Reciprocal Interaction of Serotonin and Neuronal Activity in Regulation of cAMP-Responsive Element-Dependent Gene Expression

Melissa A. Mahgoub, Yildirim Sara,1 Ege T. Kavalali, and Lisa M. Monteggia
Departments of Psychiatry (M.A.M., L.M.M.) and Physiology (E.T.K.), Center for Basic Neuroscience (Y.S., E.T.K.), University of Texas Southwestern Medical Center, Dallas, Texas
Received October 13, 2005; accepted December 23, 2005

ABSTRACT
Neuronal activity triggers multiple signal transduction pathways and potently regulates gene expression in the brain. In the central nervous system, in addition to the synaptic input, neurons are subject to neuromodulatory influences that can activate the same signaling elements. However, the principles that govern the interaction of neuromodulators and neuronal activity in the regulation of gene expression are unclear. Here, we examine how serotonergic neuromodulation interacts with neuronal activity in the regulation of gene expression in hippocampal neurons. We show that cAMP-response element binding protein (CREB) phosphorylation and gene expression were stimulated by serotonin (5-HT) in the absence of neuronal activity. In contrast, in the presence of neuronal activity, 5-HT inhibited gene expression down to the baseline, although neuronal activity alone was sufficient to maximally activate gene expression. The ability of 5-HT to stimulate CREB phosphorylation in the absence of neuronal activity or inhibit CREB phosphorylation during activity was due to a tight balance between protein kinases and phosphatases that could be physiologically tilted by different serotonergic receptors or exogenously influenced by phosphatase and kinase inhibitors. Taken together, these results suggest a reciprocal inhibitory interaction between neuronal activity and 5-HT in the regulation of cAMP response element-dependent gene expression in hippocampal neurons.

Neuronal activity can exert a profound influence on downstream gene expression. In the hippocampus, activity-dependent changes occur in a number of genes that contain cAMP-responsive elements (CREs), including c-fos (Sassone-Corsi et al., 1988) and somatostatin (Montminy and Bilezikjian, 1987). CREs are specific sequences present within the regulatory changes occur in a number of genes that contain cAMP-responsive elements (CREs), including c-fos (Sassone-Corsi et al., 1988) and somatostatin (Montminy and Bilezikjian, 1987). CREs are specific sequences present within the regulatory regions of many genes on which the transcriptional factor, cAMP response element binding protein (CREB), binds and mediates effects on gene expression (Montminy, 1997; Shaywitz and Greenberg, 1999). The function of CREB is determined by its phosphorylation state. Studies have demonstrated that phosphorylation of CREB at Ser-133 is necessary for CRE-mediated transcription (Montminy et al., 1990). Studies of CREB phosphorylation at Ser-133 have shown that protein kinase A (PKA), mitogen-activated protein kinases, and calcium/calmodulin-dependent protein kinases (CaMks) can each phosphorylate this residue depending on the stimulus (West et al., 2001).

Hippocampal activity is regulated by several neuromodulators secreted by nerve terminals that originate from mult...
tiple brain regions, including the hippocampus (Cooper et al., 1996). These neuromodulators can activate the same signal transduction pathways as neuronal activity and exert profound changes on downstream gene expression. These neuromodulators can also influence neuronal activity by regulating the activity of ion channels as well as directly influencing neurotransmitter release machinery in the mammalian brain. How then do neuromodulators interact with neuronal activity to regulate gene expression at the cellular level? The interaction between neuromodulators and neuronal activity could be linearly additive; that is, the outcome in gene expression could be predicted from the negative or positive effect of the two stimuli on different targets within the signal transduction pathway. Or, alternatively, the two extrinsic stimuli could have a more complex nonlinear interaction.

