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Reciprocal interaction of 5-HT and neuronal activity in regulation of CRE-dependent gene expression

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Running title: CREB Regulation by 5-HT and Activity

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; CRE, cAMP responsive element; CREB, cAMP response-element binding protein; pCREB, phosphorylated CREB at Ser133; TTX, tetrodotoxin; PKA, protein kinase A; MAPK, mitogen-activated protein kinases; CaMK, calcium/calmodulin-dependent protein kinases; OA, okadaic acid; 8-OH DPAT, 8-hydroxy-dipropylaminotetralin; WAY 100635, N-{2-[4-(2-methyoxyphenyl)-1-piperazinyl]ethyl}-N-2-pyridinylcyclo-hexanecarboxamide; KT5720, (9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester; PD 98059, 2'-Amino-

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3'methoxyflavone; KN92, 2-[N-(4′-Methoxybenzenesulfonyl)]amino-N-(4’-chlorophenyl)-2-propenyl-N-methylbenzylamine phosphate; KN93, N-[2-[[[3-(4'-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4'-methoxybenzenesulfonamide phosphate salt; SB269970, (*R*)-3-[2-[2-(4-Methylpiperidin-1-yl)ethyl]pyrrolidine-1-sulfonyl]phenol hydrochloride; 5-CT, 5-Carboxamidotryptamine maleate salt.

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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 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 CRE-dependent gene expression in hippocampal neurons.

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Introduction

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 (CRE's), including c-fos (Sassone-Corsi et al., 1988) and somatostatin (Montminy and Bilezikjian, 1987). CRE's are specific sequences present within the regulatory region 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 (MAPK) and calcium/calmodulin-dependent protein kinases (CaMK) 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 multiple brain regions including the hippocampus (Cooper, 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 non-linear 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

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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 hours. 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 $5HT_{1A}$ and $5\text{-}HT_7$ receptors can tilt this balance towards CREB phosphorylation, whereas in the presence of activity 5-HT tilts this balance towards dephosphorylation by its action through $5\text{-}HT_7$ 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.

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Methods

Cell culture. Dissociated hippocampal cultures were prepared from 1-2 day-old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) as described (Kavalali et al., 1999; Mozhayeva et al., 2002). All experiments were performed with approval of the Institutional Animal Care and Research Advisory Committee. All pharmacological treatments were carried out with parallel drug-free vehicle controls on at least three culture batches for each condition. All cultures were at least 14 days in vitro unless otherwise states. In a given culture batch, typically 6-12 coverslips were subjected to pharmacological treatment. Before detailed analysis, homogeneity of the cell numbers over all coverslips in a batch was visually verified.

Drugs Treatments. Drugs were obtained from and used at the following concentrations unless otherwise stated, 10 µM 5-HT (Sigma, St. Louis, MO); 10 µM KT5720 (Calbiochem, La Jolla, CA); 50 µM PD 98059 (Calbiochem); 100 nM Staurosporine (Sigma, St. Louis, MO); 30 µM KN92 or KN93 (Seikagaku, Tokyo, Japan); 1 µM FK-506 (Calbiochem); 20 nM and 2 µM Okadaic Acid (Calbiochem); 10 µM WAY 100635 (Sigma); 10 µM SB269970 (Sigma); 10 µM 5-CT (Sigma); 10 µM 8-OH DPAT (Sigma).

Electrophysiology. For neuronal recordings, after at least 10 days old in culture coverslips were placed in a recording chamber and superfused with extracellular solution containing (in mM): 150 NaCl, 4 KCl, 2 $MgCl_2$, 10 Glucose, 10 HEPES and 2 $CaCl_2$, (pH 7.4, 310 mOsm at room temperature). To detect spontaneous neurotransmitter release 1 µM Tetrodotoxin (TTX, Sankyo, Tokyo, Japan) was added to suppress action potential firing. Pyramidal neurons were whole-cell voltage-clamped to –70 mV by using pipettes filled with (in mM): 115 Cs-$MeSO_3$, 10 CsCl, 5 NaCl, 10 HEPES, 0.6 EGTA, 20 tetraethylammonium chloride, 4 Mg-ATP, 0.3 Na_2GTP, 10 QX-314 (Sigma) (pH 7.38, 295 mOsm). For

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current clamp experiments pipettes were filled with (in mM): 135 K-Gluconate, 10 KCl, 5 NaCl, 10 HEPES, 0.6 EGTA, 4 Mg-ATP, 0.3 Na_2GTP (pH 7.38, 295 mOsm).

