Chromatin remodeling and neuronal response: multiple signaling pathways induce specific histone H3 modifications and early gene expression in hippocampal neurons

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Summary

Plasticity in gene expression is achieved by a complex array of molecular mechanisms by which intracellular signaling pathways directly govern transcriptional regulation. In addition to the remarkable variety of transcription factors and co-regulators, and their combinatorial interaction at specific promoter loci, the role of chromatin remodeling has been increasingly appreciated. The N-terminal tails of histones, the building blocks of nucleosomes, contain conserved residues that can be post-translationally modified by phosphorylation, acetylation, methylation and other modifications. Depending on their nature, these modifications have been linked to activation or silencing of gene expression. We wanted to investigate whether neuronal stimulation by various signaling pathways elicits chromatin modifications that would allow transcriptional activation of immediate early response genes. We have analysed the capacity of three drugs – SKF82958 (a dopaminergic receptor agonist), pilocarpine (a muscarinic acetylcholine receptor agonist) and kainic acid (a kainate glutamate receptor agonist) – to induce chromatin remodeling in hippocampal neurons. We show that all stimulations induce rapid, transient phosphorylation of histone H3 at serine 10. Importantly, the same agonists induce rapid activation of the mitogen-activated protein kinase pathway with similar kinetics to extracellular-regulated-kinase phosphorylation. In the same neurons where this dynamic signaling cascade is activated, there is induction of c-*fos* transcription. Histone H3 Ser10 phosphorylation is coupled to acetylation at the nearby Lys14 residue, an event that has been linked to local opening of chromatin structure. Our results underscore the importance of dynamic chromatin remodeling in the transcriptional response to various stimuli in neuronal cells.

Key words: Chromatin, c-*fos*, MAPK, Hippocampus, Phosphorylation, Acetylation

Introduction

Short- and long-term changes in gene expression are likely to play a key role in neuroplasticity in the adult animal (Walton et al., 1999; Hardingham et al., 2001; Tartaglia et al., 2001). These processes are governed by the specific activation of several signaling pathways. Indeed, the generation of physiological responses to environmental stimuli coincides with the rapid, transient transcriptional induction of a set number of genes. It is generally thought that these transcriptional responses depend on the modulation of transcription-factor activity. The underlying mechanisms by which signaling pathways regulate gene expression have been partly deciphered in several systems (Brivanlou and Darnell, 2002).

Essential advances have been made in recent years towards the understanding of how chromatin remodeling might influence – and determine – gene expression. Growing evidence indicates that distinct post-translational modifications occurring on histone tails are primary events contributing to the dynamic process of chromatin remodeling (Cheung et al., 2000a; Strahl and Allis, 2000). The N-terminal tails of core histones are poorly structured and are thought to confer secondary and more flexible contacts with DNA that allow dynamic changes in the accessibility of the underlying genome (Luger et al., 1997; Wolffe and Kurumizaka, 1998). The N-terminal tails are fully conserved from yeast to human and are substrates of modifications including acetylation, methylation, ubiquitination and phosphorylation (Cheung et al., 2000a). In particular, position-specific modifications of the histone H3 N-terminal tail have been associated with distinct chromatin-based outputs, such as transcriptional regulation (Lys9/Lys14 acetylation, Ser10 phosphorylation), transcriptional silencing (Lys9 methylation), histone deposition (Lys9 acetylation) and chromosome condensation/segregation (Ser10/Ser28 phosphorylation) (for reviews, see Cheung et al., 2000a;

Jenuwein and Allis, 2001). Among these modifications, phosphorylation on serine 10 of histone H3 offers the best example of a direct link between signal transduction and histone modification (Cheung et al., 2000a). Mitogenic stimulation induces rapid, transient phosphorylation of histone H3 (Mahadevan et al., 1991; Chadee et al., 1999; Sassone-Corsi et al., 1999), which has been associated with transcriptional activation of immediate-early genes (IEGs) (Cheung et al., 2000b; Nowak and Corces, 2000).

Histone H3 phosphorylation at Ser10 has a dual 'personality' (Cheung et al., 2000a). It is observed at metaphase as a hallmark for condensing chromatin at mitosis. In mammals, all H3 molecules are phosphorylated during mitosis by a kinase of the Aurora family (Crosio et al., 2002). By contrast, during interphase, mitogenic signals induce a rapid, transient phosphorylation of only a small subset of H3 molecules at the same Ser10 residue (Sassone-Corsi et al., 1999). Mitogenic phosphorylation of H3 in response to epidermal growth factor (EGF) or 12-*O*-tetradeconyl-phorbol-13-acetate (TPA) involves the mitogen-activated protein kinase (MAPK) cascade and the consequent activation of the extracellular-regulated kinases (ERKs). The MAPK-activated RSK-2 was identified as an epidermal growth factor (EGF)-induced histone H3 kinase (Sassone-Corsi et al., 1999), whereas the same Ser10 site can also be phosphorylated by the mitogen- and stress-induced kinases MSK-1 and MSK-2 (Thomson et al., 1999). Indeed, in a similar manner to the Ser133 phosphoacceptor site in the transcription factor CREB (De Cesare et al., 1999; Lonze and Ginty, 2002), the H3 Ser10 residue seems to constitute a converging site of multiple intracellular pathways and kinases. Interestingly, chromatin remodeling via H3 phosphorylation also occurs in the mammalian nervous system in response to a photic stimulation in clock neurons within the suprachiasmatic nucleus (SCN), and after γ-aminobutyric acid (GABA) stimulation of neurons within the supraoptic nucleus (SON). Notably, light stimulation also induces phosphorylation of ERKs (Obrietan et al., 1998; Crosio et al., 2000), phosphorylation of CREB (Travnickova-Bendova et al., 2002) and a robust induction of IEGs transcription in the SCN (Crosio et al., 2000).

An important feature of histone modifications is that they can occur in concert on the same histone tail (Cheung et al., 2000b; Clayton et al., 2000) or on tails of different histones (Turner et al., 1992; Zeitlin et al., 2001; Sun and Allis, 2002). These combinatorial possibilities indicate the presence of a 'histone code', in which specific combinations of modifications could correspond to various states of remodeled chromatin or to the activation of distinct sets of genes. In particular, in different experimental systems, phosphorylation at Ser10 has been shown to facilitate concurrent Lys14 acetylation (Cheung et al., 2000b; Lo et al., 2000; Lo et al., 2001), a modification elicited by histone acetyltransferases (HATs) such as the CREB-binding protein CBP (McManus and Hendzel, 2001). Importantly, the enzymatic activities eliciting acetylation and phosphorylation are also coupled, because CBP and RSK-2 have been found to be associated in a signaling-dependent fashion (Merienne et al., 2001).

