Cellular/Molecular

Coregulation of Glutamate Uptake and Long-Term Sensitization in *Aplysia*

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In *Aplysia*, long-term facilitation (LTF) at sensorimotor synapses of the pleural–pedal ganglia is mediated by an increase in the release of a neurotransmitter, which appears to be glutamate. Glutamate uptake also is increased in sensory neurons 24 hr after the induction of long-term sensitization (Levenson et al., 2000b). The present study investigated whether the same signaling pathways were involved in the long-term increase in glutamate uptake as in the induction of LTF. Thus, roles for cAMP, PKA (cAMP-dependent protein kinase), MAPK (mitogen-activated protein kinase), and tyrosine kinase in the regulation of glutamate uptake were tested. We found that 5-HT increased cAMP and activated PKA in sensory neurons. Exposure of pleural–pedal ganglia to analogs of cAMP or forskolin increased glutamate uptake 24 hr after treatments. Inhibitors of PKA (KT5720), MAPK (U0126 and PD98059), and tyrosine kinase (genistein) blocked the long-term increase in glutamate uptake produced by 5-HT. In addition, bpV, a tyrosine phosphatase inhibitor, facilitated the ability of subthreshold levels of 5-HT to increase glutamate uptake. Inhibition of PKC, which is not involved in LTF, had no effect on the long-term increase in glutamate uptake produced by 5-HT. Furthermore, activation of PKC by phorbol-12,13-dibutyrate did not produce long-term changes in glutamate uptake. The results demonstrate that the same constellation of second messengers and kinases is involved in the long-term regulation of both glutamate release and glutamate uptake. These similarities in signaling pathways suggest that regulation of glutamate release and uptake during formation of long-term memory are coordinated through coregulation of these two processes.

Key words: glutamate uptake; cAMP–PKA; MAPK; tyrosine kinase; *Aplysia*; sensitization

Introduction

In *Aplysia*, expression of long-term facilitation (LTF) at sensorimotor synapses of the pleural–pedal ganglia is mediated by an increase in the release of a neurotransmitter (Dale et al. 1988), which appears to be glutamate (Dale et al., 1988; Lin and Glanzman, 1994; Zhu et al., 1997; Armitage and Siegelbaum, 1998; Levenson et al., 2000a; Chin et al., 2002). Glutamate uptake also is increased in sensory neurons 24 hr after the induction of long-term sensitization (LTS) and LTF (Levenson et al., 2000b). In concert with increased glutamate uptake, neuronal glutamine uptake is also increased 24 hr after induction of LTS and LTF (Levenson et al., 2000b). In mammals, increased glutamate uptake is observed in area CA1 of the hippocampus after induction of long-term potentiation (LTP) (Levenson et al., 2002). In both *Aplysia* and mammals, the long-term increase in glutamate uptake during LTS and LTP appears to be attributable to an increase in the number of high-affinity glutamate transporters in neuronal membrane (Levenson et al., 2000b; Collado et al., 2002). Thus, glutamate uptake appears to be regulated along with changes in synaptic efficacy that are associated with the formation of memory in both vertebrates and invertebrates.

One mechanism by which regulation of glutamate and glutamine uptake could be coordinated with the formation of long-term memory would be through common signaling pathways. Previous data in *Aplysia* indicate that the transmitter 5-HT mimics the effects of sensitization training on the tail siphon withdrawal reflex (Alberini et al., 1994; Hegde et al., 1997; Levenson et al., 2000b) and the long-term increase in glutamate and glutamine uptake (Levenson et al., 2000b). Furthermore, the increase in glutamate uptake, like the induction of LTS, requires transcription and translation (Levenson et al., 2000b). These parallels between LTS and the long-term increase in glutamate uptake suggest that glutamate release and glutamate uptake may be coregulated with LTS; the same signaling pathways that produce LTS may also produce the increase in glutamate uptake. We have tested this coregulation hypothesis by examining the signaling pathways involved in the induction of LTS. The cAMP–PKA (cAMP-dependent protein kinase), MAPK (mitogen-activated protein kinase), and tyrosine kinase signaling pathways all play a role in the induction of long-term sensitization (Ocorr et al., 1986; Greenberg et al., 1987; Purcell et al., 2003; Sharma et al., 2003). We found that analogs of cAMP induced long-term in-

creases in glutamate uptake, and PKA, MAPK, and tyrosine kinase inhibitors blocked the 5-HT–induced increase in glutamate uptake. In addition, bpV, a tyrosine phosphatase inhibitor, facilitated the ability of subthreshold levels of 5-HT to increase glutamate uptake. We also tested the involvement of the PKC signaling pathway, which is not required for the induction of LTF (for review, see Byrne and Kandel, 1996). Activation of PKC by phorbol-12,13-dibutyrate (PDBu) had intermediate but not long-term effects on glutamate uptake. Furthermore, inhibition of PKC by chelerythrine had no effect on the long-term increase in glutamate uptake produced by 5-HT. The results of this study indicate that the same signaling pathways induce LTS and the long-term increase in glutamate uptake.