Immunocytochemistry. Cells were immunostained following standard protocol (Kavalali et al., 1999). Briefly, cultures at least 14 days in vitro were incubated with Tyrode's solution (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES) for 2 hours. The cells were stimulated either in the presence or absence of 1 µM tetrodotoxin for 2 hours unless otherwise stated. All stimulations were carried out in the absence of serum. Cells were fixed for 30 min with 4% paraformaldehyde in phosphate buffered saline (PBS) containing 4 mM EGTA, washed twice with 1X PBS containing 100 mM glycine, blocked in 2% goat serum (Vector Labs, Burlingame, CA) and 0.4% saponin (Sigma), then incubated with primary antibody, anti-pCREB (1:1000, Upstate, Lake Placid, NY), anti-cfos (1:200, Oncogene, San Diego, CA), or anti-somatostatin (1:200, Chemicon, Temedula, CA) overnight at 4ºC. Coverslips were washed twice with 1X PBS containing 100 mM glycine, incubated with secondary antibody, goat-anti-rabbit (1:200; Molecular Probes, Eugene, OR), washed with 1X PBS containing 100 mM glycine, then mounted onto frosted uncharged slides and viewed on an Olympus BX51 upright microscope with an epifluorescent light source. Images were captured with a Sony DXC-9000 color video camera attached to the microscope, and translated to a Scion Image program to determine fluorescent measurements for individual cells by an observer blind to the treatments. Averages from three random images were taken for each slide and plotted as cumulative histograms. Statistical significance was determined using the Kolmogorov-Smirnov test (Figure 2) or by using analysis of variance (ANOVA) followed by the Tukey post hoc test to identify significant differences among groups. A P value <0.05 was considered to be statistically significant.

Western Blot Analysis. Following stimulation, cells were scraped in lysis buffer (25mM Hepes, 150mM NaCl, 2 mM EDTA, 1 mM DTT, .1% NP-40, 1 µM aprotinin, 1 µM leupeptin, 10 mM NaF, 1X Protease Inhibitor) and protein concentration determined by the modified Lowry method (Biorad). 25

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µg of total protein 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 1X TBS with 0.1% Tween 20 and 10% nonfat dry milk for 1 hour at room temperature. The blots were then incubated in the presence of the rabbit anti-pCREB (Upstate; 1:1000 dilution), rabbit anti-CREB (Upstate, NY; 1:1000), or mouse anti-actin (ICN, Irvine, CA; 1:10,000) in fresh blocking solution overnight at 4^{o}C. The blots were washed three times for 10 minutes in 1X TBS with 0.1% Tween 20 at room temperature and then incubated for 1 1/2 hours at room temperature with a peroxidase-labeled goat anti-rabbit IgG (Vector Labs, Burlingame, CA; 1:2500) or goat anti-mouse IgG (Vector Labs; 1:20,000). Bands were visualized using enhanced chemiluminescence (Amersham, Little Chalfont, UK). Densitometry of the immunoreactivity was quantitated with the Scion Image Program.

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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 display 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 to 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. 1A, 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, that 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 CNQX) (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.

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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 trancriptionally activated by the binding of phosphorylated CREB (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 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 to treatment with tetrotoxin, as detected by a clear shift in the number of neurons exhibiting enhanced c-fos and somatostatin immunofluorescence **(**Fig. 2A, B**)** ($p<0.001$, Kolmogorov-Smirnov test). To test the potential influence of 5-HT on this activity-dependent gene expression, we added 10 µM 5-HT for 2 hours 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. 2A, 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 in hippocampal cultures and recorded in current clamp (to detect action potentials) and in voltage-clamp (to detect synaptic responses in isolation) modes **(**Fig. 3A, 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

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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 is consistent with earlier studies which indicate that 5-HT can cause hyperpolarization in hippocampal neurons through activation of a G protein-coupled inward rectifier K^+ channel (GIRK) (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 pCREB immunofluorescence using an antibody specific for phosphorylated CREB (pCREB) at Ser-133 site (Ginty et al., 1993). In the presence of tetrodotoxin, pCREB was detectable at very 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 % 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**)**.

