of age or training with footshock, along with significant effect in at least one post-hoc planned comparison; Brain regions analyzed were dentate gyrus (DG), area CA1, and area CA3 of the hippocampus, basolateral (BLA) and lateral (LA) amygdala, and piriform cortex (PIR).