Altered Regulation of Protein Kinase A Activity in the Medial Prefrontal Cortex of Normal and Brain-Injured Animals Actively Engaged in a Working Memory Task

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Abstract

Cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) signaling is required for short- and long-term memory. In contrast, enhanced PKA activity has been shown to impair working memory, a prefrontal cortex (PFC)-dependent, transient form of memory critical for cognition and goal-directed behaviors. Working memory can be impaired after traumatic brain injury (TBI) in the absence of overt damage to the PFC. The cellular and molecular mechanisms that contribute to this deficit are largely unknown. In the present study, we examined whether altered PKA signaling in the PFC as a result of TBI is a contributing mechanism. We measured PKA activity in medial PFC (mPFC) tissue homogenates prepared from sham and 14-day postinjury rats. PKA activity was measured both when animals were inactive and when actively engaged in a spatial working memory task. Our results demonstrate, for the first time, that PKA activity in the mPFC is actively suppressed in uninjured animals performing a working memory task. By comparison, both basal and working memory-related PKA activity was elevated in TBI animals. Inhibition of PKA activity by intra-mPFC administration of Rp-cAMPS into TBI animals had no influence on working memory performance 30 min postinfusion, but significantly improved working memory when tested 24 h later. This improvement was associated with reduced glutamic acid decarboxylase 67 messenger RNA levels. Taken together, these results suggest that TBI-associated working memory dysfunction may result, in part, from enhanced PKA activity, possibly leading to altered expression of plasticity-related genes in the mPFC.

Key words: CREB; memory; PKA signaling; prefrontal cortex; traumatic brain injury

Introduction

MEMORY IMPAIRMENTS are one of the primary complaints of persons with traumatic brain injury (TBI).^{1–4} These impairments, which can occur in the absence of overt brain damage, can influence daily activities and affect quality of life. A number of studies have examined the cellular and molecular mechanisms underlying memory impairments and have suggested that altered neuronal plasticity may be a key contributor.^{5–11} One mechanism implicated in the memory impairments associated with TBI is a reduction in signaling through the cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway in the hippocampus.¹² It has been demonstrated that whereas short-term memory formation involves PKA-mediated phosphorylation of substrate proteins, such as ion channels and membrane receptors, long-term memory requires PKA-dependent phosphorylation of the transcription factor, cAMP response element-binding protein (CREB) and CREB-mediated gene expression.^{13,14} In contrast to its role in short- and long-term memory, activation of PKA in the prefrontal cortex

(PFC) has been shown to impair working memory, a transient form of memory critical for goal-directed behavior.^{15,16} For example, normal aging is associated with working memory dysfunction, elevated basal PKA activity, and CREB phosphorylation in the PFC.¹⁷ Interestingly, inhibition of PKA within the PFC using ((R)-Adenosine, cyclic 3',5'-(hydrogenphosphorothioate) triethylammonium) (Rp-cAMPS) ameliorates age-associated working memory dysfunction.

Working memory dysfunction is often observed in persons who have sustained a TBI.^{18–20} This dysfunction can occur in the absence of overt damage to the PFC, suggesting that impaired neuronal function and/or altered circuitry in this brain region may be involved.^{18,21} Deficits in working memory function in the subacute stage of experimental brain injury (days to weeks after the insult) have been associated with enhanced catecholamine synthesis and signaling in the medial PFC (mPFC).²² Because catecholamine receptors can be Gs-coupled, it is possible that enhanced signaling through these receptors would result in elevated cAMP and PKA activity, possibly contributing to working memory dysfunction.

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Further, given that PKA is a major kinase responsible for CREB phosphorylation, and the phosphorylation of the CREB has been reported to be elevated in the mPFC during the acute phase of injury, it is plausible that sustained elevated expression of CREB-responsive genes may alter neuronal properties and negatively affect working memory.^{22,23}

Although pharmacological activation of prefrontal PKA has been demonstrated to impair working memory, whether PKA activity is regulated during working memory has not been examined. We therefore examined whether PKA activity is altered when animals are engaged in a working memory task. We present data to show that, in uninjured animals, PKA activity is suppressed in animals performing a working memory task. In brain-injured animals, PKA activity in the mPFC was found to be elevated, compared to uninjured controls. However, inhibition of PKA by intra-mPFC infusions of Rp-cAMPS did not immediately improve working memory in brain-injured animals. Interestingly, working memory was enhanced in a delayed fashion, suggesting that altered gene expression as a result of sustained PKA activity may be an underlying mechanism for the observed working memory deficits.