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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 was indistinguishable from the levels observed following forskolin 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**)**. Since 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 hrs of stimulation **(**Fig. 5B**)**. The ability of 5-HT to inhibit pCREB, in the presence of activity, took approximately 2 hours 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 hrs 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-HT_{1A} and 5-HT_7 receptor subtypes since 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 Tyrodes solution for 2 hours, then treated with the selective antagonists 10 μM WAY-100635 (5-HT_{1A} antagonist), 10 μM SB269970 (5-HT_7 antagonist), or 10 μM ketanserin (5-HT_2 antagonist) thirty seconds 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

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while 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-HT_{1A} and 5-HT_7 stimulation could mediate CREB phosphorylation, we treated cultures with varying concentrations (1, 10 or 100 μM) of either the 5-HT_{1A} agonist 8-hydrox-N,N-dipropyl-aminotetralin (8-OH-DPAT), or the 5- $HT_{1A/7}$ agonist 5-carboxamidotryptamine maleate (5-CT) in the presence of tetrodotoxin. Currently, there are no selective 5-HT_7 agonists. We found that both 8-0H-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 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 protein kinase A (PKA) (Gonzalez et al., 1989), protein kinase C (PKC) (Xie and Rothstein, 1995) different forms of Ca^{2+}/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 5-HT stimulation and remained in the solution during stimulation. We found that KT5720, PD98059, and KN93 all blocked the induction of CREB phosphorylation by 5-HT **(**Fig. 7A**)**. These effects were specific since the inactive KN93 derivative, KN92, did not prevent 5-HT's ability to induce pCREB. We also found that the non-selective kinase inhibitor, staurosporine, significantly reduced the ability of 5-HT to stimulate CREB phosphorylation, although it did not fully prevent the effect, in agreement with previous work (Bito et al., 1996).

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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 I (PPI), protein phosphatase 2A (PP2A), protein phosphatase 4 (PP4), protein phosphatase 5 (PP5), and a Ca^{2+}/CaM-dependent protein phosphatase (PP2B also called calcineurin (CaN). 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 acic (OA, 20 nM) which will inhibit PP2A, PP4, and PP5, the effect of high doses of OA (2 μM) which inhibits PP1, and FK506 (1 μM) a specific inhibitor of CaN 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 FK506 significantly blocked the reduction of CREB phosphorylation by 5-HT **(**Fig. 7B).

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. 7A, 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 FK506, to our cultures in the presence of tetrodotoxin and then stimulated with 5-HT for three minutes. We chose a three-minute 5-HT stimulation since this time point normally produces only a minimal increase in pCREB **(**Fig. 5B**)**. We found that 5-HT in the presence of OA and FK506 produced a significant increase in the amount of CREB phosphorylation **(**Fig. 8A**)**.

Since 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 three minutes 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**)**.

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Discussion

The results presented in this study show that 5-HT regulation of CREB phosphorylation and downstream CRE-dependent gene expression is 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 towards CREB dephosphorylation whereas in the absence of activity the kinase pathway wins over time and leads to CREB phosphorylation. This premise is further supported the 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, MAPK or CaMK (during serotonergic stimulation in the presence of activity) each produces almost a complete blockade of CREB phosphorylation. These data suggests that inhibition of any of these kinases diminishes pCREB down to baseline levels due to the shift in the balance towards 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

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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). NMDA-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-HT_{1A} and 5-HT_7 receptor results in increased CREB phosphorylation. This finding was rather unexpected since 5-HT_{1A} receptors couple to G_i to inhibit the cAMP pathway while 5-HT_7 receptors couple to G_s 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 to their properties in heterologous expression systems. Interestingly, activation of the 5-HT_{1A} and 5-HT_7 receptors have both been suggested to stimulate CREB phosphorylation (Nishi and Azmitia, 1999; Johnson et al., 2003). Clearly the co-activation 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