The hippocampus is a cortical structure that has been strongly implicated in the processes of memory acquisition and retention (Sprick, 1995; Chen and Tonegawa, 1997; Shapiro and Eichenbaum, 1999). Disregulation in dopaminergic, acetylcholinergic and glutamatergic neuromodulation has been implicated – in different ways – in deficits of learning, memory and behavior (Frey et al., 1991; Bortolotto and Collingridge, 1993; Huerta and Lisman, 1993; Otani et al., 1993; Blokland, 1995). Systemic administration of pilocarpine (a muscarinic acetylcholine receptors agonist) or kainate (KA; an analog of the excitatory amino acid L-glutamate) constitute classical animal models of epilepsy (Clifford et al., 1987; Ben-Ari and Cossart, 2000). Induction of seizure and seizure-related brain damage by these compounds causes neuron loss in different hippocampal areas within 2-3 days. Neurodegeneration affects mainly neurons in CA subfields of hippocampus, whereas granule cells in the dentate gyrus (DG), which are resistant to neurodegeneration, initiate new synaptic contacts and, in some cases, display an increase in neurogenesis (Clifford et al., 1987; Ekdahl et al., 2001; Zagulska-Szymczak et al., 2001). Dopaminergic afferences to the hippocampus seem to be involved in learning reinforcement (Otmakhova and Lisman, 1996; Spanagel and Weiss, 1999) and dopamine receptor agonists have been shown to contribute in increasing the release of glutamate (Bouron and Reuter, 1999). Indeed, convergent interactions between both glutamate and acetylcholine signaling with the dopaminergic system have been proposed to participate in the individual susceptibility to epilepsy (Starr, 1996). In this respect, D1 and D2 dopamine receptors seem to play opposite roles in the regulation of the threshold for seizures. In particular, a neuroprotective role of D2 receptors against KA- and pilocarpine-induced cell death has been demonstrated using mice with a deletion of the D2 receptor gene (Bozzi et al., 2000; Bozzi and Borrelli, 2002).

Chromatin remodeling has been mainly studied in the context of cell proliferation, where it is associated with gene regulation (Berger, 2002). Given the fact that neurotransmitters and neuromodulators act by inducing short- and long-term changes in gene expression (Walton et al., 1999; Hardingham et al., 2001; Tartaglia et al., 2001), we evaluated the capacity of three drugs (SKF82958, pilocarpine and kainic acid) to induce chromatin remodeling in hippocampal neurons; these drugs are selective agonists of dopamine (DA) (Mottola et al., 1996), muscarinic acetylcholine (mACh) (Clifford et al., 1987) and ionotropic glutamate (GLU) receptors (Hollmann and Heinemann, 1994), respectively. We have studied histone modifications and, in particular, H3 phosphorylation on serine 10, a hallmark of induced early gene expression. Our findings reveal a tight link between neuronal activation and plasticity in chromatin remodeling in hippocampal neurons.

Materials and Methods

Animals and tissue preparation

In all experiments presented, wild-type 129/Sv mice were used, although analogous results were obtained in other mouse strains (not shown). All animals were 7-12-week-old males. Food and water were available ad libitum. Animal care was conducted in accordance with standard ethical guidelines. SKF82958 (0.2 mg kg^{-1}, 1 mg kg^{-1} or 5 mg kg^{-1}) (RBI-Sigma, Saint Louis, MI), pilocarpine (30 mg kg^{-1}, 100 mg kg^{-1} or 300 mg kg^{-1}) (Torcis, Ballwin, MO) and kainic acid (10 mg kg^{-1}, 20 mg kg^{-1} or 35 mg kg^{-1}) (A.G. Scientific, San Diego, CA) dissolved in 0.9% NaCl, or saline solution alone, were administered intraperitoneally. Mice were sacrificed by CO_2 euthanasia at the indicated time and dissected. For immunohistochemistry (IHC) and in situ hybridization (ISH), brains were placed in optimal cutting temperature compound (OCT), frozen on

dry ice and 10-μm-thick coronal cryosections were prepared. Tissues to be assayed by western blot analysis were homogenized in boiling Laemmli buffer.

Antibodies

A panel of antibodies targeting various modifications of histone H3 was used in the present study. These were rabbit antibody against histone H3 phosphorylated on Ser10 (P.H3, 1:500 final dilution; Upstate Biotechnology, Lake Placid, NY), rabbit antibody against histone H3 acetylated on Lys9 and Lys14 ($Ac_{9/14}$.H3, 1:500 final dilution; Upstate Biotechnology), rabbit antibody against histone H3 acetylated on Lys14 (Ac_{14}.H3, 1:100 final dilution; Upstate Biotechnology), rabbit antibody against histone H3 acetylated on Lys9 (Ac_9.H3, 1:100 final dilution, New England Biolabs, Beverly, MA), rabbit antibody against histone H3 phosphorylated on Ser10 and acetylated on Lys14 (P-Ac_{14}.H3, 1:100 final dilution; Upstate Biotechnology), rabbit antibody against histone H3 phosphorylated on Ser10 and acetylated on Lys9 (P-Ac_9.H3, 1:100 final dilution, New England Biolabs), rabbit and mouse antibodies against ERK phosphorylated Thr202/Thr204 ERK peptide (P.ERK, 1:1000 and 1:100 final dilution, respectively; New England Biolabs), mouse antibody against digoxygenine (DIG, 1:100; Roche Molecular Biochemicals, Meylan, France). Secondary antibodies (peroxidase, alkaline-phosphatase, Cy3 or FITC conjugated) against both mouse and rabbit were obtained from the Jackson Laboratories (West Grove, PE) and used at a 1:1000 final dilution.

Immunohistochemistry

Brain sections were fixed in 1× PBS (pH 7.5) containing 4% paraformaldehyde. Antigen unmasking was performed by boiling slides for 3 minutes in 10 mM sodium citrate buffer. The slides were then allowed to cool for 20 minutes at room temperature and endogenous peroxidase activity was blocked by incubation in 1% H_2O_2 in 1× PBS. After 1 hour of blocking with 5% bovine serum albumin (BSA) in 1× PBS/0.05% Tween-20, incubation with the primary antibody was performed overnight at 4°C. Sections were analysed by the peroxidase-labeled detection method according to manual instruction (DAKO, Glostrup, Denmark). For double-labeling experiments, sections were first incubated with a mouse P.ERK antibody and the rabbit P.H3 antibody, washed, incubated with the appropriate secondary antibodies and then revealed by Nitro Blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) and diaminobenzidine (DAB) staining.