Methods

Materials

The PKA inhibitor, Rp-cAMPS, and the CaMKII inhibitor, KN-67 (4-[(2S)-2-[(5-isoquinolylsulfonyl) methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)propyl] phenyl isoquinolinesulfonic acid ester), were purchased from Tocris Bioscience (Ellisville, MO). PKA regulatory (PKA RI- α /B) and catalytic (PKA C- α) antibodies (Abs) were purchased from Cell Signaling Technology (Danvers, MA).

Controlled cortical impact injury

Male Sprague-Dawley rats (260–300 g) were purchased from Harlan (Indianapolis, IN). All protocols involving the use of animals were in compliance with the National Institutes of Health's (NIH's) *Guide for the Care and Use of Laboratory Animals* and were approved by the institutional animal care and use committee. An electric controlled cortical impact device (Virginia Commonwealth University Custom Design & Fabrication, Richmond, VA) was used to administer a unilateral brain injury, as previously described.^{24–26} Male Sprague-Dawley rats were anesthetized with 4% isoflurane and a 2:1 mixture of N₂O/O₂, then mounted in a stereotaxic frame. The head was held in a horizontal plane, and a 7-mm craniectomy was performed on the right cranial vault. The center of the craniectomy was placed at 3.0 mm posterior of the bregma and 3.5 mm lateral to the mid-line. Animals received a single impact of 3.3 mm deformation with an impact velocity of 4.0 m/sec at an angle of 10 degrees from the vertical plane using a 6-mm-diameter impactor tip. Impact was delivered onto the parietal association cortex. Body temperature was maintained at 37°C by the use of a heating pad.

Working memory

All behavioral tests were performed by an experimenter blind to the treatment groups. The delay match-to-place working memory task was carried out as previously described using five pairs of location-match trials.^{27–29} For tissue preparation, after locating the platform in the fifth location trial, the animal was allowed to remain on it for 10 sec, then removed from the tank and immediately killed (within 20 sec). mPFC tissue was removed and used for PKA activity measurements. For testing the influence of intra-mPFC drug infusions, rats were bilaterally implanted with sterile stainless-steel guide cannulae aimed at the dorsal border of the mPFC using a stereotaxic device (bregma 3.2 mm, lateral $\pm$ 0.75 mm, and depth 2.5 mm). Implantations were carried out immediately after TBI.

Infusions (0.25 μ L/min for 4 min) were carried out 30 min before working memory assessment.

Protein kinase A activity assay

Activity of PKA was measured using a MESACUP protein Kinase Assay Kit (MBL International Co., Woburn, MA) as recommended by the manufacturer. Briefly, 25 μ g of mPFC proteins were used for the assay using the PS peptide (RFARKGSLRQKNV, with S being the site of phosphorylation) as a substrate. Because we have observed that freezing the tissue causes a marked loss in PKA activity, tissue homogenates were prepared from freshly dissected mPFC. The assay was carried in 25 mM of Tris (pH 7.0), 3 mM of MgCl₂, 0.1 mM of adenosine triphosphate, 0.5 mM of ethylenediaminetetraacetic acid, 1 mM of ethylene glycol tetraacetic acid, and 5 mM of 2-mercaptoethanol. Phosphorylation of the serine residue was detected with a biotinylated monoclonal Ab and peroxidase-conjugated streptavidin, and colorization of a peroxidase substrate. The assays were performed with and without 20 μ M of cAMP to compare fully stimulated PKA activity and the unstimulated activity. A purified PKA catalytic subunit (Sigma-Aldrich, St. Louis, MO) was used to generate standard curve for PKA activity assay.