One important neuromodulator that can influence the activity of hippocampal neurons is the neurotransmitter serotonin (5-HT). The hippocampus, in particular the dentate gyrus, receives widespread serotonergic innervation from the median raphe nucleus (Moore and Halaris, 1975). This serotonergic innervation can alter hippocampal activity and may have a significant role in the regulation of psychiatric disorders such as mood disorders and schizophrenia (Lopez-Ibor, 1992). 5-HT's role in the physiology and/or pathophysiology of the hippocampus has been attributed to the long-term changes it exerts on neuronal circuitry presumably directed by its influence on neuronal gene expression, as has previously been shown for Aplysia (Pittenger and Kandel, 2003). Indeed, serotonergic receptors that can activate or inhibit the cAMP signaling cascade, and ultimately downstream gene expression, are present on hippocampal neurons. However, activation of serotonergic receptors can also directly influence membrane excitability by activating K+ channels. Therefore, the potential interaction of 5-HT and neuronal activity in the regulation of gene expression in the hippocampus may be quite complex.

To examine the interaction of 5-HT and neuronal activity in the regulation of gene expression, we examined CRE-dependent gene expression and CREB phosphorylation in dissociated hippocampal cultures. Using this approach, we show that CRE-dependent gene expression and its trigger, CREB phosphorylation, can be stimulated by 5-HT in the absence of spontaneous activity. Although neuronal activity alone was sufficient to maximally activate gene expression, 5-HT in the presence of neuronal activity inhibited gene expression down to background levels. Surprisingly, the serotonergic stimulation of CREB phosphorylation in the absence of neuronal activity, as well as serotonergic inhibition of CREB phosphorylation in the presence of activity, displayed a slow time course reaching maximal levels (stimulation or inhibition) within 2 to 3 h. Using specific as well as broad-spectrum inhibitors of protein kinases and phosphatases, we show that this slow time course is due to a tight balance between the activity of kinases and phosphatases. In the absence of neuronal activity, 5-HT acting through 5HT1A and 5-HT7 receptors can tilt this balance toward CREB phosphorylation, whereas in the presence of activity, 5-HT tilts this balance toward dephosphorylation by its action through 5-HT7 receptors. Overall, these results suggest a complex reciprocal interaction between neuronal activity and 5-HT in the regulation of CRE-dependent gene expression in hippocampal neurons.
**Western Blot Analysis.** After stimulation, cells were scraped in lysis buffer (25 mM HEPES, 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40, 1 μM aprotinin, 1 μM leupeptin, 10 mM NaF, and 1× protease inhibitor), and protein concentration was determined by the modified Lowry method (Bio-Rad, Hercules, CA; Lowry et al., 1951). Total protein (25 μg) in reducing loading dye was boiled for 3 min, then electrophoresed on an 8% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane, then blocked in 1× Tris-buffered saline with 0.1% Tween 20 and 10% nonfat dry milk for 1 h at room temperature. The blots were then incubated in the presence of the rabbit anti-pCREB (1:1000 dilution; Upstate), rabbit anti-CREB (1:1000; Upstate), or mouse anti-actin (1:10,000; ICN, Irvine, CA) in fresh blocking solution overnight at 4°C. The blots were washed three times for 10 min in 1× Tris-buffered saline with 0.1% Tween 20 at room temperature and then incubated for 1.5 h at room temperature with a peroxidase-labeled goat anti-rabbit IgG (1:2500; Vector Labs) or goat anti-mouse IgG (1:20,000; Vector Labs). Bands were visualized using enhanced chemiluminescence (Amersham, Little Chalfont, Buckinghamshire, UK). Densitometry of the immunoreactivity was quantitated with the Scion Image Program.

**Results**

**Dissociated Hippocampal Cultures Display Spontaneous Network Activity.** To investigate the interaction of neuronal activity and 5-HT in the regulation of CRE-dependent gene expression, we used a dissociated hippocampal culture system obtained from dentate gyrus (DG) and CA3 regions of neonatal rat hippocampus (Kavalali et al., 1999). Neurons in this culture system form an active network, which displays multiple forms of functional and structural plasticity (Baranes et al., 1996; Kavalali et al., 1999). We first wanted to characterize the properties of spontaneous network activity in these DG-CA3 cultures to see whether it is comparable with the activity seen in other dissociated culture systems such as cultured cortical neurons or retinal neurons (Harris et al., 2002; Opitz et al., 2002).