In situ hybridization

MKP-1 (nucleotides 538-1103 of open reading frame), MKP-3 (nucleotides 450-693 of open reading frame) and c-*fos* (nucleotides 1-336 of open reading frame) ^{35}S-labeled antisense riboprobes were prepared by in vitro transcription (Promega, Madison, WI). Hybridization was performed as described (Mellstrom et al., 1993).

In situ immuno hybridization using a DIG-labeled c-*fos* riboprobe and the anti-P.H3 antibody on cryostat sections was as described (Strahle et al., 1993), with the following modifications: hybridization was at 65°C; the final (fifth) post-hybridization wash was in MABT (100 mM maleic acid, 150 mM NaCl, 0.1% Tween 20); sections were blocked in 5% BSA/MABT and then incubated overnight at 4°C with anti-DIG antibody and anti-P.H3 antibody. Slides were washed, incubated with the appropriate secondary antibodies.

Western blot analysis

Protein extracts were resolved by standard SDS/PAGE. Samples were electroblotted onto Protan nitrocellulose (Schleicher & Schuell, Dassel, Germany). Membranes were incubated in PBS with 5% low-fat milk and specific antibody for 12 hours at 4°C. Donkey anti-rabbit horseradish-peroxidase-conjugated antibodies were used to reveal immune complexes by enhanced chemiluminescence (Pierce, Rockford, IL).

Chromatin immunoprecipitation

One hour after injection of KA (35 mg kg^{-1}) or saline solution, mice were sacrificed and hippocampal tissues were quickly dissected and held in ice-cold 1× PBS containing 5 mM NaF, 1 mM Na_3VO_4, 10 mM sodium butyrate, 1 mM phenylmethylsulfonyl fluoride and 1 μg ml^{-1} each of aprotin, pepstatin A and leupeptin. Tissues were fixed in 1% picric acid-formaldehyde (PAF) in 1× PBS for 5 minutes to crosslink histones with their associated genomic DNA. Immunoprecipitation and washing were performed as described (Cheung et al., 2000b). Immunoprecipitated DNA samples were resuspended in H_2O and a fraction used in PCR with the oligonucleotides CACGGCCGGTCCCTGTTGTTC / GTCGCGG-TTGGAGTAGTAGGCG for c-*fos*, TCAGCGGGGAG-TTTTTGTG / CTGTGAGTGACCCTCAAAGTGG for MKP-1 and GACACCG-CATGCAAAGAATAGCTG / CTTTCCCAAGGCCTTT-ACCACC for H4, as previously described (Cheung et al., 2000b; Li et al., 2001).

Data analysis

Tissue sections were examined under the light microscope. Cell count was performed on six images of adjacent fields and the number of positive neurons within the image was counted. Typically, 200-400 neurons were counted for each field in a given image. Data are presented as the proportion of positive nuclei. Data were analysed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Statistical comparisons were performed from six independent animals obtained in three independent experiments.

Results

Kinetics of histone H3 phosphorylation in response to various signaling systems in hippocampal neurons

A schematic representation of the nucleosomal chromatin structure and a blow-up of the histone H3 N-terminal tail are shown in Fig. 1A. The conserved residues whose covalent modification by acetylation and phosphorylation has been coupled to activation of gene expression are indicated. We have studied phosphorylation of histone H3 at Ser10 by immunocytochemistry using a specific antibody (P.H3) on brain cryosections. Stimulation of neuronal activity in the hippocampus through the action of different neuromodulators was obtained by systemic injection of naive mice with 5 mg kg^{-1} of SKF82958 (a DA receptor agonist), 300 mg kg^{-1} of pilocarpine (a mACh receptor agonist) and 35 mg kg^{-1} of kainic acid (a GLU receptor agonist) (Brooks-Kayal et al., 1998; Chiang et al., 2001; Nisenbaum et al., 1998; Ruskin et al., 1998). After injection, mice were returned to their home cages for 15 minutes, 1 hour or 3 hour before dissection. There was no detectable phosphorylation of H3 in the hippocampus after saline injection, but stimulation of the neuronal zactivity via activation of DA, mACh and GLU receptors causes a powerful induction of H3 phosphorylation in different subfields of the hippocampus. Histone H3 Ser10 phosphorylation displays kinetics that parallel that of immediate early genes, being rapid and transient. H3 phosphorylation is detectable by 15 minutes, peaks at 1 hour and returns to basal levels 3 hours after induction (Fig. 1B). In the DG, almost all nuclei are intensely P.H3 positive, whereas only a subset of neurons in the CA3 region show a P.H3 signal, which is also significantly lower than the one in the DG, regardless of the stimulus used. Higher magnification of the DG (Fig. 1C, first