Western blotting

Equal amounts of mPFC protein extracts were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, then transferred to Immobilon-P membranes (Millipore, Billerica, MA). After overnight blocking in 5% bovine serum albumin (BSA), membranes were incubated for 3 h in primary Abs (0.1–0.5 μ g/mL) at room temperature. Membranes were washed, incubated with species-specific, alkaline-phosphatase-conjugated secondary Abs and immunoreactivity detected using a chemiluminescence substrate. Bands were quantified using ImageJ software (freely available through NIH).

Immunohistochemistry

To assess the extent of Rp-cAMPS diffusion, rats were infused into the mPFC as described above with either saline or Rp-cAMPS, then killed 1 or 24 h later. Brains were removed, cut into 2-mm slabs, and drop-fixed overnight in ice-cold 4% paraformaldehyde/15% picric acid in phosphate-buffered saline (PBS). Cryoprotected brains were sectioned on a cryostat. Free-floating slices (40 μ m in thickness) that spanned the mPFC or dorsal hippocampus were incubated overnight in primary Ab (0.5–1.0 μ g/mL) in PBS plus 0.25% Triton X-100 containing 2% BSA and 2.5% normal goat serum. After extensive washing, immunoreactivity was detected using species-specific secondary Abs coupled to Alexa Fluors. Immunofluorescence results were corroborated in separate sections using horseradish-peroxidase-conjugated secondary Abs and diaminobenzadine. Sections were visualized and photographed using a Zeiss Axiovert 2 (Carl Zeiss AG, Jena, Germany) microscope.

Quantitative real-time polymerase chain reaction

Messenger RNA (mRNA) was extracted from mPFC tissue with biotinylated poly-thymidine and streptavidin paramagnetic particles using a PolyATract System 1000 mRNA extraction kit (Promega, Madison, WI) as recommended by the manufacturer. The concentration of the mRNA was determined using a RiboGreen RNA quantification kit (Invitrogen, Carlsbad, CA). A 20-ng sample of mRNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). The glutamic acid decarboxylase 67 (Gad67) mRNA level, relative to the neuron-specific β -tubulin mRNA level, in each sample was quantified by amplification of the complementary DNA using TaqMan probes and the comparative threshold cycle (C_T) method (Schmittgen and Livak, 2008)³⁰ in a

StepOne real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA). Sequences of the primers and TaqMan probes were as follows: Gad67: forward primer 5'-CAAACCTCAGCGGCATAGAAA-3', reverse primer 5'-GAAGA GGTAGCCTGCACACA-3', probe 5'-TGCTGCTCCAGTGTCTTCTGCCA-3'; neuron-specific β -tubulin: forward primer 5'-CTCA ACCACCTTGTGTCT-3', reverse primer 5'-CATGAAGAAA TGCAAGCG-3', probe 5'-ATGAGCGGAGTCACCACC-3'.

Statistical analysis

Student's *t*-tests were used for the analysis of western blot data, and one-way analyses of variance (ANOVAs) were used to analyze changes in PKA activity over time. Two-way ANOVA was used for evaluating working memory performance and the effect of training on PKA activity. Results were considered significant at $p < 0.05$, with a Holm-Sidak method for multiple comparisons as the post-hoc test to isolate the groups with differences. Data are presented as the mean $\pm$ standard error of the mean (SEM).

Results

Protein kinase A activity is increased in the medial prefrontal cortex of brain-injured animals

Previous studies have shown that pharmacological activation of PKA within the mPFC of normal animals impairs working memory.^{15,16} We therefore questioned whether the working memory dysfunction observed after TBI is associated with enhanced PKA activity in the mPFC. Figure 1A shows the relative position of the mPFC (comprised of the prelimbic [PL] and infralimbic [IL] cortices) and the measurements used for tissue dissection. Figure 1B shows that mPFC tissue homogenates obtained from brain-injured animals have significantly increased PKA activity at both 7 and 14 days postinjury, compared to that observed in sham controls ($n = 6$ /condition; $p = 0.009$ by one-way ANOVA). The increase in PKA activity may have resulted from either an increase in the levels of the catalytic subunit, or a decrease in the levels of the regulatory subunit, after injury. To assess these possibilities, we measured total PKA activity (i.e., activity in the presence of 20 μ M of cAMP) and carried out western blots to examine subunit levels. The results presented in Figure 1C show that the inclusion of 20 μ M of cAMP in the assay mixture results in PKA activity that did not differ across groups, suggesting no change in total PKA activity. Examination of the levels of the catalytic subunit of PKA (C- α) by western blot did not reveal any significant changes between sham and TBI groups ($p = 0.414$; Fig. 1D). Further, no significant change in the levels of the regulatory subunit (RI- α/β) in the mPFC were detected after injury ($p = 0.459$; Fig. 1D).