Whole-cell patch clamp recordings revealed that spontaneous synaptic transmission starts 5 days after plating of DG-CA3 hippocampal cultures and occurs naturally without pharmacological or electrical intervention (Fig. 1, A and B). The increase in the frequency of spontaneous miniature synaptic events was strongly correlated with the increased frequency and periodicity of the spontaneously evoked synaptic currents (Fig. 1C). The developmental increase in the frequency of spontaneous miniature synaptic events closely parallels continual formation and maturation of synapses in culture (Mozhayeva et al., 2002). Therefore, the correlation between spontaneous miniature event frequency and spontaneous activity suggests that continual synapse formation and maturation in the culture results in assembly of a dense functional network, which results in spontaneous network activity. This spontaneous network activity is characterized by action potential firing in individual neurons (as evidenced by its sensitivity to tetrodotoxin) and propagates in the culture through synaptic contacts (as evidenced by its sensitivity to the glutamate receptor antagonist 6-cyano-2,3-dihydroxy-7-nitroquinoxaline) (data not shown). The spontaneous network activity increases as the cultures mature and reaches maximal level by 14 days after plating at a frequency around 5 Hz.

**Spontaneous Network Activity Influences 5-HT Regulation of CRE-Dependent Gene Expression.** How does this spontaneous network activity influence CRE-dependent gene expression in these cultures? To examine CRE-dependent gene expression, we quantified the levels of c-fos and somatostatin, two CRE-containing genes that are transcriptionally activated by the binding of pCREB at Ser-133. We examined c-fos and somatostatin levels by fluorescent immunocytochemistry in mature (14 days) hippocampal cultures with or without concomitant spontaneous network activity. We treated primary hippocampal cultures with near-physiological Tyrode’s solution (4 mM K+) alone or with 1 μM tetrotoxin to block spontaneous network activity. As expected from previous studies, activity (4 mM K+) significantly increases gene expression, compared with treatment with tetrotoxin, as detected by a clear shift in the number of neurons exhibiting enhanced c-fos and somatostatin immunofluorescence (Fig. 2, A and B) (p < 0.001, Kolmogorov-Smirnov test).

A

To test the potential influence of 5-HT on this activity-dependent gene expression, we added 10 μM 5-HT for 2 h to hippocampal cultures in the presence of or absence of spontaneous network activity. Surprisingly, we found that 5-HT in the presence of tetrotoxin produced a robust increase in c-fos and somatostatin immunocytochemistry. However, 5-HT in the presence of spontaneous network activity decreased c-fos and somatostatin immunocytochemistry below the level seen with spontaneous activity alone (Fig. 2, A and B) (p < 0.001, Kolmogorov-Smirnov test).

**5-HT Inhibits Spontaneous Network Activity in Hippocampal Cultures.** How does 5-HT affect spontaneous network activity in these cultures to regulate CRE-dependent gene expression? To begin addressing this question, we first examined the effect of 5-HT on spontaneous network activity...
activity in hippocampal cultures and recorded in current clamp (to detect action potentials) and in voltage-clamp (to detect synaptic responses in isolation) modes (Fig. 3, A and B). 5-HT inhibited synaptic responses driven by network activity (Fig. 3D). However, it did not have any effect on the frequency and amplitude of miniature excitatory postsynaptic currents observed in the presence of tetrodotoxin, as well as uptake and release of styryl dye FM1-43 measuring the size of total recycling vesicle pool per synapse (data not shown). In contrast, spontaneous action potentials detected in current clamp mode were significantly reduced in frequency after 5-HT application [from 4.8 ± 1.0 Hz (n = 9) control to 0.3 ± 0.1 Hz after 5-HT (n = 7)] (Fig. 3D). The reduction in action potential firing frequency was also associated with a 12-mV hyperpolarization induced by 5-HT application (Fig. 3C). Our results on the action of 5-HT on spontaneous activity are consistent with earlier studies that indicate that 5-HT can cause hyperpolarization in hippocampal neurons through activation of a G protein-coupled inward rectifier K⁺ channel (Sodickson and Bean, 1998).