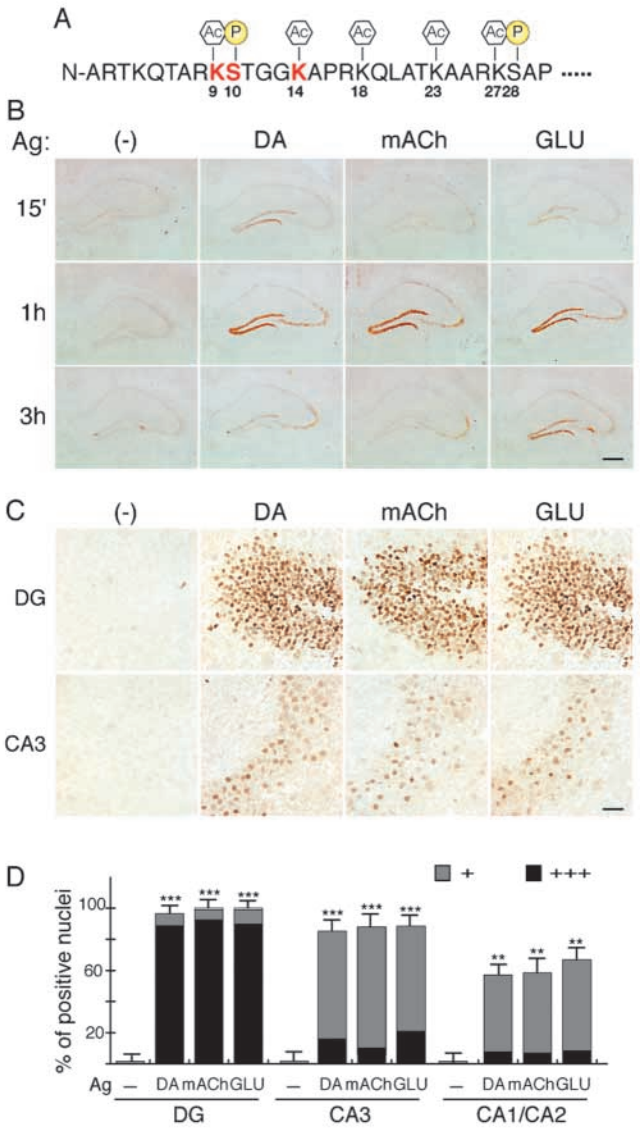

Fig. 1. Phosphorylation of histone H3 is induced in the hippocampus by stimulation of DA, mACh and GLU receptors. (A) Schematic representation of the histone H3 N-terminal tail. The key residues within the H3 tail where covalent regulatory modifications occurs are indicated. These include acetylation at lysines and phosphorylation at conserved serine residues. (B) Immunohistochemistry on mouse hippocampal cryosections using P.H3 antibody. In order to stimulate DA, mACh or GLU receptors, mice were injected with SKF82958 (5 mg kg^{-1}), pilocarpine (300 mg kg^{-1}) or kainic acid (35 mg kg^{-1}), respectively, and sacrificed after15 minutes, 1 hour or 3 hour. Control animals, indicated with the symbol (–), were injected with saline solution. (C) Phosphorylation of histone H3 in the DG and in the CA3 after stimulation of DA, mACh and mGLU receptors.
(D) Quantification of the data in (A,B). A total of six animals were analysed from three independent experiments. Cell counts were performed under the light microscope. Statistical comparisons were performed with one-way ANOVA on the total number of positive cells (independently from the intensity of the staining that is indicated on the graph in gray and black) followed by Bonferroni's post hoc test. ***, $P<0.001$; **, $P<0.01$. Scale bars, 300 μM (B); 70 μM (C).

row) and CA3 region (Fig. 1C, second row) stained with P.H3 antibody confirms this notion. A quantification of the results is reported in Fig. 1D. To extend our analysis, we injected mice with decreasing doses of each agonist (5 mg kg^{-1}, 1 mg kg^{-1} and 0.2 mg kg^{-1} SKF82958; 300 mg kg^{-1}, 100 mg kg^{-1} and 30 mg kg^{-1} pilocarpine; 35 mg kg^{-1}, 20 mg kg^{-1} and 10 mg kg^{-1} kainic acid). As shown in Fig. 2, the level of histone H3 phosphorylation is directly proportional to the dose of drug administered.

Some drugs have long-term effects on neuronal degeneration and cell death, so we also examined the effect of activating DA, mACh and GLU receptors at later times after injection on H3 phosphorylation. No P.H3 positive cells were observed at 16 hours, 24 hours, 48 hours and 72 hours after injection in any hippocampal area (data not shown).

Phosphorylation of histone H3 and ERK occur in the same neurons

The MAPK/ERK kinase cascade, implicated in histone H3 phosphorylation during mitogenic stimulation (Sassone-Corsi et al., 1999; Thomson et al., 1999), appears to have a crucial role in models of neuronal plasticity, such as long-term facilitation in *Aplysia* sensory neurons (Martin et al., 1997), long-term potentiation (LTP) in CA1 of the hippocampus (English and Sweatt, 1996; English and Sweatt, 1997; Impey et al., 1998) and some models of learning (Atkins et al., 1998; Blum et al., 1999). In hippocampal primary cultures, MAPK/ERK activation is regulated by signaling via a rich diversity of neuromodulators and neurotransmitters, including DA, GLU and mACh receptors (for a review, see Sweatt, 2001). We performed IHC on cryosections using an antibody raised against the phosphorylated Thr202/Thr204 ERK peptide (P.ERK). Interestingly, the kinetics of ERK phosphorylation parallels that observed for Ser10 phosphorylation on histone H3. There is a basal level of ERK phosphorylation in neurons of the CA3 region in saline-treated mice. This phosphorylated ERK is largely cytoplasmic. Upon stimulation by the various agents, there is a significant induction of ERK phosphorylation (Fig. 3A), mainly in DG neurons but also in CA1, CA2 and CA3 subfields, paralleling the induction pattern of H3 phosphorylation (Fig. 1B). Interestingly, and as previously observed (Fiore et al., 1993; Ortiz et al., 1995), only a subset of phosphorylated ERK molecules is translocated to the nucleus after induction, underlining the presence of cytoplasmatic substrates for ERKs.

To investigate whether the induced phosphorylation of H3 and ERKs occurred concomitantly in the same hippocampal neurons, we performed double labeling with specific phospho-antibodies on cryosections obtained from mice stimulated with DA, mACh and GLU receptor agonists. Analysis at higher magnification of DG neurons stained for the phosphorylated histone H3 (BCIP/NBT staining; Fig. 3B, top) and phosphorylated ERK (DAB staining, Fig. 3B, middle) or both (Fig. 3B, bottom) showed that a large proportion of neurons positive for H3 phosphorylation also exhibited ERK phosphorylation. This result indicates that the two signaling events occur concomitantly in the same neurons. It is noteworthy that, although all three inducers elicit ERK and histone H3 phosphorylation with a similar pattern in the DG, their effects seem to be different depending on which subfield of the hippocampus is analysed. A quantification of the data in Fig. 3B is presented in Fig. 3D. Indeed, ERK and histone H3 phosphorylations are more evident in the CA1 after DA

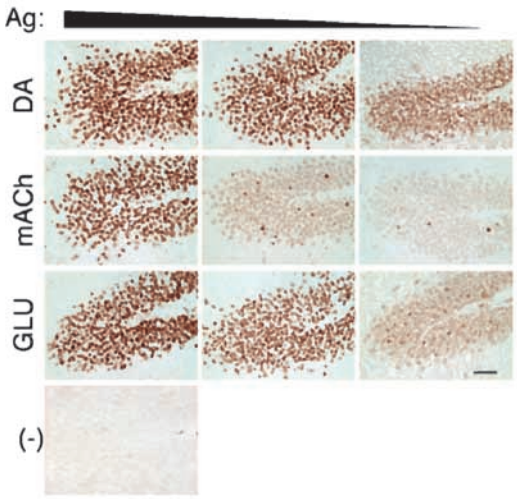

Fig. 2. Dose-dependent histone H3 phosphorylation. Mice were injected with 5 mg kg^{-1}, 1 mg kg^{-1} or 0.2 mg kg^{-1} SKF82958, 300 mg kg^{-1}, 100 mg kg^{-1} or 30 mg kg^{-1} pilocarpine, or 35 mg kg^{-1}, 20 mg kg^{-1} or 10 mg kg^{-1} kainic acid, and sacrificed after 1 hour. Control animals, indicated with the symbol (–) were injected with saline solution. A portion of the DG is shown. Scale bar, 70 μM.

stimulation (Fig. 3D, top), whereas DA and mACh stimulation results in the same type of activation in the CA2 (Fig. 3D, middle). Finally, the most significant phosphorylation of ERK and histone H3 is observed in the CA3 after kainate stimulation (Fig. 3D, bottom). These results underscore the notion that there is a spatial specificity in chromatin remodeling in various hippocampal subfields.