Working memory decreases protein kinase A activity in the medial prefrontal cortex

Although activation of PKA has been found to impair working memory in normal animals, its inhibition has been reported to be inconsequential.¹⁵ This suggests that PKA activity may be suppressed during working memory, though this possibility has not been tested. To examine this, uninjured (sham) animals ($n = 4$ /group) were trained in the delay match-to-place task on day 14 postsurgery. On the fifth trial, animals were given only the location trial, after which they were quickly killed and mPFC tissues dissected (Fig. 2A). TBI animals were similarly tested on day 14 postinjury, with half the animals ($n = 4$) receiving working memory training and the other half ($n = 4$) remaining untrained. Homogenates from freshly dissected mPFC tissues were used to measure PKA activity, as described in the *Methods* section. When analyzed using a two-way ANOVA

($F_{(1,12)} = 5.69$; $p = 0.03$), PKA activity was found to be markedly suppressed ($p = 0.01$) in uninjured animals performing the working memory task, as compared to untrained sham controls (Fig. 2B). PKA activity was also found to be significantly suppressed ($p = 0.002$) as a result of working memory training in TBI rats, although not to the level observed in trained sham animals.

Intramedial prefrontal cortex Rp-cAMPS infusion improves working memory in traumatic brain injury rats

To examine whether suppressing the elevated mPFC PKA activity observed in TBI animals can improve working memory, Rp-cAMPS (2 nmole/side) or vehicle (saline) was infused bilaterally into the mPFC of injured animals ($n = 8$ /group) on day 14 postinjury. Performance in the delay match-to-place task was assessed 30 min and 24 h later to assess both the acute and long-term consequences of PKA inhibition. Figure 3A shows that vehicle-infused, injured animals are incapable of performing the working memory task, as indicated by similar latencies to find the hidden platform during the location and match trials. Rp-cAMPS infusion did not improve this performance (interaction of treatment and trial; $F_{(1,13)} = 1.03$; $p = 0.33$) when tested 30 min postinfusion. However, when the same animals were retested 24 h later, Rp-cAMPS-infused, injured animals were found to have significantly improved performance, as compared to vehicle-infused injured controls ($F_{(1,13)} = 5.29$; $p = 0.04$; Fig. 3B). The lack of an improvement in working memory 30 min after Rp-cAMPS could have resulted from an inability of the animals to recover from the infusion by this time point. To test this possibility, we bilaterally infused KN-67 (1 μ g/side), an inhibitor of calcium/calmodulin-dependent protein kinase II (CaMKII), into the mPFC of a separate group of day 14 postinjury animals and tested working memory 30 min later. This drug was chosen because previous studies have shown that working memory in normal animals is enhanced after infusion of a CaMKII inhibitor into the mPFC.³¹ Figure 3C shows that injured animals infused with KN-67 perform the working memory task significantly better than simultaneously tested vehicle controls. After the completion of behavioral training, infusion needle placement was assessed in a representative group of animals. Figure 3D shows a representative cresyl-violet-stained tissue section demonstrating that the infusion needle tracks terminated at the dorsal border of the mPFC (arrows).