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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_7 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_7 receptor splice variants (5-HT_{7a}, 5-HT_{7b}, 5-HT_{7d}, and 5-HT_{7g}), which share a high degree of homology but differ at their carboxy terminus. It is possible that 5-HT_7 receptor splice variants couple to different intracellular signal transduction cascades. Indeed, a recent report has demonstrated that the 5-HT_{7b} splice variant produces a significantly higher level of constitutive adenylyl cyclase following forskolin stimulation than the 5-HT_{7a} and 5-HT_{7d} splice variants (Krobert and Levy, 2002). Another possibility may be the involvement of 5-HT_7 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 CNS the A kinase anchoring protein 79 (AKAP79) 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 (Klauck 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 (Colledge et al., 2000; Tavalin et al., 2002). Interestingly, some isoforms of the 5-HT_7 receptor (5-HT_{7b} and 5-HT_{7g}) appear to have putative PDZ (Class II) domains, which may suggest an association with PDZ –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_7 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 NMDA 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.

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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 CNS, 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 subsequent gene expression, 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 serotonergic input. Therefore, the coincidence of neuronal activity and serotonergic 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

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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.

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Footnotes

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Figure Legends

Fig. 1. Dissociated hippocampal neurons in culture display increasing levels of spontaneous network activity starting at 5 days after plating. Representative traces depicting the increase in the frequency of spontaneous miniature synaptic events detected in the presence of tetrodotoxin (TTX) in developing hippocampal cultures (a). Recording traces illustrate the evoked synaptic activity detected in the same cultures (b). This activity is recorded in the presence of intracellularly applied QX-314 to block action potential firing in the cell. The time course of increase in the frequency of miniature synaptic activity parallels the development of network activity (c) (n=4-8 for each symbol).

Fig. 2. 5-HT stimulates expression of c-fos and somatostatin in an activity dependent manner. Primary hippocampal neurons were treated with 4 mM K^+ and tetrodotoxin, 5-HT and tetrodotoxin, 4 mM K^+ in the absence of tetrodotoxin, and 5-HT without tetrodotoxin. Cumulative probability histogram of c-fos immunoreactivity shows that 5-HT in the presence of tetrodotoxin stimulates c-fos expression while 5-HT in the absence of tetrodotoxin inhibits expression (a**)** ($p<0.001$, Kolmogorov-Smirnov test). Cumulative probability histogram of somatostatin immunoreactivity shows a similar shift in increased expression following 5-HT stimulation in the presence of tetrodotoxin but inhibition of expression following 5-HT stimulation in the absence of tetrodotoxin **(**b**)** ($p<0.001$, Kolmogorov-Smirnov test).

Fig. 3. 5-HT inhibits spontaneous activity in hippocampal cultures. (a, b) Recording illustrates the effect of 10 µM 5-HT application in current clamp mode used to detect (a) action potentials and (b) voltage-clamp mode (+QX-314) to detect postsynaptic currents. 5-HT inhibited spontaneous action potential firing as well as resulting synaptic responses. (c) 5-HT application caused an increase in membrane conductance indicating activation of a current resulting in ~12 mV hyperpolarization. (d**)** 5-HT application significantly reduced the frequency of network activity as well as spontaneous action potentials detected in current clamp mode (from 4.8±1.0 to 0.3±0.1 Hz after 5-HT n=9).

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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 while 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 while 5-HT in the absence of tetrodotoxin inhibits pCREB in primary hippocampal cultures **(**d**)**.

Fig. 5. Dose-response and time-response characteristics of CREB phosphorylation during 5-HT stimulation. Increasing concentrations of 5-HT (1, 10 or 100 μM) were added to cultures either in the presence or absence of tetrodotoxin for 2 hours **(**a**)**. In the presence of tetrodotoxin, 10 μM 5-HT produced a maximal increase of CREB phosphorylation. In the absence of tetrodotoxin, 10 μM 5-HT inhibited 5-HT back to baseline conditions. 10 μM 5-HT in the presence or absence of tetrodotoxin was added to cultures for varying amounts of times (3, 30, 60, 90, 120 or 180 minutes) **(**b**)**. 5-HT in the presence of tetrodotoxin produced a robust increase in CREB phosphorylation following 2 hours of stimulation. In the absence of tetrodotoxin, 5-HT inhibited pCREB to near background levels following 2 hours of stimulation.

