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ALTERED ADRENERGIC RECEPTOR SIGNALING FOLLOWING TRAUMATIC BRAIN INJURY CONTRIBUTES TO WORKING MEMORY DYSFUNCTION

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Abstract—The prefrontal cortex is highly vulnerable to traumatic brain injury (TBI) and its structural and/or functional alterations as a result of TBI can give rise to persistent working memory (WM) dysfunction. Using a rodent model of TBI, we have described profound WM deficits following TBI that are associated with increases in prefrontal catecholamine (both dopamine and norepinephrine) content. In this study, we examined if enhanced norepinephrine signaling contributes to TBI-associated WM dysfunction. We demonstrate that administration of α1 adrenoceptor antagonists, but not α2A agonist, at 14 days post-injury significantly improved WM performance. mRNA analysis revealed increased levels of α1A, but not α1B or α1D, adrenoceptor in the medial prefrontal cortex (mPFC) of brain-injured rats. As α1A and 1B adrenoceptor promoters contain putative cAMP response element (CRE) sequences, we therefore examined if CRE-binding protein (CREB) actively engages these sequences in order to increase receptor gene transcription following TBI. Our results show that the phosphorylation of CREB is enhanced in the mPFC at time points during which increased α1A mRNA expression was observed. Chromatin immunoprecipitation (ChIP) assays using mPFC tissue from injured animals indicated increased phospho-CREB binding to the CRE sites of α1A, but not α1B, promoter compared to that observed in uninjured controls. To address the translatability of our findings, we tested the efficacy of the FDA-approved α1 antagonist Prazosin and observed that this drug improves WM in injured animals. Taken together, these studies suggest that enhanced CREB-mediated expression of α1 adrenoceptor contributes to TBI-associated WM dysfunction, and therapies aimed at reducing α1 signaling may be useful in the treatment of TBI-associated WM deficits in humans.

Key words: catecholamines, neurotransmitter signaling, protein kinase A, CREB-mediated gene expression, chromatin immunoprecipitation.

The prefrontal cortex (PFC) is highly vulnerable to traumatic brain injury (TBI), and damage to this structure can cause cognitive deficits including problems with working memory (WM). WM, the ability to transiently hold information in mind in order to guide goal-directed behavior, is critical for many higher cognitive functions. While overt damage (as detected using brain imaging) results in WM deficits, many people with TBI show WM dysfunction in the absence of detectable PFC damage. Identification of the mechanisms underlying these deficits is an intense area of investigation. Using a rodent model, we have shown that TBI can cause persistent WM problems in the absence of detectable neuronal loss in the medial prefrontal cortex (mPFC), a structure anatomically and functionally equivalent to the dorsolateral prefrontal cortex (dlPFC) in humans and non-human primates (Moghaddam and Homayoun, 2008). Interestingly, these deficits were found to be associated with increased PFC catecholamine levels, suggesting that exaggerated dopamine and/or norepinephrine signaling may contribute to the WM deficits seen after TBI. We have previously shown that both systemic and intra-mPFC administration of D1 receptor antagonists to brain-injured animals reduce WM dysfunction (Kobori et al., 2006; Kobori and Dash, 2006). The consequence of elevated norepinephrine on WM dysfunction following TBI has not been examined.

It has been shown that both excessive and insufficient norepinephrine levels impair WM, indicating that an optimal amount of norepinephrine is required for normal WM function (Arnsten and Li, 2005). For example, the WM dysfunction caused by chronic stress is associated with increased norepinephrine levels, and this deficit can be reversed by administration of the α1 antagonist urapidil (Birnbaum et al., 1999; Southwick et al., 1999). In contrast, the WM deficits observed as a result of normal aging are associated with decreased norepinephrine levels. These deficits can be relieved by administration of the α2A agonist guanfacine, an effect thought to be due to stimulation of post-synaptic α2A receptors (Arnsten and Goldman-Rakic, 1985). Since α2A receptors are Gi coupled, this suggests that stimulation of these receptors would result in a decrease in cAMP signaling.