5-HT Stimulates CREB Phosphorylation in an Activity-Dependent Manner. We next monitored CREB phosphorylation, the principle target of signal transduction cascades that trigger CRE-dependent gene expression, under the same conditions. To monitor CREB phosphorylation, we incubated mature hippocampal cultures, at least 14 days in vitro, with 4 mM K⁺ in the presence or absence of tetrodotoxin. We measured nuclear CREB immunofluorescence using an antibody specific for pCREB at Ser-133 site (Ginty et al., 1993). In the presence of tetrodotoxin, pCREB was detectable at low levels (Fig. 4A). In contrast, neuronal activity in the absence of tetrodotoxin produces a strong induction in pCREB immunofluorescence (Fig. 4A). To determine the effect of 5-HT on CREB phosphorylation, we stimulated cultures with 10 μM 5-HT in the presence of tetrodotoxin and observed strong pCREB immunofluorescence (Fig. 4A). However, 5-HT in the absence of tetrodotoxin inhibited CREB phosphorylation to similar levels observed with 4 mM K⁺ in the presence of tetrodotoxin (Fig. 4A). We quantitated the level of pCREB immunofluorescence and then graphed the data as cumulative histograms (Fig. 4B). We found that in the presence of tetrodotoxin, 5-HT produces a maximal shift in CREB phosphorylation similar to that seen when hippocampal cultures are active. However, in the absence of tetrodotoxin, 5-HT inhibits pCREB back to baseline levels. The immunofluorescence data for all subsequent experiments is show as the percent change immunofluorescence relative to baseline (4 mM K⁺ and TTX) conditions (Fig. 4C). We confirmed the immunofluorescence data with immunoblots using the phospho-Ser-133 CREB antibody and showed that the change in pCREB was not due to a change in total levels of CREB (Fig. 4D).

Next, we investigated the effect of various 5-HT concentrations on pCREB levels. We stimulated hippocampal cultures with increasing concentrations of 5-HT (1, 10, or 100 μM) in the presence or absence of tetrodotoxin. We found that in the presence of tetrodotoxin, 5-HT increased pCREB with maximal stimulation observed at 10 μM 5-HT (Fig. 5A). The levels of pCREB observed following 5-HT stimulation were indistinguishable from the levels observed following forskolin
We initially focused on the 5-HT1A and 5-HT7 receptor subtypes are involved in mediating the activity-dependent pCREB levels. Therefore, we stimulated cells with 5-HT, in the presence or absence of tetrodotoxin, for 2 h in this study unless otherwise specified.

Fig. 4. Effect of 5-HT on CREB phosphorylation. Fluorescent staining of CREB phosphorylation in 4 mM K+ and tetrodotoxin, 5-HT in the presence of tetrodotoxin, 4 mM K+ without tetrodotoxin, and 5-HT in the absence of tetrodotoxin (A). Cumulative histogram of pCREB immunofluorescence following 5-HT stimulation showed that 5-HT in the presence of tetrodotoxin stimulates pCREB levels, whereas 5-HT in the absence of tetrodotoxin inhibits pCREB (B). The percent change of pCREB immunofluorescence is plotted relative to 4 mM K+ in the presence of tetrodotoxin (C). Western blot analysis confirms that 5-HT in the presence of tetrodotoxin stimulates pCREB, whereas 5-HT in the absence of tetrodotoxin inhibits pCREB in primary hippocampal cultures (D).

We next investigated which 5-HT receptor subtypes because they have been suggested to play a role in mediating 5-HT’s effect on CREB phosphorylation (Duman, 1996), a pp90rsk (Bohm et al., 1995), and Ras-dependent protein kinases (Sheng et al., 1991; Bito et al., 1998) and are highly expressed in the hippocampus (Ruat et al., 1993; Kia et al., 1996). To better understand the biochemical mechanism underlying 5-HT’s activation of pCREB and downstream gene expression, we focused on identifying the kinases involved in this process. Many Ser/Thr kinases can phosphorylate CREB Ser-133, including PKA (Gonzalez et al., 1989), protein kinase C (Xie and Rothstein, 1995), different forms of Ca2+/calmodulin-dependent protein kinases (Sheng et al., 1991; Bito et al., 1996), a pp90rsk (Bohm et al., 1995), and Ras-dependent p105 kinase (Ginty et al., 1994). To delineate the kinases involved in 5-HT-mediated CREB phosphorylation, we used a broad array of kinase inhibitors, including a PKA inhibitor (KT5720, 10 μM), a MEK inhibitor (PD98059, 50 μM), a CaM kinase inhibitor (KN-93, 30 μM; and its inactive analog KN-92, 30 μM), and the nonselective kinase inhibitor staurosporine (100 nM). Kinase inhibitors were added 15 min prior to treatment or forskolin treatment in the presence of 5-HT, suggesting that 5-HT stimulation is producing a maximal increase in pCREB levels (data not shown). We also found that in the absence of tetrodotoxin, 5-HT inhibited pCREB with maximal inhibition at 10 μM 5-HT (Fig. 5A). Because 10 μM 5-HT produced the maximal amount of pCREB stimulation (in the presence of tetrodotoxin) and inhibition (in the absence of tetrodotoxin), we use this concentration for all experiments in this study.