Fig. 6. 5-HT receptor subtypes that stimulate and inhibit CREB phosphorylation. The 5HT-$_{1A}$ selective antagonist, WAY-100635, the 5-HT_7 selective antagonist, SB269970, or the 5-HT_2 selective antagonist, ketanserin, were added 30 seconds prior to 5-HT stimulation in the presence of tetrodotoxin **(**a**)**. Both WAY-100635 and SB269970, but not ketanserin, significantly 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-$HT_{1A/7}$ agonist, 5-CT (10 μM), and the 5-HT_{1A} selective agonist, 8-OH

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DPAT (10 μM) were added to the hippocampal cultures for 2 hrs 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_7 but not the 5-HT_{1A} or the 5-HT_2 receptor subtype.

Fig. 7. Multiple kinases and phosphatases are involved in mediating 5-HT's ability to phosphorylate and dephosphorylate CREB in hippocampal cultures. The PKA inhibitor, KT5720 (10 μM), the MEK inhibitor, PD98059 (50 μM), and the CaMK inhibitor, KN93 (30 μM), all significantly blocked 5-HT's ability to phosphorylate CREB **(**a**)** ($p<0.01$). The inactive analog of KN93, KN92 (30 μM), however, did not prevent 5-HT from stimulating CREB phosphorylation. The non-specific kinase inhibitor, staurosporine (100nM), also significantly blocked 5-HT's ability to stimulate pCREB ($p<0.05$). Okadaic acid (OA; 2 μM) and FK-506 (1 μM) both significantly prevented the ability of 5-HT to dephosphorylate CREB in the absence of activity **(**b**)** ($p<0.01$). However, while the combination of OA and 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.

Fig. 8. 5-HT's ability to phosphorylate and dephosphorylate CREB is tightly controlled. A three-minute application of 5-HT in the presence of tetrodotoxin has little effect on CREB phosphorylation **(**a**)**. However, a three-minute application of 5-HT in the presence of the phosphatase inhibitors OA (2 μM) and FK-506 (1 μM) significantly increased the level of CREB phosphorylation ($p<0.05$). A three-minute application of 5-HT in the absence of tetrodotoxin has little effect on dephosphorylating CREB (b). However, in the presence of the kinase inhibitors, KT5720 and PD98059, a three-minute stimulation of 5-HT produced a significant dephosphorylation of CREB ($p<0.01$).

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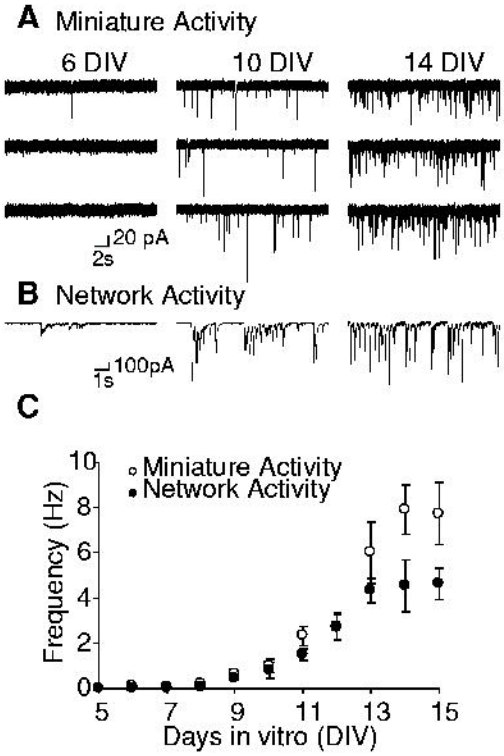

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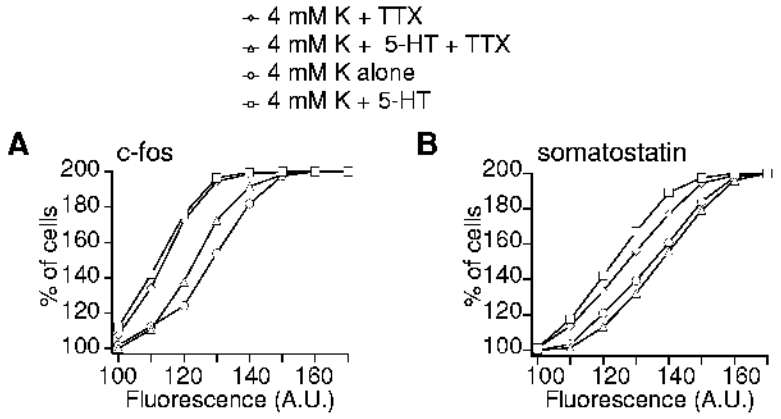