In this study, we investigated if excessive norepinephrine in the mPFC following TBI contributes to WM dysfunction. Our data show that administration of α1 antagonists improves WM function in brain-injured animals. We further demonstrate that TBI increases the levels of α1 adrenoceptor mRNA within the mPFC, that was associated with enhanced binding of phosphorylated cAMP response ele-

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Abbreviations: aCSF, artificial cerebrospinal fluid; AP-1, activating protein-1; ChIP, chromatin immunoprecipitation; CRE, cAMP response element; CREB, cAMP response element binding protein; DAB, 3,3′-diaminobenzadine; GAD67, glutamic acid decarboxylase 67; mPFC, medial prefrontal cortex; PFC, prefrontal cortex; PKA, protein kinase A; qPCR, quantitative polymerase chain reaction; TBI, traumatic brain injury; TH, tyrosine hydroxylase; WM, working memory.

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ment binding protein (CREB) to the α1A adrenoceptor promoter regions. Our findings suggest that therapies aimed at modulating norepinephrine signaling may have benefit in the treatment of TBI-induced WM dysfunction.

EXPERIMENTAL PROCEDURES

Materials

Phospho-CREB (Ser^{133}) and pan-specific CREB antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Monoclonal anti-glutamic acid decarboxylase 67 (GAD67) and monoclonal anti-NeuN antibodies were obtained from Millipore (Billerica, MA, USA). The α_1 adrenoceptor antagonist prazosin (*1-(4-Amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furanylcarbonyl) piperazine hydrochloride*), α1 adrenoceptor antagonist HEAT (*2-{[b-(4-Hydroxyphenyl)ethyl]aminomethyl}-1-tetralone hydrochloride*), and the α_{2A} adrenoceptor agonist guanfacine (*[(2,6-Dichlorophenyl)acetyl]guanidine hydrochloride*) were purchased from Tocris Bioscience (Ellisville, MO, USA).

Brain injury

All protocols involving the use of animals were in compliance with NIH's *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (260–300 g) were purchased from Harlan (Indianapolis, IN, USA). An electromagnetic controlled cortical impact (CCI) device (Virginia Commonwealth University Custom Design and Fabrication) was used to administer unilateral brain injury as previously described (Lyeth et al., 1990; Dixon et al., 1991; Smith et al., 1995). Rats were anesthetized with 4% isoflurane and a 2:1 mixture of N_2O:O_2, 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 midline. Animals received a single impact of 3.3 mm deformation with an impact velocity of 4.0 m/s at an angle of 10° from the vertical plane using a 6 mm diameter impactor tip. The impact was delivered onto the parietal association cortex. The body temperature was maintained at 37 °C by the use of a heating pad.

Western blotting

Fourteen days post-injury, animals were sacrificed and brains were dissected while submerged under ice-cold artificial CSF (10 mM HEPES pH 7.2, 1.3 mM NaH_2PO_4, 3 mM KCl, 124 mM NaCl, 10 mM dextrose, 26 mM $NaHCO_3$ and 2 mM $MgCl_2$). The mPFC tissues ipsilateral to the side of impact were quickly removed and snap-frozen on dry ice. Sham-operated animals were sacrificed at 14 days following surgery and used as controls. The mPFC brain tissue was homogenized using a motorized tissue grinder in a lysis-buffer containing 10 mM Tris pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF, and 0.1 μM okadaic acid, followed by centrifugation at 10,000×*g* for 10 min. The pelletized material was rinsed twice with the lysis-buffer, then re-suspended in lysis-buffer containing 0.5% Triton X-100. Following sonication (Sonic Dismembrator 100; Fisher Scientific, Pittsburgh, PA, USA), the Triton X-100 soluble fraction was collected and used as a membrane and nuclear fraction extract. The protein concentration was measured using a NanoOrange protein quantification kit (Invitrogen, Carlsbad, CA, USA). Extracts were denatured in 1× Laemmli buffer, resolved in a SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Bedford, MA, USA) followed by blocking overnight in TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20) plus 5% BSA. Membranes were then incubated with primary antibody (0.2 μg/ml) for 3 h at room temperature. Following incubation with the primary antibody, membranes were washed three times and immunoreactivity was assessed by an alkaline phosphatase-conjugated secondary antibody and a CDP-Star chemiluminescent substrate (Cell Signaling Technology). The optical density of the immunoreactive bands was measured utilizing ImageJ software (http://rsb.info.nih.gov/ij/index.html). Prior to reprobing, blots were stripped by two 10-min washes in 50 mM NaOH at room temperature. The membranes were then washed extensively with TBST and reblocked for an hour in 2% BSA prior to immunodetection.