We then examined the time course of 5-HT-mediated effects on CREB phosphorylation. 5-HT (10 μM) elicited a slight shift in the phosphorylation state of CREB within 30 min but a robust effect was not observed until after 2 h of stimulation (Fig. 5B). The ability of 5-HT to inhibit pCREB, in the presence of activity, took approximately 2 h to bring pCREB levels back to baseline (Fig. 5B). Therefore, we stimulated cells with 5-HT, in the presence or absence of tetrodotoxin, for 2 h in this study unless otherwise specified.

5-HT Receptor Subtypes Involved in Regulating pCREB Levels. We next investigated which 5-HT receptor subtypes are involved in mediating the activity-dependent changes on CREB phosphorylation in hippocampal neurons. We initially focused on the 5-HT1A and 5-HT7 receptor subtypes because they have been suggested to play a role in mediating 5-HT’s effect on CREB phosphorylation (Duman, 1998) and are highly expressed in the hippocampus (Ruat et al., 1993; Kia et al., 1996). Cells were pretreated in Tyrode’s solution for 2 h, then treated with the selective antagonists 10 μM WAY-100635 (5-HT1A antagonist), 10 μM SB269970 (5-HT7 antagonist), or 10 μM ketanserin (5-HT2 antagonist) 30 s prior to 5-HT stimulation. We found that in the presence of tetrodotoxin, WAY-100635 significantly attenuated 5-HT’s ability to stimulate CREB phosphorylation, whereas SB269970 completely blocked this effect, with ketanserin having no effect (Fig. 6A). However, in the absence of tetrodotoxin, only SB269970 was able to significantly prevent the 5-HT-mediated dephosphorylation of CREB (Fig. 6B).

To confirm that both 5-HT1A and 5-HT7 stimulation could mediate CREB phosphorylation, we treated cultures with varying concentrations (1, 10, or 100 μM) of either the 5-HT1A agonist 8-OH DPAT or the 5-HT1A/7 agonist 5-CT in the presence of tetrodotoxin. Currently, there are no selective 5-HT7 agonists. We found that both 8-OH DPAT and 5-CT stimulate CREB phosphorylation in the presence of tetrodotoxin, with maximal phosphorylation observed at 10 μM concentration (Fig. 6A).

5-HT Activates Parallel Intracellular Signaling Pathways to Phosphorylate/Dephosphorylate CREB. To confirm that both 5-HT1A and 5-HT7, stimulation could mediate CREB phosphorylation, we treated cultures with varying concentrations (1, 10, or 100 μM) of either the 5-HT1A agonist 8-OH DPAT or the 5-HT1A/7 agonist 5-CT in the presence of tetrodotoxin. Currently, there are no selective 5-HT7 agonists. We found that both 8-OH DPAT and 5-CT stimulate CREB phosphorylation in the presence of tetrodotoxin, with maximal phosphorylation observed at 10 μM concentration (Fig. 6A).
5-HT stimulation and remained in the solution during stimulation. We found that KT5720, PD98059, and KN93 all blocked the ability of 5-HT to stimulate CREB phosphorylation \((p < 0.05)\), suggesting the involvement of the 5-HT\(_{1A}\) and 5-HT\(_{7}\) receptor subtypes. To confirm this finding, the 5-HT\(_{1A}\)-selective agonist 5-CT (10 \(\mu\)M) and the 5-HT\(_{1A}\)-selective agonist 8-OH DPAT (10 \(\mu\)M) were added to the hippocampal cultures for 2 h in the presence of tetrodotoxin. Both 5-CT and 8-OH DPAT stimulated CREB phosphorylation in the presence of tetrodotoxin. In the absence of tetrodotoxin, WAY-100635 does not block 5-HT's ability to dephosphorylate CREB (B). In contrast, SB269970 significantly prevents the ability of 5-HT to CREB dephosphorylate CREB \((p < 0.05)\), suggesting the involvement of the 5-HT\(_{1A}\) or the 5-HT\(_{1C}\) receptor subtype.