Intramedial prefrontal cortex infusion of Rp-cAMPS reduces cyclic adenosine monophosphate response element-binding protein phosphorylation in the medial prefrontal cortex, but not the hippocampus

In addition to the mPFC, the spatial working memory task we employed is also dependent on the function of the hippocampus, suggesting that diffusion of the drug to this structure may have contributed to the results we observed. To examine the extent of Rp-cAMPS diffusion, we measured phospho-CREB immunoreactivity. Although some suppression in general immunoreactivity was observed along the needle tracks of all animals examined (data not shown), Figure 4 shows that phospho-CREB (Ser¹³³) immunoreactivity was markedly reduced proximal to the infusion site by 1 h postinfusion of Rp-cAMPS in the mPFC. This time point was chosen because it approximately corresponds to the time from infusion to the completion of the working memory testing shown in Figure 3A. The differential staining intensity of cells within the core, compared to those unaffected by the Rp-cAMPS, makes it appear that there is a complete absence of phospho-CREB immunoreactivity. However, the high magnification inset shows that

FIG. 1. Traumatic brain injury (TBI) increases basal protein kinase A (PKA) activity in the medial prefrontal cortex (mPFC). Rats ($n=6$ /time point) were subjected to controlled cortical impact injury and mPFC tissues removed at 7 and 14 days postinjury. Sham-operated animals were used as controls. **(A)** Representative photomicrograph showing a cresyl-violet-stained tissue section and the relative positions of the corpus callosum (cc), cingulate (Cg), prelimbic (PL), and infralimbic (IL) cortices of rat mPFC. Illustration of a coronal section of rat PFC indicating the measurements used for tissue dissection. Relative level of PKA activity measured in mPFC extracts in the **(B)** absence and **(C)** presence of excess cAMP. Basal PKA activity in the mPFC was found to be significantly increased at both 7 and 14 days postinjury. **(D)** Representative images of western blots for the catalytic (PKAcat) and regulatory (PKAreg) subunits of PKA from sham and 14-day post-TBI animals. Quantification ($n=3$ /group) did not reveal any significant changes in the levels of PKA subunits in response to injury. Data are presented as the mean $\pm$ SEM. $*p<0.05$. d, day. Color image is available online at www.liebertpub.com/neu

weakly immunopositive nuclei can be seen in the core of the infusion. By 24 h after infusion, phospho-CREB immunoreactivity had returned to normal levels. When tissue sections were immunoreacted for total CREB, no visible change was observed between the groups. In the hippocampus, phospho-CREB immunoreactivity was unaffected by Rp-cAMPS infusion into the mPFC, with similar levels of staining observed in the dentate gyrus, CA1/

CA2, and the CA3. To ensure that the decrease in phospho-CREB staining in the mPFC we observed was not the result of a general effect on phosphoimmunoreactivity, sections were also immunostained for phosphorylated extracellular signal-regulated kinase1/2 (Erk1/2; on Thr²⁰²/Tyr²⁰⁴). No obvious difference in phospho-Erk1/2 immunoreactivity was observed in the mPFC of Rp-cAMPS-infused animals, compared to vehicle-infused controls.

FIG. 2. Protein kinase A (PKA) activity in the medial prefrontal cortex (mPFC) is suppressed in animals performing a spatial working memory task. PKA activity in the mPFC was assessed in sham and 14-day postinjury rats that were trained in the working memory task ($n=4/\text{group}$). Untrained sham and injured animals were used as controls ($n=4/\text{group}$). (A) Schematic representation of the training paradigm and the time of euthanasia. (B) Summary data for PKA activity in trained and untrained injured and sham animals. Data are presented as the mean $\pm$ SEM. *1, significant difference ($p<0.05$) between trained and untrained sham animals; *2, significant difference ($p<0.05$) between trained and untrained traumatic brain injury (TBI) animals. WM, working memory.

Intramedial prefrontal cortex infusion of Rp-cAMPS decreases traumatic brain injury-induced glutamic acid decarboxylase 67 expression

The above-described results suggest that Rp-cAMPS may have improved working memory 24 h postinfusion by decreasing CREB-responsive gene expression. We have previously shown that CCI increases Gad67 protein expression (the rate-limiting enzyme for gamma-aminobutyric acid [GABA] synthesis).²¹ Because Gad67 expression can be regulated by CREB, quantitative real-time PCR was employed to measure Gad67 mRNA levels in the mPFC after injury and in response to Rp-cAMP infusion.³² Figure 5A shows that TBI causes a significant increase in Gad67 mRNA that is maximal by 24 h postinjury and remains significantly elevated by 14 days postinjury (one-way ANOVA; $F_{(4,20)}=91.1$; $p<0.001$). When 14-day postinjury animals were infused with Rp-cAMPS into the mPFC, significant reductions in Gad67 mRNA levels were observed at both the 30-min and 24-h time points (Fig. 5B).