Histone H3 phosphorylation and early gene expression in hippocampal neurons

Compelling evidence in several biological systems indicates that distinct histone modifications can elicit chromatin remodeling at specific loci, an essential prerequisite for the activation of IEG transcription (Cheung et al., 2000a; Berger and Felsenfeld, 2001). Interestingly, the activation of IEGs has been suggested to be linked to genomic events that control long-term changes within the nervous system (Berridge, 1986; Goelet et al., 1986; Wisden et al., 1990). To establish a direct link between H3 phosphorylation and IEG expression after stimulation of DA, mACh and GLU receptors, we used in situ hybridization to analyse the expression of c-*fos*, a representative member of the IEG family. Cryosections obtained from treated mice were hybridized with a c-*fos* riboprobe. After stimulation of DA, mACh and GLU receptors, c-*fos* expression is already detectable by 15 minutes (not shown), peaks 1 hour after induction (Fig. 4A) and is no longer detectable 3 hours later (not shown) in all the regions of hippocampus and cortex. Induction of H3 phosphorylation always appeared to precede induction of c-*fos* transcription.

To establish whether induction of H3 phosphorylation and c-*fos* expression were occurring concomitantly in the same hippocampal neurons in response to the various stimulations, we performed an in situ immunostaining on cryosections using a riboprobe that was DIG labeled for c-*fos* (Cy3 staining) and the antibody against phosphorylated histone H3 (FITC

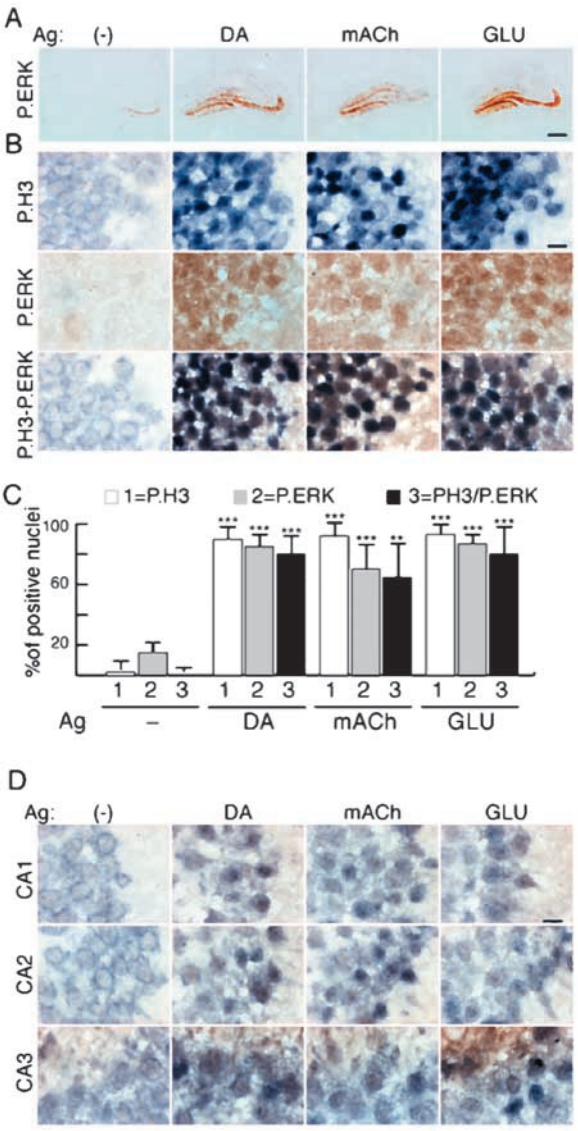

Fig. 3. Histone H3 phosphorylation and ERK phosphorylation occur in the same hippocampal neurons. (A) Immunohistochemistry using P.ERK antibody on mouse hippocampal cryosections obtained from mice sacrificed 1 hour after saline, SKF82958, pilocarpine or kainic acid injection. (B) IHC was performed using anti-P.H3 (NBT/BCIP staining, top), anti-P.ERK (DAB staining, middle) and a mix of these two antibodies (bottom). The DG regions are shown. (C) Quantification of the data in (B). Cell counts were performed under the light microscope. Statistical comparisons were performed with one-way ANOVA followed by Bonferroni's post hoc test. ***, $P<0.001$. (D) High magnifications of CA1, CA2 and CA3 regions revealed with both anti-P.H3 and anti-P.ERK antibodies. Scale bars, 300 μM (A); 20 μM (B,D).

staining). For all the agonists, almost all DG neurons positive for H3 phosphorylation exhibited c-*fos* expression (Fig. 4B), providing strong evidence that the two events are intimately coupled in most hippocampal neurons.

To extend our results we analysed the expression of two other IEGs of interest, the MAP kinase phosphatases 1 (MKP-1) and 3 (MKP-3). The analysis of these two genes is relevant because the rapid dephosphorylation of the MAPK/ERK proteins

observed in the hippocampus after stimulation (Fig. 2) suggests a possible negative feedback control exerted by an ERK-specific, inducible phosphatase. Moreover, it has been previously shown that transcriptional activation of *MKP-1* in response to stress is associated with histone-H3 modification (Li et al., 2001). Our results show that the timing of induction of both *MKP-1* and *MKP-3* closely parallels that of c-*fos* (Fig. 4C), although there are some interesting differences. All three agonists used in this study stimulate *MKP-3* expression in a similar extent in all regions of the hippocampus. By contrast, *MKP-1* is predominantly induced in the DG and in the CA3 by stimulation of DA and GLU receptors, and in CA2, CA3 and the cortex upon stimulation of mACh receptors. The fact that the two phosphatases display a pattern of expression that only partially overlaps indicates a fine cell-specific regulation and possibly different physiological functions. In this context, it is useful to remember that MKP-3 is known to have a predominantly cytoplasmic localization (Camps et al., 1998a,b).