We next investigated the phosphatases involved in the dephosphorylation of CREB following 5-HT stimulation of hippocampal neurons. There are several types of phosphatases expressed in the brain, including protein phosphatase 1, protein phosphatase 2A, protein phosphatase 4, protein phosphatase 5, and a Ca\(^{2+}\)/CaM-dependent protein phosphatase (also called calcineurin). Phosphatase inhibitors were added 15 min prior to 5-HT and remained in the solution during stimulation. We examined the effect of low doses of okadaic acid (OA; 20 nM), which will inhibit protein phosphatase 2A, protein phosphatase 4, and protein phosphatase 5, the effect of high doses of OA (2 \(\mu\)M), which inhibits PP1, and FK-506 (1 \(\mu\)M), a specific inhibitor of calcineurin in our cell culture system. We found that low doses of OA had no effect on preventing the ability of 5-HT to reduce CREB phosphorylation; however, high doses of OA as well as FK-506 significantly inhibited the ability of 5-HT to dephosphorylate CREB \((p < 0.01)\), it did not fully block the effect, suggesting that other phosphatases may be involved.

### Inhibition of Kinases or Phosphatases Shifts the Time Course of 5-HT-Induced CREB Phosphorylation

Although 5-HT's ability to phosphorylate or dephosphorylate
CREB involved multiple intracellular pathways (Fig. 7, A and B), the time course to mediate these effects was rather slow (Fig. 5B). We were interested to determine whether 5-HT stimulation activates both kinases and phosphatase at the same time that then compete to produce a rather slow delay on CREB phosphorylation. To test this, we added the phosphatase inhibitors OA (2 μM) and FK-506 to our cultures in the presence of tetrodotoxin and then stimulated with 5-HT for 3 min. We chose a 3-min 5-HT stimulation because this time point normally produces only a minimal increase in pCREB (Fig. 5B). We found that 5-HT in the presence of OA and FK-506 produced a significant increase in the amount of CREB phosphorylation (Fig. 8A).

Because we could speed up the time it took for 5-HT to stimulate pCREB levels by blocking phosphatases, we wondered whether we could speed up 5-HT's ability to dephosphorylate CREB by blocking specific kinases. We stimulated cultures with 5-HT for 3 min in the presence of the kinase inhibitors KT5720 and PD98059 and found that blocking PKA and MEK resulted in a faster dephosphorylation of CREB (Fig. 8B).

Discussion

The results presented in this study show that 5-HT regulation of CREB phosphorylation and downstream CRE-dependent gene expression are influenced by the activity state of neurons. When neurons are inactive, 5-HT stimulates CREB phosphorylation and CRE-dependent gene expression. In the presence of neuronal activity, however, 5-HT inhibits CREB phosphorylation to the levels seen without any activity. In addition, 5-HT also inhibits spontaneous network activity present in these cultures. The inhibitory effect of 5-HT cannot be attributed to its inhibition of spontaneous activity because the inhibition by 5-HT brings the action potential firing frequency down to 1 Hz, which normally is sufficient to trigger CREB phosphorylation (Deisseroth et al., 1996). Furthermore, serotonergic stimulation of CREB phosphorylation seen in the absence of activity is not detectable when spontaneous activity is permitted. Rather, our findings indicate that this dual action of 5-HT is due its simultaneous activation of phosphatase and kinase pathways. The tight balance between the two pathways leads to a slow time course of 5-HT action on CREB (within hours). The presence of neuronal activity shifts the balance toward CREB dephosphorylation, whereas in the absence of activity the kinase pathway wins over time and leads to CREB phosphorylation. This premise is further supported by the rapid action of 5-HT (within minutes) on CREB in the presence of kinase or phosphatase inhibitors. These findings also reveal that even at rest, the phosphorylation state of CREB is tightly controlled by a balance between the activity of kinases and phosphatases.