Discussion

Working memory impairments are thought to be key contributors to a number of cognitive and behavioral deficits that are often observed in people who have sustained a TBI.^{33,34} Clinical and

experimental studies have shown that TBI can profoundly alter working memory in the absence of overt damage to the PFC, suggesting impaired function of neurons and/or plasticity.²¹ Because enhanced PKA activity in the mPFC has been shown to impair working memory, we measured its activity in the mPFC of TBI animals and examined its contribution to TBI-induced working memory dysfunction.¹⁵ The results from the present study revealed four important findings: 1) PKA activity is significantly reduced in uninjured animals actively engaged in a spatial working memory task; 2) basal PKA activity is markedly increased in the mPFC of brain-injured rats, as compared to sham controls; 3) although injured animals can suppress PKA activity during working memory, residual PKA activity persists; and 4) working memory in brain injured animals is improved when tested 24 h, but not 30 min, after intra-mPFC infusion of the PKA inhibitor, Rp-cAMPS.

Previously, it has been reported that enhancing PKA activity by direct infusion of the activator, Sp-cAMP, into the mPFC of normal rodents impairs working memory.¹⁵ In contrast, inhibition of PKA activity by infusion of Rp-cAMPS had no demonstrable influence on working memory performance, suggesting that either basal mPFC PKA activity does not play a role in working memory or that PKA activity is suppressed when working memory is required.¹⁵ To distinguish between these possibilities, we examined PKA activity

FIG. 3. Intra-mPFC (medial prefrontal cortex) administration of Rp-cAMPS improves working memory performance in a delayed manner. Fourteen days after traumatic brain injury, rats were bilaterally infused into the mPFC with either Rp-cAMPS or vehicle ($n = 8/\text{group}$). Working memory was tested using the delay match-to-place task. **(A)** When tested 30 min postinfusion, no significant difference was detected between the vehicle- and Rp-cAMPS-treated injured animals. **(B)** When tested 24 h postinfusion, Rp-cAMPS-infused injured animals performed significantly better than vehicle-treated injured controls, as indicated by a significantly reduced latency to find the hidden platform in the match trials. **(C)** Bilateral intra-mPFC infusion of the CaMKII inhibitor, KN-67, to 14-day postinjury animals improves working memory when tested 30 min later. **(D)** Representative photomicrograph of a cresyl-violet-stained tissue section showing the infusion track within the mPFC of an animal killed 1 h after infusion (left side) and approximate corresponding panel from Paxinos and Watson (right side). Data are presented as the mean $\pm$ SEM. *Significant interaction of group and trial by two-way ANOVA ($p < 0.05$). Loc, location; PL, prelimbic; IL, infralimbic; cc, corpus callosum. Color image is available online at www.liebertpub.com/neu

FIG. 4. Intra-mPFC (medial prefrontal cortex) administration of Rp-cAMPS decreases cAMP response element-binding protein (CREB) phosphorylation in the mPFC, but not in the hippocampus. Representative photomicrographs of phospho-CREB, CREB, and phospho-Erk (extracellular signal-regulated kinase) immunoreactivity in mPFC tissue sections taken from a vehicle-infused, a 1-h post-Rp-cAMPS-infused, and a 24-h post-Rp-cAMPS-infused rat mPFC. A dramatic reduction in phospho-CREB (p-CREB) immunoreactivity can be seen in the 1-h post Rp-cAMP infusion tissue that resolves by 24 h. Inset: High magnification photomicrograph from within the core of the mPFC infusion site showing weakly phospho-CREB immunopositive cells. Scale bar represents 200 μ m for p-CREB and CREB in the mPFC, and 50 μ m for phospho-ERK (p-ERK). Scale bar for images of the hippocampus represents 1 mm. Color image is available online at www.liebertpub.com/neu

during the “delay” interval of a spatial working memory task and found that it was markedly suppressed in animals performing the task, as compared to untrained controls. To our knowledge, this is the first study to report that PKA activity is reduced in the mPFC when animals are engaged in a working memory task and may explain the absence of effect of PKA inhibitors on working memory in normal animals.