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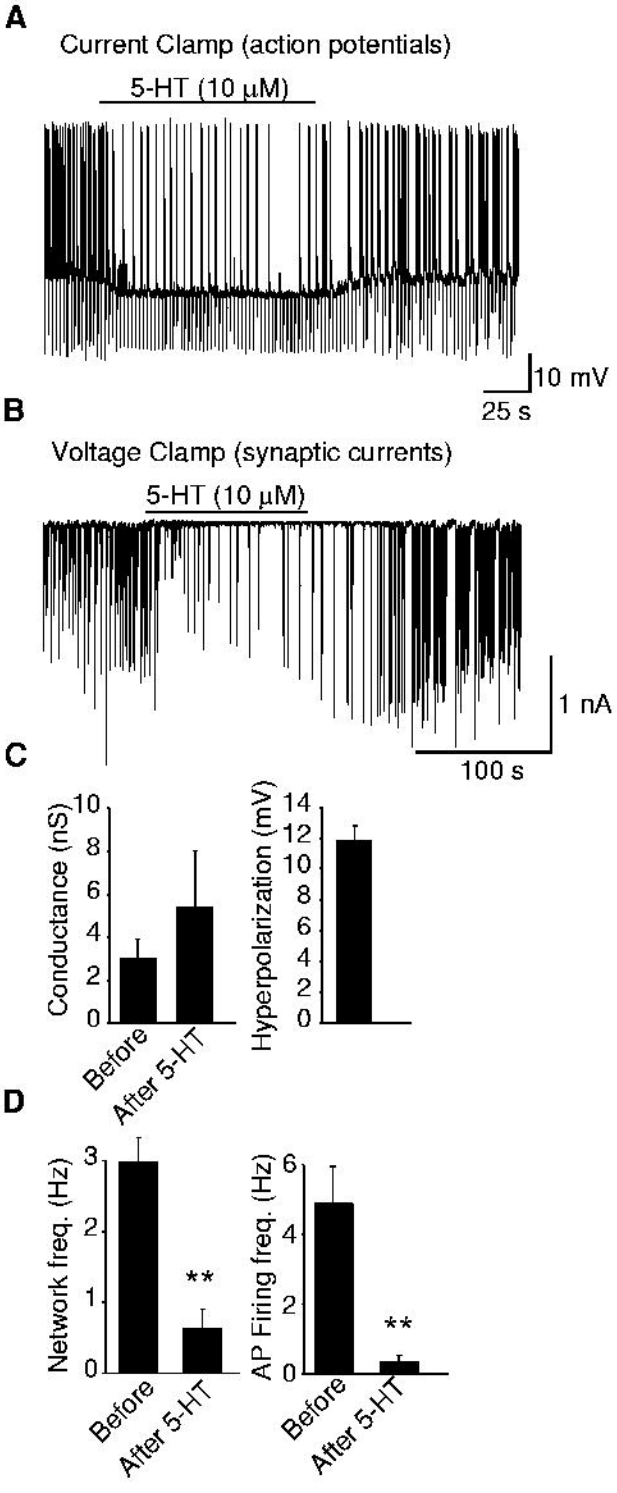