This tight coupling between kinases and phosphatases is further supported by our results that inhibition of PKA, mitogen-activated protein kinase, or CaMK (during serotonergic stimulation in the presence of activity) each produces almost a complete blockade of CREB phosphorylation. These data suggest that inhibition of any of these kinases diminishes pCREB down to baseline levels due to the shift in the balance toward phosphatases that can then dephosphorylate CREB within this time frame when kinases are disadvantaged. Conversely, the inhibition of either PP1 or calcineurin (during serotonergic stimulation in the absence of activity) results in a significant increase in the amount of CREB phosphorylation, mirroring the previous shift.

The activity-dependent shift between regulation of kinase and phosphatase signaling that we see here shares similarities with the tight regulation of signaling pathways that has been implicated in the triggering of long-term potentiation (LTP) and long-term depression (LTD) (Malinow and Malenka, 2002). N-Methyl-D-aspartate-dependent LTD in the hippocampus occurs under conditions of low-frequency stimulation (akin to what we observe after inhibition by 5-HT, see Fig. 3) in which small increases in intracellular
calcium lead to activation of calcineurin and PP1, a necessary step in the induction of LTD. However, following stronger stimulation and larger increases in intracellular calcium, CaMKII is activated, which is necessary for the induction of LTP (Lisman et al., 2002). This analogy with the signaling cascades involved in synaptic plasticity may shine light onto the mechanisms that underlie 5-HT and neuronal activity interaction in the regulation of gene expression.

This tight interaction of 5-HT and neuronal activity in the regulation of CREB phosphorylation is also coupled to specific 5-HT receptor subtypes. In the absence of activity, stimulation of either the 5-HT1a receptor or 5-HT7 receptor results in increased CREB phosphorylation. This finding was rather unexpected because 5-HT1a receptors couple to G<sub>i</sub> to inhibit the cAMP pathway, whereas 5-HT7 receptors couple to G<sub>s</sub> to stimulate the cAMP pathway in cell lines overexpressing these receptor subtypes (Liu and Albert, 1991; Baker et al., 1998). It is possible that the coupling of these receptor subtypes in vivo in central neurons may be more complex compared with their properties in heterologous expression systems. Interestingly, activations of the 5-HT1a and 5-HT7 receptors have both been suggested to stimulate CREB phosphorylation (Nishi and Azmitia, 1999; Johnson et al., 2003). Clearly, the coactivation of activity- and 5-HT-dependent pathways may lead to complex interactions between different signaling cascades, which net effect on CREB phosphorylation would be hard to predict without direct knowledge of intracellular calcium and second messenger levels (e.g., cAMP). Therefore, future experiments measuring the intracellular levels of cAMP and calcium following activity and/or serotonin stimulation, preferably with specific 5-HT receptor agonists, will be instrumental in dissecting the relative contribution of these pathways in promoting CREB phosphorylation.

One perplexing question is how activation of 5-HT<sub>7</sub> can both stimulate intracellular pathways to increase CREB phosphorylation (in the absence of activity) and inhibit CREB phosphorylation (in the presence of activity). One possibility may involve the 5-HT<sub>7</sub> receptor splice variants (5-HT<sub>7</sub>x, 5-HT<sub>7</sub>y, 5-HT<sub>7</sub>z, and 5-HT<sub>7</sub>z), which share a high degree of homology but differ at their carboxy terminus. It is possible that 5-HT<sub>7</sub> receptor splice variants couple to different intracellular signal transduction cascades. Indeed, a recent report has demonstrated that the 5-HT<sub>7</sub> splice variant produces a significantly higher level of constitutive adenylyl cyclase following forskolin stimulation than the 5-HT<sub>7</sub>x and 5-HT<sub>7</sub>y splice variants (Krobert and Levy, 2002). Another possibility may be the involvement of 5-HT<sub>7</sub> receptors with scaffolding proteins that place protein kinases and phosphatases close to their effectors and ensure a high level of specificity in intracellular signaling pathways. For instance, in the central nervous system, the A kinase anchoring protein 79 is known to be an important scaffolding protein that binds to protein kinase A, protein kinase C, and calcineurin in the regulation of AMPA receptors (Klauk et al., 1996). Targeting of these kinases and phosphatases to the AMPA-type glutamate receptors plays an important role in regulating AMPA receptor function during LTP and LTD (Collede et al., 2000; Tavalin et al., 2002). Interestingly, some isoforms of the 5-HT<sub>7</sub> receptor (5-HT<sub>7</sub>x and 5-HT<sub>7</sub>y) appear to have putative postsynaptic density 95/disc-large/zona occludens (class II) domains, which may suggest an association with postsynaptic density 95/disc-large/zona occludens domain containing scaffolding proteins and ultimately protein kinase and phosphatases that contribute to the tight balance between phosphorylation and dephosphorylation we observed following 5-HT<sub>7</sub> activation. Further work will be necessary to investigate this possibility.