One weakness of the present study is that it does not reveal the mechanism by which PKA activity is suppressed as a result of working memory. The PKA holoenzyme consists of a catalytic subunit dimer (composed of α , β , or γ homodimers) and a

regulatory subunit dimer (composed of RI and RII homo or heterodimers). Binding of cAMP to the regulatory subunits causes their dissociation from the catalytic subunits, allowing the catalytic subunits to carry out substrate phosphorylation. Thus, working memory may have suppressed PKA activity by decreasing the levels of cAMP either by reducing its synthesis (by Gi-protein-mediated inhibition of adenylyl cyclase) or by enhancing its degradation (by activation of cAMP phosphodiesterase). In addition to altering cAMP availability, working memory activity could have influenced the phosphorylation of PKA itself. For example, phosphorylation of the catalytic subunit (e.g., Thr197 in the activation

FIG. 5. Intra-mPFC (medial prefrontal cortex) administration of Rp-cAMPS reduces glutamic acid decarboxylase 67 (Gad67) messenger RNA (mRNA) in the mPFC after injury. **(A)** Time course for changes in Gad67 mRNA levels in the mPFC after traumatic brain injury TBI; $n = 5$ /time point. **(B)** Gad67 mRNA in the mPFC is significantly reduced in 14-day postinjury animals treated with Rp-cAMPS at both the 30-min and 24-h time points. Data are presented as the mean $\pm$ SEM. * $p < 0.05$. d, day; Veh, vehicle.

loop) is required for maximal PKA activity. Although this site is unusually resistant to phosphatase-mediated inactivation, its dephosphorylation as a result of working memory training would be anticipated to reduce PKA activity.³⁵ Finally, phosphorylation of the regulatory subunits has been demonstrated to negatively influence their reassociation with the catalytic subunits.³⁶ Dephosphorylation of the regulatory subunit as a result of working memory training could therefore enhance holoenzyme assembly and suppress kinase activity. Future studies will aim to determine the mechanism underlying the working-memory-related suppression of PKA activity we observed.

Our results that PKA activity is suppressed as a result of working memory training is consistent with the premise that a tilt in the balance between protein kinase and protein phosphatase activity, in favor of phosphatase, is critical for working memory.¹⁶ We have previously shown that working memory enhances the activity of the protein phosphatase, calcineurin, and that inhibition of its activity impairs working memory function.³¹ Although, at present, it is not clear whether the suppression of PKA activity and enhancement of calcineurin activity occur in the same cells, recent studies have shown that these signaling molecules interact with anchoring proteins that place them in close proximity. For example, A-kinase anchoring protein 79/150 (AKAP79/150) has been shown to bind to both the regulatory subunit of PKA and calcineurin, where they can regulate the phosphorylation of targets such as L-type calcium channels.^{37,38} Similarly, the synaptic incorporation of GluR1 has been shown to be regulated by the opposing actions of AKAP-150-anchored PKA and calcineurin³⁹ as has the plasticity associated with GABA_A receptors.⁴⁰ Although the complexity of membrane anchoring, and how this conveys target selectivity, is only beginning to be understood, our results suggest that, similar to longer-lasting forms of plasticity, PKA and calcineurin may have opposing roles in the regulation of the neuronal activity required for working memory.

The results presented herein show that PKA activity is increased in the mPFC after TBI. The TBI-associated increase in PKA activity does not appear to have resulted from altered expression of PKA C- α or PKA RI- α/β , although the levels of other isoforms were

not examined. Further, there was no significant difference between sham and TBI animals when total PKA activity was assessed. These results suggest that enhanced levels of cAMP within the mPFC may be the underlying mechanism. Although we did not measure tissue cAMP levels, TBI has been shown to increase mPFC catecholamine levels as well as alter dopamine neurotransmission in other brain regions.^{21,29,41–43} It is plausible that activation of G_s-coupled receptors (e.g., dopamine D1) by increased catecholamine signaling may result in increased cAMP levels and the enhanced PKA activity we observed. Future experiments will aim to determine the mechanism of PKA activation and the role, if any, enhanced catecholamine synthesis plays.