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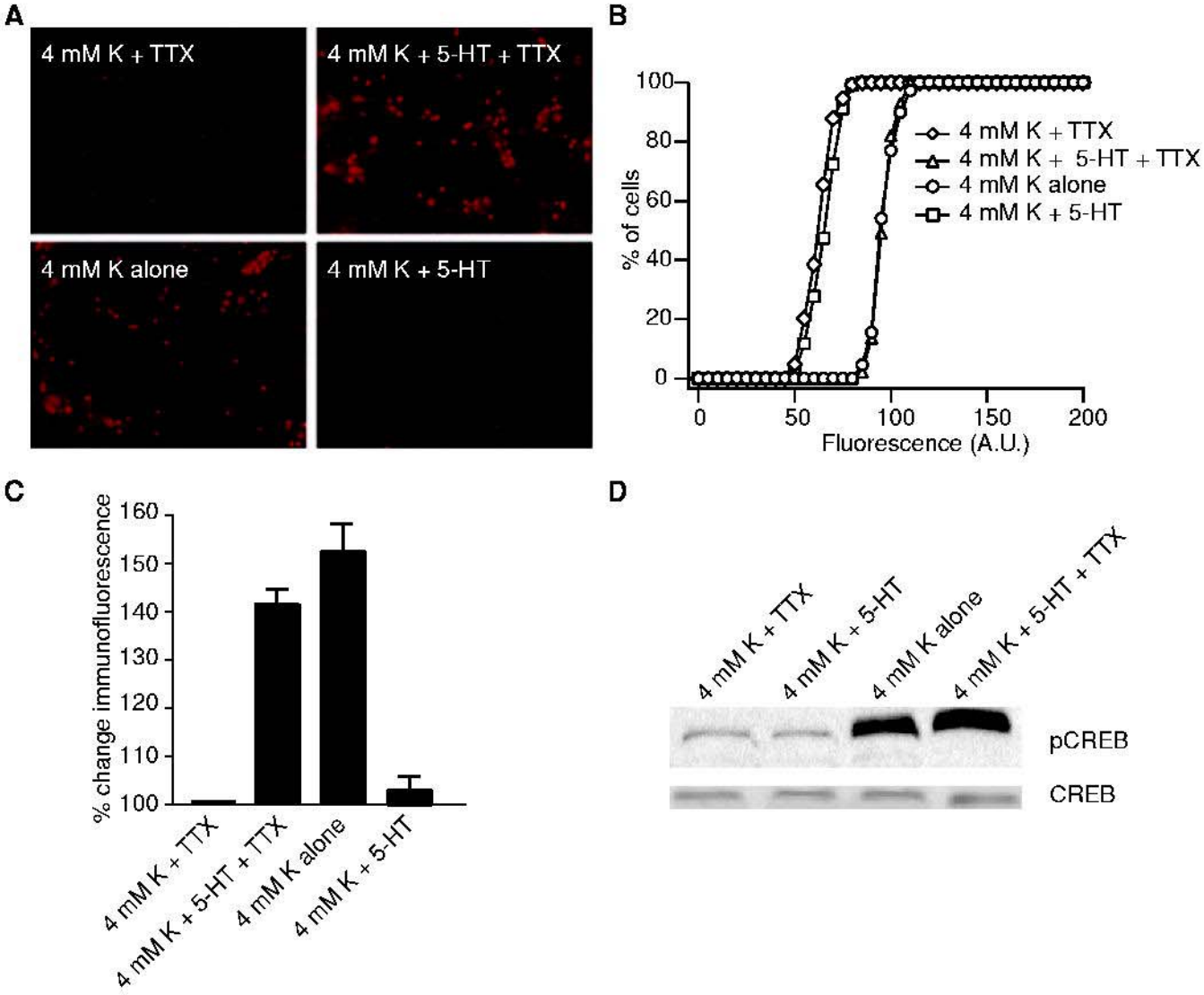

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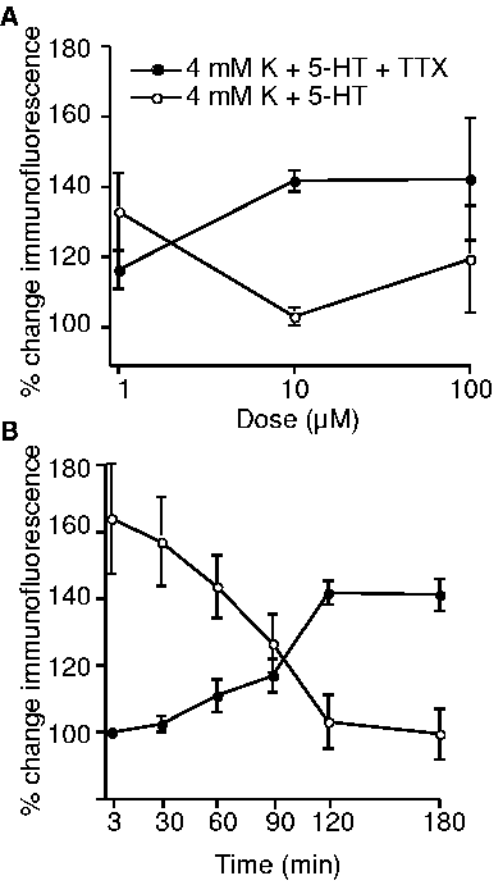

