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Immunohistochemical analysis of histone H3 acetylation and methylation—Evidence for altered epigenetic signaling following traumatic brain injury in immature rats*

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Abstract

Posttranslational modifications (PTMs) of histone proteins may result in altered epigenetic signaling after pediatric traumatic brain injury (TBI). Hippocampal histone H3 acetylation and methylation in immature rats after moderate TBI were measured and decreased only in CA3 at 6 h and 24 h with persistent methylation decreases up to 72 h after injury. Decreased histone H3 acetylation and methylation suggest altered hippocampal CA3 epigenetic signaling during the first hours to days after TBI.

Keywords

Epigenetic; Hippocampus; Pediatric; Posttranslational modification; Acetylation; Methylation

Changes in epigenetic gene expression influence normal neuroplasticity, learning, and memory (Levenson and Sweatt, 2005), and thus may be important in brain cognitive dysfunction after traumatic brain injury (TBI). Despite altered gene expression in adult and pediatric TBI models (Dash et al., 2004; Griesbach et al., 2002; Petrov et al., 2001), the signaling events mediating such changes are unknown. Epigenetics has been defined as “the study of the processes that mediate metastable and somatically heritable states of gene expression without altering the DNA sequence” (Jaenisch and Bird, 2003). Epigenetic processes are but one means by which the environment interacts with the genome via cell signaling cascades producing persistent and heritable changes in gene expression. While epigenetic signaling occurs during differentiation and development (Levenson and Sweatt, 2005), it also occurs in adulthood (Jaenisch and Bird, 2003), but should be especially important in the injured developing brain.

The two primary mechanisms responsible for epigenetic gene expression are histone posttranslational modifications (PTMs) and DNA methylation (Jaenisch and Bird, 2003). The patterns of these modifications represent the epigenome (Dunn et al., 2003). Histone tails are

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targets for several PTMs, including acetylation, phosphorylation, methylation, glycosylation, ribosylation, ubiquitination, and sumoylation (de la Cruz et al., 2005; Felsenfeld and Groudine, 2003), which in turn, modify the structure and pattern of chromatin condensation and gene regulation (Berger, 2001; Jenuwein and Allis, 2001). We demonstrate, in this first examination of epigenetic signaling after TBI, that selectively vulnerable changes in hippocampal CA3 histone H3 acetylation and methylation occur after experimental pediatric TBI.

The immunohistochemical levels of hippocampal CA3 and CA1 histone H3 acetylation or methylation were evaluated in injured or sham postnatal day (PND) 17 rats after moderate controlled cortical impact (CCI) as previously described (Jenkins et al., 2002) (velocity of 4 m/s, brain deflection depth of 2.0 mm, 6 mm tip). Anesthetized (2.0% isoflurane in N₂O/O₂ (2:1)) male Sprague–Dawley rat pups (Harlan, 35–40 g) were either injured or sham injured and allowed 6, 24, or 72-h survival ($n = 5$ /group). Core temperature was maintained at 37.5–38 °C throughout surgery, injury, and recovery. All studies conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals from the U.S. Department of Health and Human Services and were approved by the University of Pittsburgh Medical Center Institutional Animal Care and Use Committee.

After postinjury survival, rats were perfused with 4% paraformaldehyde and sections of the mid-dorsal hippocampus were stained with hematoxylin and eosin (H&E) for neuronal density or reacted with antibodies for immunohistochemistry. Immunohistochemical staining was performed by an avidin–biotin–peroxidase method (ABC) using an acetyl–histone H3 (lys 9) antibody (40 µg/ml, New England Biolabs), a histone H3 antibody (100 µg/ml, Upstate Biotechnology), and a dimethyl (lys 9 and lys 4) histone H3 antibody (20 µg/ml, Upstate Biotechnology). Regional relative optical density was used for statistical analysis (one-way ANOVA analysis followed by a Bonferroni post hoc analysis). Antibody specificity was examined in naive rats using Western blot analyses (1 µg/ml of each antibody). The ipsilateral hippocampal CA3 and CA1 sectors are selectively vulnerable to TBI (Clark et al., 1997) and were the focus of the analysis.

Routine H&E staining revealed no overt hippocampal neuronal loss after injury (Fig. 1A) which unlikely impacted the densitometry measurements for immunohistochemistry. Immunohistochemical analysis of CA3 and CA1 showed selective staining of pyramidal neuronal nuclei regardless of antibody (Fig. 1B). Significant decreases in histone H3 acetylation occurred in CA3 at 6 h (5-fold, $P < 0.05$) and 24 h (3-fold, $P < 0.05$) following CCI (Figs. 1C–F, R). No significant changes were found in CA1 histone H3 acetylation. Assessment of histone H3 methylation revealed decreases in CA3 at 6 h (6-fold, $P < 0.05$), 24 h (1.5-fold, $P < 0.05$), and 72 h (3-fold, $P < 0.05$) after injury (Figs. 1H–K, S). Again, no significant changes were found in CA1. No changes in H3 histone protein levels were seen in either CA3 or CA1 after injury (Figs. 1M–P, T). Western blot analysis of all antibodies revealed suitable specificity at the appropriate molecular mass (Figs. 1G, L, and Q).