In addition, neuronal activity may also influence the subcellular localization of protein kinases and phosphatases or the subcomposition of scaffolding proteins, thereby influencing the downstream signal transduction pathways. For instance, it has recently been shown that N-methyl-D-aspartate receptor activation necessary to induce LTD can recruit PP1 to synapses (Morishita et al., 2001). The protein kinase, CaMKII, has also been shown to be redirected to synapses in an activity-dependent manner.

Our electrophysiological experiments indicate that 5-HT application has no significant direct effect on synaptic transmission (i.e., neurotransmitter release machinery), but 5-HT could decrease action potential firing by activating a hyperpolarizing current in hippocampal neurons, which in turn inhibits neurotransmission driven by these action potentials. Thus, the 5-HT effect on spontaneous network activity was predominantly inhibitory by decreasing the resting membrane potential. Can the 5-HT-dependent inhibition of gene expression be attributed to the inhibition of spontaneous activity by 5-HT? The answer to this question is no because of two reasons. First, the inhibition by 5-HT brings activity down to 1 Hz (Fig. 3D), a frequency that by itself is still sufficient to trigger CREB phosphorylation (Deisseroth et al., 1996). Second, although 5-HT inhibits spontaneous activity, it stimulates CRE-dependent gene expression in the absence of any activity. Therefore, to explain this effect, we need to postulate that somehow in the presence of activity the stimulatory effect of 5-HT is lost and turns into a fully inhibitory influence.

Why does 5-HT work in an opposite fashion to cellular activity in mediating downstream gene expression? In the central nervous system, neurons show multiple forms of activity in response to synaptic inputs at various frequencies. CREB phosphorylation has been suggested to act as an integrator of the activity history of cells (Bito et al., 1996). Under these circumstances, if 5-HT's actions were simply additive with the influence of activity, then the level of pCREB and CREB-2 would be easily saturated. In contrast, our results provide an answer to this conundrum and indicate that even in the presence of activity, CREB phosphorylation can be turned off by serotonin. Therefore, the coincidence of neuronal activity and serotonin input of CREB phosphorylation forms a molecular switch to control gene expression.

Our results indicate that 5-HT mediates biphasic control of cellular excitability and downstream gene expression. It is unclear whether other neuromodulators act in a similar fashion as 5-HT or if 5-HT is specific in this regard. However, there are examples of dopamine (DA) exerting a state-dependent modulatory effect on the excitability of cortical, caudate-putamen, and nucleus accumbens neurons (O'Donnell, 2003). These cells display an intrinsic “up”/depolarized state when they can generate impulses and a “down”/hyperpolarized state when they are silent, and the transition between these states can in turn be regulated by DA (Peters et al., 2000). It will be of interest to see whether DA's ability to alter the...
state dependence of these neurons results in downstream gene expression akin to that observed with 5-HT. Taken together, this study on the interaction between 5-HT and neuronal activity in the regulation of CREB phosphorylation and, ultimately, gene expression may provide a framework by which neuromodulators activate signal transduction pathways to trigger gene expression.

Acknowledgments

We thank M. Mozayane, E. Nelson, and T. Virmani for sharing cultures and T. Gemelli for technical assistance. We also thank M. Collinge and E. Nestler for critical reading of this manuscript.

References


Address correspondence to: Lisa M. Monteggia, Department of Psychiatry, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9070. E-mail: lisa.monteggia@utsouthwestern.edu