Neuronal activity and global chromatin modification

Different lines of evidence indicate that a combination of modifications on histone N-termini, rather than a single phosphorylation or an acetylation event, is required to induce changes in the chromatin environment. Both acetylation on Lys14 and phosphorylation on Ser10 of histone H3 tail have been associated with activation of transcription in yeast, mammals and *Drosophila* (Cheung et al., 2000b; Clayton et al., 2000; Lo et al., 2000; Thomson et al., 2001). We wanted to extend these observations to non-proliferating hippocampal neurons using non-mitogenic stimuli. We performed IHC on brain cryosections of animals killed 1 hour after stimulation of DA, mACh and GLU receptors, using antibodies that recognize the histone H3 tail when it is phosphorylated on Ser10 (P.H3), acetylated on Lys14 (Ac_{14}.H3) or when phosphorylation on Ser10 and acetylation on Lys14 (P-Ac_{14}.H3) are combined (Fig. 5A).

Interestingly, although histone H3 is strongly phosphorylated at Ser10 upon stimulation by each of the inducers, levels of H3 acetylated at Lys14 did not increase significantly after any treatment. Because Lys14 acetylation has been directly coupled to induced gene expression in proliferating cultured cells (Cheung et al., 2000a), there might be different requirements for chromatin modification in non-proliferating hippocampal cells in vivo, in response to neuroendocrine, non-mitogenic signals. In addition, H3 acetylation at Lys14 has been generally associated with widely transcriptionally active euchromatin, although the activation of IEGs reported here in response to an acute stimuli represents only a small subset of the genome. Moreover, analogous results were obtained when using antibodies that recognize H3 when acetylated on Lys9 (Ac_9.H3, a modification mainly involved in histone deposition) (Loyola et al., 2001) or di-acetylated on Lys9 and Lys14 ($Ac_{9/14}$.H3). By contrast, upon stimulation, we observe an important induction of H3 phospho-acetylation when using both the antibodies P-Ac_{14}.H3 and P-Ac_9.H3. Representative fields of the DG stained with the above mentioned antibodies are shown in Fig. 5A, and P-Ac_{14}.H3 staining in CA1, CA2 and CA3 regions is shown in Fig. 5B.

To validate these results further, we performed western blot analyses on total protein extracts obtained from isolated hippocampi of mice treated as described above. Equal amounts of proteins were loaded on SDS/PAGE gels and modifications were revealed with antibodies against ERK, P.ERK, P.H3, Ac_{14}.H3 and P-Ac_{14}.H3 (Fig. 6A). This analysis confirms the results obtained by IHC for histone-H3 modifications. Interestingly, and in accordance with previous results (Yan et al., 1999; Sweatt, 2001), all stimuli elicit fairly selective activation of the p42 isoform of ERK, whereas the p44 isoform is mostly unaffected. It will be interesting to determine the basis of this selective activation, because it is not a general property of the MAPK system in other cell types.

We therefore investigated whether KA-induced phosphorylation of histone H3 is associated with c-*fos* and *MKP-1* gene activation. A chromatin immunoprecipitation (ChIP) assay was used to measure the levels of phosphorylated and phospho-acetylated histone H3 physically associated with c-*fos* and *MKP-1* genes. After chromatin isolation from hippocampi dissected from KA- or saline-treated mice, ChIP assays were carried out using P.H3 and P-Ac_{14}.H3 antibodies. Genomic DNA present in the immunoprecipitates was extracted and analysed by PCR. We monitored the specificity and accuracy of these assays by performing mock ChIP reactions in the absence of the antibody and executing PCR amplifications using the DNA from the templates. A reproducible twofold increase in the levels of phosphorylated histone H3 on c-*fos* and *MKP-1* chromatin was observed in samples from KA-treated mice (Fig. 6B). Taken together, these

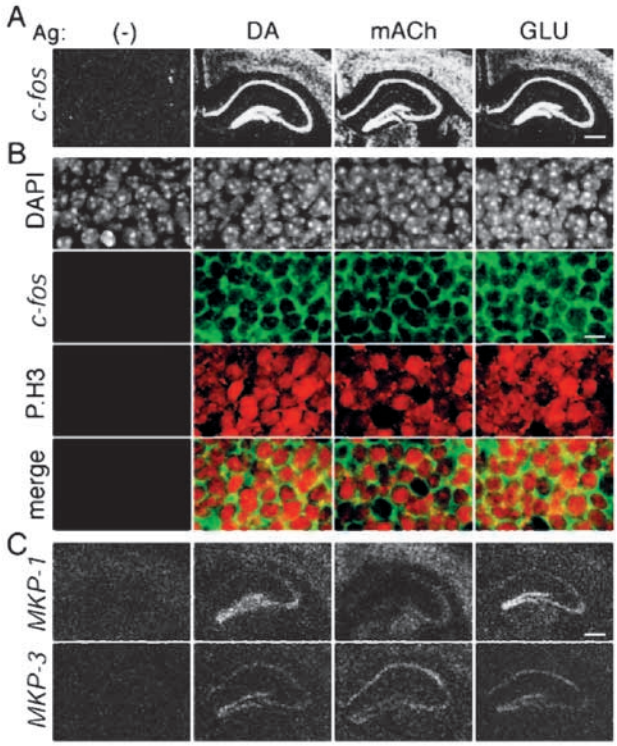

Fig. 4. Correlation of histone H3 phosphorylation and IEG induction in the hippocampus. Cryosections obtained from mice sacrificed 1 hour after saline, SKF82958, pilocarpine or kainic-acid injection were used for in situ hybridization experiments using c-*fos* (A), *MKP-1* (C, top) and *MKP-3* (C, bottom), S^{35}-labeled riboprobes or in situ-immuno hybridization (B), using c-*fos* DIG-labeled riboprobe (FITC staining) and P.H3 antibody (Cy3 staining). Nuclear localization was assessed by DAPI staining. Scale bars, 300 μM (A,C); 20 μM (B).