These data provide new evidence that altered epigenetic signaling occurs after experimental pediatric TBI. Regional differences in both histone H3 acetylation and methylation were seen between hippocampal sectors in the first hours to days after moderate pediatric CCI. Decreases in CA3 histone H3 acetylation occurred at 6 to 24 h after injury, but decreases in CA3 histone H3 methylation were more persistent lasting up to 72 h following pediatric CCI. No significant changes in either histone H3 acetylation or methylation were found in the CA1 sector confirming regional hippocampal differences in epigenetic signaling after TBI. Based on regional analysis of epigenetic modifications, regional gene expression in selectively vulnerable hippocampal regions such as CA3 and CA1 is important to compare after experimental pediatric TBI as performed in a recent study of adult rodent TBI (Shimamura et al., 2005).

Combined with ATP-dependent chromatin remodeling, DNA methylation and histone PTMs are the principle epigenetic mechanisms by which tissue-specific gene expression patterns are established and maintained (Jaenisch and Bird, 2003; Jenuwein and Allis, 2001). Acetylation of a core histone by a histone acetyl transferase leads to a loss of histone positive charges and histone–DNA affinity with enhanced accessibility of the associated DNA to transcription factors or other DNA binding proteins. The regulation of gene expression by histone PTMs is complex involving combinations of different PTMs such as phosphorylation, ribosylation, ubiquitination, or sumoylation at multiple sites on different histone proteins (H2A, H2B, H3, and H4) that interact to activate or repress differential gene expression (de la Cruz et al., 2005; Felsenfeld and Groudine, 2003; Jenuwein and Allis, 2001). This has led to the ‘histone code’ theory that PTMs of multiple histones determine the expression status of individual genes dependent upon their chromatin localization, and affect protein interactions that determine chromatin protein binding domains (de la Cruz et al., 2005; Jenuwein and Allis, 2001).

The proximal signaling mechanisms responsible for these injury-induced epigenetic changes are presently unknown, but changes in histone acetylation occur in relation to mitogenactivated protein kinase (MAPK) pathway activation during learning (Swank and Sweatt, 2001). Moreover, glutamatergic, cholinergic, or dopaminergic agonists induce rapid and transient changes in hippocampal histone acetylation and phosphorylation through G protein-coupled-neurotransmitter-receptors possibly via MAPK pathways (Crosio et al., 2003). Acute neurotransmitter surges (including excitotoxins) due to impact depolarization of the brain and secondary insults have been documented following TBI in a number of models (Katayama et al., 1990; Kochanek et al., 2000) as has acute changes in MAPK pathway activation (Dash et al., 2002; Otani et al., 2002; Mori et al., 2002; Hu et al., 2004). Furthermore, numerous brain stress pathways have been shown to be induced following TBI (Matzilevich et al., 2002), which activate multiple MAPKs.

In summary, experimental pediatric CCI resulted in altered epigenetic signals reflected by changes in hippocampal CA3 histone H3 acetylation and methylation for hours to days after injury. These changes are likely related to documented upstream excitotoxic and stress cascades after TBI. A significant role in cognitive function is evolving for histone acetylation as histone deacetylase (HDAC) inhibitors have provided functional benefit in some models of cognitive dysfunction (Korzus et al., 2004; Levenson and Sweatt, 2005). HDAC inhibitors may also provide a new therapeutic approach for treating pediatric TBI.

Abbreviations

PTMs, posttranslational modifications; HDAC, histone deacetylase; CCI, controlled cortical impact.

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Fig. 1.

(A) The lack of overt hippocampal CA3 and CA1 neuronal death seen 72 h after injury. H&E stain, $\times 8$. (B) Nuclear staining of CA1 pyramidal neurons in the stratum pyramidal layer (SP) by the acetyl histone H3 antibody with scattered staining in the stratum oriens basal dendritic layer (SO), and the apical dendritic layers of the stratum radiatum (SR) and the stratum lacunosum-molecularis (SLM), $\times 120$. (C–F) Immunohistochemical analysis using the histone H3 acetylation antibody revealed significant decreases at 6 h and 24 h following CCI, $\times 10$. Representative regional analysis boundaries for CA3 used in this study are shown in blue and CA1 in red. (G) Analysis of the histone H3 lysine-9 acetylation antibody showed good specificity. (H–K) Immunohistochemical analysis using the dimethyl histone H3 antibody

revealed significant decreases in CA3 at 6, 24 and 72h after injury, $\times 10$. (L) Western blot analysis with the dimethyl histone H3 antibody ($1\mu\text{g/ml}$) revealed two bands. The lighter upper band is the proper mass to be a dimer of histone H3 and thus could also represent specific binding to the histone H3 protein by the dimethyl antibody. (M–P) Immunohistochemical analysis using the histone H3 antibody at 6, 24 and 72 h after CCI revealed no change from sham levels, $\times 10$. (Q) Western blot analysis of the histone H3 antibody ($1\mu\text{g/ml}$) showed good specificity. (R–T) Densitometry data for the histone H3 acetylation antibody (mean $\pm$ SD) showed significant CA3 differences ($P < 0.05$) from sham levels at 6 and 24 h after injury. Significant CA3 differences ($P < 0.05$) for the dimethyl histone H3 antibody (mean $\pm$ SD) were also found at 6, 24, and 72h compared to sham levels after injury. Densitometry data for the histone H3 antibody (mean $\pm$ SD) showed no significant differences in protein levels.