Chromatin immunoprecipitation (ChIP)

The mPFC tissue of injured (ipsilateral to the injury) and sham animals were dissected at 14 days after the surgery as described in western blots section. The tissue was minced in small pieces and incubated in 1% paraformaldehyde in PBS at room temperature for 15 min to crosslink DNA binding proteins to the DNA. The tissue was then sonicated on ice for 5 s×5 times with 70% power using Sonic Dismembrator 100 (Fisher Scientific, Pittsburgh, PA, USA). This sonication condition was pre-determined to fragment the majority of genomic DNA to 200 to 600 base pair lengths. DNA concentration in samples was measured using a PicoGreen assay kit (Invitrogen) and a CytoFluor Multi Well Plate Reader (Applied Biosystems) and was normalized prior to carrying out the ChIP assay. ChIP was carried out using EZ-ChIP assay kit with procedures recommended by the manufacturer (Upstate, VA, USA). The sonicated lysates was incubated with either pan-specific anti-CREB or anti-phospho CREB antibodies in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.1, 167 mM NaCl) at 4 °C overnight. Precipitation was performed by incubating the samples with Protein G agarose for an hour at 4 °C. After brief centrifugation, the precipitated materials were washed sequentially with Low Salt Immune Complex Washing Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 150 mM NaCl), High Salt Immune Complex Washing Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.1, 500 mM NaCl), LiCl Immune Complex Washing Buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 20 mM Tris–HCl pH 8.1) for one time each, and TE Buffer two times. The immunoprecipitated material was eluted from Protein G in elution buffer (1% SDS, 100 mM $NaHCO_3$) for 15 min at room temperature two times. For negative control, IP was performed in an absence of antibody or using IgG non-immune antibody (Santa Cruz Biotechnology, CA, USA). After the completion of IP, the crosslink between DNA and protein was reversed by incubating the DNA-protein complex in 200 mM NaCl at 65 °C for 6 h, followed by degradation of RNA and proteins with RNase A and proteinase K, respectively. The DNA was purified using a spin column system provided as part of the EZ-ChIP kit. IP was performed in triplicate and final output DNA (purified DNA) was pooled for each sample.

RNA extraction and quantification

The mPFC tissue ipsilateral to the brain injury was removed as described above. The tissue was sonicated in Trizol (Invitrogen) and total RNA was extracted according to the manufacturer's protocol. The concentration of the total RNA was determined using RiboGreen RNA quantification kit (Invitrogen) and a CytoFluor Multi Well Plate Reader (Applied Biosystems). A 1 μg sample of total RNA was reverse transcribed for 2 h at 36 °C in a 20 μl reaction containing 50 mM Tris pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 2.5 μM random hexamer, 1 mM each dNTP, 40 U RNase inhibitor, and 400 U Superscript II reverse transcriptase (Invitrogen). The level of expression of each target mRNA was quantified by amplification of the cDNA in triplicate using a *StepOne real-time PCR* system (Applied Biosystems) as described below. Sequences of primers for α_{1A}, α_{1B}, and α_{1D} adre-