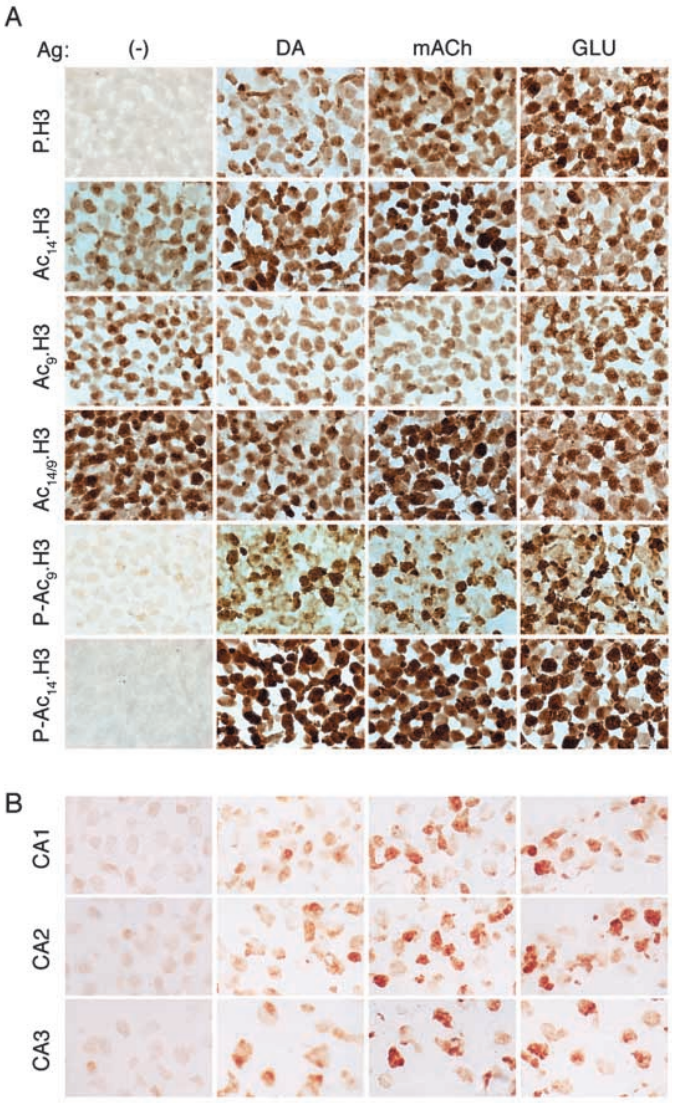

Fig. 5. Phospho-acetylation of histone H3 after stimulation of DA, mACh and GLU receptors. IHC was performed using (from top to bottom) anti-P.H3 (P), anti-Ac_{14}.H3 (Ac_{14}), anti-Ac_9.H3 (Ac_9), anti-$Ac_{9/14}$.H3 ($Ac_{9/14}$), anti-P-Ac_9.H3 (P-Ac_9) and anti-P-Ac_{14}.H3 (P-Ac_{14}) antibodies on cryosections obtained from mice treated as in Fig. 1B. (A) High magnifications of the DG revealed with all these antibodies. (B) High magnifications of the CA1, CA2 and CA3 regions revealed with anti-P-Ac_{14}.H3 (P-Ac_{14}) antibody. Scale bar, 15 μM.

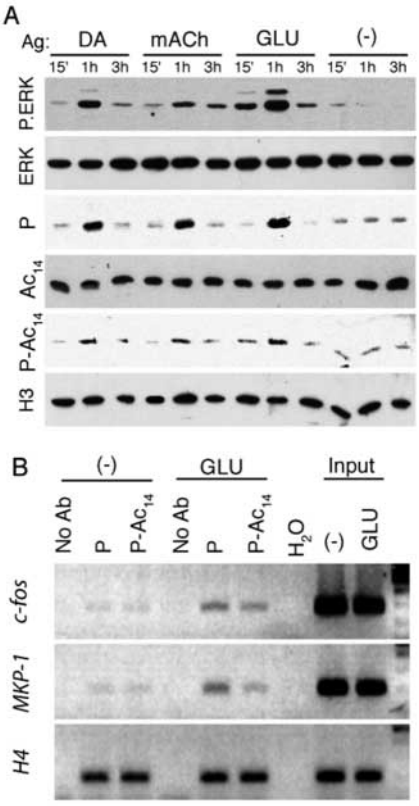

Fig. 6. (A) Western blot analysis on total protein extracts obtained from mice sacrificed 15 minutes, 1 hour or 3 hour after saline, SKF82958, pilocarpine or kainic-acid injection. 15 μg of proteins were separated on SDS/PAGE gels and revealed using anti-P.ERK, anti-panERK (ERK), anti-P.H3 (P), anti-Ac_{14}.H3 (Ac_{14}) and anti-P-Ac_{14}.H3 (P-Ac_{14}) antibodies. (B) Phosphorylation-acetylation of histone H3 on c-*fos* and *MKP-1* chromatin. ChIP assays were performed on chromatin obtained from hippocampi of mice injected with 35 mg kg^{-1} kainic acid (GLU) or saline treated (–), using a preimmune serum (no Ab), anti-P.H3 (P), anti-Ac_{14}.H3 (Ac_{14}) or anti-P-Ac_{14}.H3 (P-Ac_{14}). The DNA recovered from the antibody-bound fraction and the DNA from the input chromatin were analysed by PCR with oligonucleotides specific for the indicated gene.

results indicate that nucleosomal changes associated with histone H3 modifications play an important role in mediating transcriptional activation of c-*fos* and *MKP-1* by stimulating DA, mAch and GLU receptors in the hippocampus.

Discussion

Chromatin constitutes the physiological template of all eukaryotic genetic information and – far from being a static structure – is subject to a diverse array of post-translational modifications that mostly involve histone N-termini (Strahl and Allis, 2000). Changes in the modification status of histone tails are thought to regulate the access by regulatory factors to the underlying DNA. Distinct histone N-terminal modifications can generate synergistic or antagonistic interactions with chromatin-associated proteins, which in turn dictate dynamic transitions between transcriptionally active or inactive chromatin. One of the goals of this study was to investigate the possibility that molecular changes in the chromatin template contribute to short- and long-term alterations in gene expression, and are responsible for the physiological effects induced by stimulation of DA, mACh and GLU receptors in the hippocampus.

The physiological effects induced by stimulation of DA, mACh and GLU receptors are thought to be determined by changes in spatiotemporal patterns of gene expression (Wisden et al., 1990; Walton et al., 1999). What are the molecular bases of these changes? Our data provide evidence of profound chromatin remodeling in hippocampal neurons in response to various signaling systems. By systemic injection of specific agonists for DA, mACh and GLU receptors, we have observed a specific, intense phosphorylation of Ser10 of H3 in different subfields of the hippocampus (Figs 1, 2, 5). The different pattern

of H3 phosphorylation in the various subfields of the hippocampus (Figs 1, 2) is likely to reflect the intrinsic physiological differences among neurons of the various regions and the selective cellular distribution of the individual receptors. For example, plasticity at the CA1/CA2 synapses, in contrast to DG and CA3 synapses, is crucial for spatial memory (Tsien et al., 1996a; Tsien et al., 1996b; Chen and Tonegawa, 1997).