Arnsten and colleagues have shown that the working memory deficits associated with aging are caused by enhanced prefrontal PKA activity, and that acute inhibition of PKA activity can improve working memory performance.⁴⁴ Contrary to these results, working memory dysfunction was not improved in TBI animals treated with Rp-cAMPS when the testing was carried out 30 min postinfusion.¹⁷ Rather, we observed an improvement in working memory performance 24 h after the infusion. Long-lasting increases in PKA activity can increase the phosphorylation of the transcription factor, CREB, leading to enhanced expression of a number of genes that can affect neuronal plasticity.⁴⁵ Consistent with this possibility, we found that intra-mPFC infusion of Rp-cAMPS resulted in decreased CREB phosphorylation and a reduction in the mRNA levels of a putative CREB target, Gad67 (Fig. 5).

Gad67 is the rate-limiting enzyme in the production of the inhibitory neurotransmitter, GABA, the levels of which have been intimately linked to both prefrontal function and dysfunction.^{46–48} We have previously reported that experimental TBI causes a lasting increase in Gad67 protein levels within the mPFC, which would increase GABA synthesis and release. Given that the balance between excitatory and inhibitory activity within the mPFC is critical for the maintenance of working-memory-related delay cell activity, enhanced GABA synthesis and release could curtail delay cell activity and cause working memory dysfunction. Consistent with this, we have shown that intra-mPFC administration of GABA_A receptor antagonists can be used to improve working memory function in brain-injured animals.²¹ The present results show that this increase in Gad67 protein is likely caused by enhanced mRNA levels and further suggest that this increase is PKA-CREB mediated. It is therefore possible that the delayed improvement in working memory we observed as a result of PKA inhibition may have resulted from a reduction in *Gad67* mRNA and protein levels. However, given that multiple genes have CREB-binding sites, altered expression of these targets as a result of Rp-cAMPS infusion may have also contributed to the results we observed.⁴⁹ Similarly, PKA has been demonstrated to phosphorylate a number of other transcription factors whose possible contributions cannot be excluded.⁵⁰

Given that the spatial working memory task we employed is dependent on the function of the hippocampus, drug diffusion into this structure could have contributed to an improvement in working memory performance. However, our experiments to examine diffusion utilizing the phosphorylation of CREB as a marker of PKA activity indicate that the influence of Rp-cAMPS was restricted to the mPFC. Further, studies using the phosphodiesterase inhibitor, rolipram, have indicated that enhancing, rather than inhibiting, PKA activity in the hippocampus after TBI is associated with improved performance in spatial memory tasks.⁵¹ Thus, diffusion of Rp-cAMPS into the hippocampus would be anticipated to have

impaired, rather than improved, performance in the spatial working memory task employed.

A number of studies have shown that PKA can phosphorylate plasticity-related targets, such as N-methyl-D-aspartate receptors, synapsin, α -amino-3-hydroxy-5-methyl-4-isoxalone propionic acid receptors, L-type voltage-gated Ca^{2+} channels, dopamine- and cAMP-regulated neuronal phosphoprotein 32, and CREB, that are required for short- and long-term memory.⁴⁵ Based on this, it has been suggested that inhibition of PKA activity as a treatment may be counterintuitive given that this would be expected to cause both short- and long-term memory dysfunction.⁵² However, our results suggest that, at least for the treatment of TBI, inhibition of PKA may be required to reverse lasting dysfunction of the prefrontal cortex. TBI can cause several behavioral changes that are thought to be the result of dysfunction of the PFC.⁵³ These include difficulties with initiating, organizing, and carrying out activities, impulsivity, compulsivity, inflexible thoughts, mood disturbances such as depression, inappropriate social behaviors, poor judgment, and impaired attention.⁵³ Although not specifically tested, our results suggest that some of these dysfunctions may also be improved by inhibiting excess PKA activity. Future studies will be directed toward evaluating the effect of PKA activity on other functions of the PFC after TBI and the persistence of the improvement observed after a single administration of a PKA inhibitor.

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