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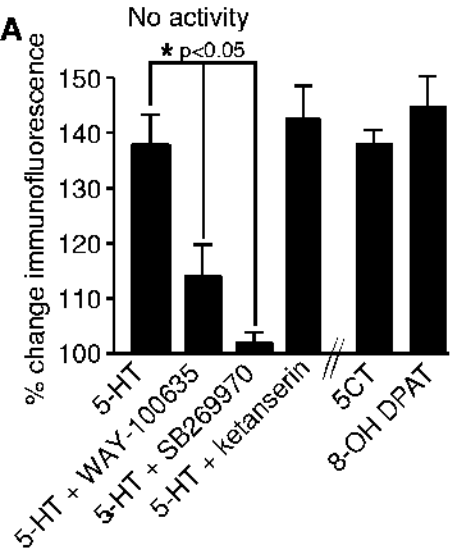

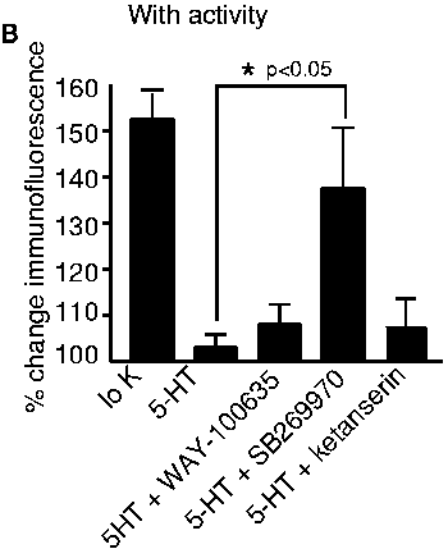

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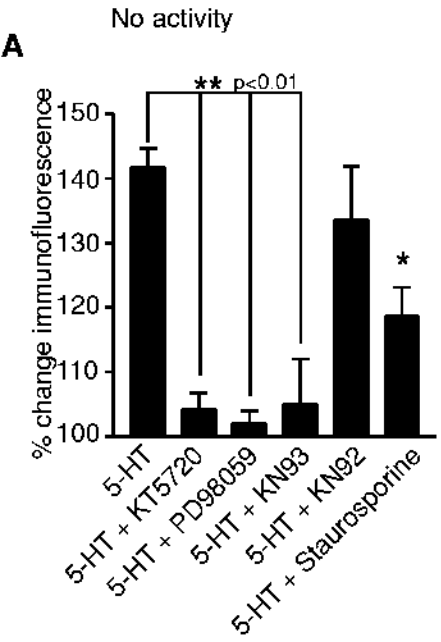

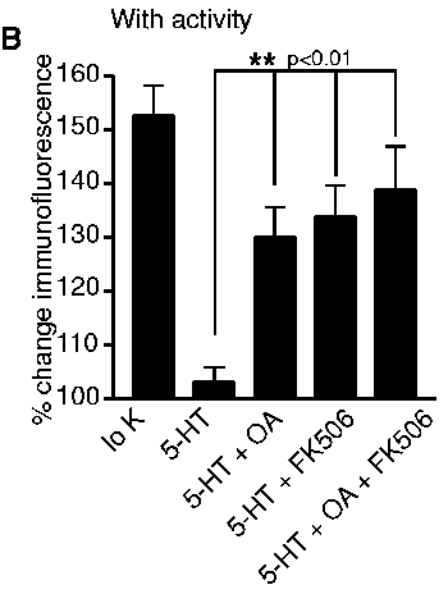

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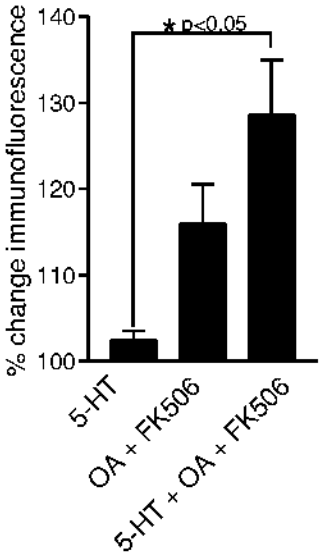
A No activity at 3 minute stimulation



B Activity at 3 minute stimulation