The kinetics of histone H3 phosphorylation parallel the induced phosphorylation of the ERKs. Double-labeling experiments using specific phospho-antibodies clearly demonstrated that these two events are concomitantly occurring in the same neurons (Fig. 3). In addition, phosphorylation of histone H3 correlates temporally with the induction of the IEGs c-*fos*, *MKP-1* and *MKP-3*. At least for c-*fos*, it is clear that H3 phosphorylation and induced gene expression occur in the same neurons (Fig. 4). Moreover, the induction of *MKP-1* and *MKP-3* expression after stimulation of DA, mACh and GLU receptors might relate to a possible feedback mechanism that could control the levels of phosphorylated ERKs and thereby the transcriptional induction of IEGs. An attractive possibility is that induced levels of MPK-1 and MPK-3 phosphatase activities could also modulate the phosphorylation state of H3, thereby contributing to chromatin remodeling events subsequent to gene induction. To date, however, there is no information about Ser10 H3 phosphatases related to transcriptional regulation and there is no evidence that phosphatases of the MKP class could dephosphorylate the H3 substrate.

Both acetylation on Lys14 and phosphorylation on Ser10 of histone H3 tail have been associated with activation of transcription in yeast, mammals and *Drosophila* (Cheung et al., 2000b; Clayton et al., 2000; Lo et al., 2000; Thomson et al., 2001). In agreement with what has been described in fibroblasts stimulated with mitogens (Cheung et al., 2000b; Thomson et al., 2001), in the hippocampus, we observe a high level of acetylation on both Lys9 and Lys14 in saline-treated animals (Fig. 5, Fig. 6A) and bulk levels of acetylated H3 did not significantly change after treatment, presumably because acetylated H3 is generally associated with active chromatin, whereas responsive genes represent only a small subset of the genome. Transcription associated with Ser10 phosphorylation, but independent from acetylation, has been described in polytene chromosomes of *Drosophila* (Nowak and Corces, 2000; Labrador and Corces, 2003). This does not seem to be the case in our experimental system. In accordance with results obtained in cell cultures, we have demonstrated the presence of phosphoacetylated histone H3 in hippocampal neurons after stimulation of DA, mACh and GLU receptors, both by immunohistochemistry and western blot analyses (Fig. 6B). Most importantly, ChIP assays have demonstrated that c-*fos* and *MKP-1* associated nucleosomes undergo preferential histone H3 phosphoacetylation upon DA, mACh and GLU stimulation (Fig. 5B), suggesting a causal link between H3 phosphorylation and IEG transcriptional activation in hippocampal neurons. Two different mechanisms have been proposed to account for the presence of phosphoacetylated H3 associated to promoters of IEGs. One possibility is that phosphorylation and acetylation are completely independent, so that indiscriminate phosphorylation of unmodified and acetylated H3 leads to accumulation of monophosphorylated and phospho-acetylated H3 (Thomson et al., 2001). Alternatively, these two modifications are sequentially linked. The present model, based on experiments in both yeasts and mammalian cells, favors a scenario in which phosphorylation occurs preferentially on unmodified H3 and that a proportion of the phosphorylated H3 is subsequently acetylated (Cheung et al., 2000b; Lo et al., 2001). Additional experiments are needed to test this hypothesis in hippocampal neurons.

Although additional evidence is needed to prove a causal link between ERK activation, H3 phosphorylation and IEG transcriptional activation in hippocampal neurons, previous studies in cultured cells indicate that the MAPK signal transduction pathway play a central role in H3 phosphorylation (Sassone-Corsi et al., 1999; Thomson et al., 1999; Cheung et al., 2000a). Interestingly, recent findings indicate that ERK activation is crucial in different models of learning and memory (reviewed in Sweatt, 2001). ERKs have been recognized as biochemical signal integrators and molecular coincidence detectors for coordinating responses to extracellular signals in neurons. Indeed, activation of several cascades induce ERK phosphorylation. For example, experiments performed on brain slices have shown that signaling through the dopamine receptor subtype D2 regulate gene expression by coupling to the Gq/PLC pathway, causing an elevation of intracellular Ca^{2+} and activation of protein kinase C, leading to the phosphorylation and activation of MAPK/ERK kinases (Yan et al., 1999). By contrast, ERK activation upon stimulation of mACh receptors seems to be mostly Src dependent, only partially dependent on phosphoinoside-3-kinase and Ca^{2+}, and independent of protein kinase C (Rosenblum et al., 2000).

An intriguing possibility is that ERK signal integration does not simply sum up signals but rather allows synergistic effects, at least in some cases. If ERK/MAPK signaling pathways constitute a key set of molecules integrating signals in the hippocampus, what is the read-out of the integrated signals? What could be the downstream effectors of ERKs that contribute to the physiological response induced by such a range of neuromodulators? A likely scenario emerging from our data identifies histone-H3 phosphorylation as another biochemical signal integrator, downstream of ERKs and coordinating responses to extracellular signals in hippocampal neurons. Activated ERKs can orchestrate the activity of other kinases that phosphorylate histone H3 at IEG loci where induced transcription occurs, as has been suggested in other biological systems for the proto-oncogenes c-*fos*, c-*jun* and c-*myc* (Chadee et al., 1999; Cheung et al., 2000b; Clayton et al., 2000), the genes induced by follicle-stimulating hormone (Salvador et al., 2001), and the gene encoding MKP-1 (Li et al., 2001). Interestingly, promoter-specific changes in histone acetylation have been observed in hippocampal neurons of rats induced into status epilepticus (Huang et al., 2002).

The superior functions that reside in the hippocampus identify this area of the mammalian brain as one of those where neuronal plasticity is the most evident. The intrinsic ability of hippocampal neurons to respond to many stimuli underscores the complex and yet versatile molecular mechanisms that must operate in order to ensure a dynamic response at the level of gene expression. The accumulating evidence that chromatin modifications are essential to ensure either activation or silencing of transcription (Berger and Felsenfeld, 2001) places these events in a privileged position to improve our

understanding of neuronal functions. Although, here, we have uniquely investigated the acute response to neuronal stimulation, our results represent an important step to unravel these mechanisms. The plasticity of chromatin modifications seems to be ideally suited to facilitate the complexity and dynamic changes in neuronal responses. In addition to the acute responses discussed here, it would fascinating to explore the contribution of epigenetic factors in determining the pathways of neuronal gene expression. It is evident that phosphorylation of histone H3 is coupled to increased neuronal activity and is directly linked to IEG transcription. Because both the transcriptional activity of c-*fos* (Tischmeyer and Grimm, 1999; Vann et al., 2000) and ERK phosphorylation (Swank and Sweatt, 2001; Sweatt, 2001) have been repeatedly shown to be induced under condition of learning, further investigation will be required to test the contribution of chromatin remodeling to this process.

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