Materials and Methods

Animal maintenance. *Aplysia californica* (100–150 gm) were obtained from several suppliers (Charles Hollahan, Santa Barbara, CA; Marinus, Long Beach, CA; and Alacrity, Redondo Beach, CA) and maintained in Instant Ocean seawater (Aquarium Systems, Mentor, OH) at 15°C under 12 hr light/dark cycles. *Aplysia* were fed 2–3 gm of romaine lettuce per animal every 3 d and maintained in the laboratory for 3 d before use in experiments.

Reagents and solutions. 5-HT, PDBu, and L15 media were purchased from Sigma (St. Louis, MO). ^{125}I-cAMP tyrosyl methyl ester was obtained from Linco Research (St. Charles, MO) and Amersham Biosciences (Piscataway, NJ). [^{14}C]Glutamate was obtained from ICN Biochemicals (Costa Mesa, CA), and [^{3}H]glutamate was obtained from PerkinElmer Life Sciences (Emeryville, CA). 8-Benzylthio-cAMP (8-bt-cAMP) was purchased from Biology Life Sciences Institute (Bremer, Germany). [γ-^{32}P]ATP was purchased from PerkinElmer Life Sciences. All other drugs were from Calbiochem (La Jolla, CA). 5-HT and the cAMP analogs were dissolved directly in isotonic L15 media. KT5720, PDBu, U0126, and PD98059 were dissolved in dimethylsulfoxide (DMSO) before being added to the culture media. Genistein was dissolved in DMSO and ethanol. Chelerythrine was dissolved in water. In the experiments in which vehicle was used, control groups were treated with similar concentrations of the vehicle as experimental groups. The final concentrations of DMSO or ethanol were always <0.025%. If no vehicle was used, control groups were subjected to media changes.

Treatments. Spaced and massed treatments over 1.5 hr are commonly used to induce LTF. For example, five 5 min pulses of 5-HT separated by 10–20 min intervals is a commonly used spaced treatment (Montarolo et al., 1986; Martin et al., 1997; Michael et al., 1998; Sharma et al., 2003), and a continuous treatment of 5-HT for 1.5 hr is a commonly used massed treatment to elicit LTF and LTS (Emptage and Carew, 1993; Zhang et al., 1997; Levenson et al., 2000b). Although spaced treatments are often more potent than massed treatments, it is often more economical and efficient to use massed treatments. For example, spaced treatments using drugs such as forskolin or 8-bt-cAMP can be very expensive because the multiple pulse treatments increase the quantities of drugs used. The use of labeled compounds can be very difficult in the spaced treatment protocol. Hence, to be consistent, we have chosen to perform all of our experiments using the massed protocol. A massed treatment of *Aplysia* for 1.5 hr with 5-HT (500 μM) is capable of producing robust LTS (Levenson et al., 2000b). Moreover, massed treatments with 1.5 hr 5-HT induce LTF (Emptage and Carew, 1993; Zhang et al., 1997). Finally, spaced (five 10 min 5-HT pulses spaced 15 min apart) and massed treatments of 50 μM 5-HT for 1.5 hr produced similar long-term increases in glutamate uptake in isolated ganglia (spaced, 62 ± 12%; *n* = 4; massed, 64 ± 15%; *n* = 4).

LTS training and testing. LTS testing and training were done as described previously (Scholz and Byrne, 1987; Cleary et al., 1998; Levenson et al., 2000b; Fernandez et al., 2003). Briefly, the tail siphon withdrawal response was elicited by electrical stimulation through a pair of electrodes implanted in either side of the tail. Before baseline testing, the threshold current required to elicit siphon withdrawal was determined for each side of the animals. Pretraining baseline siphon withdrawal du-

rations were measured using a 20 msec shock at two times threshold current. Five baseline measurements (interstimulus interval, 10 min) were made on each side. The duration of siphon withdrawal was measured as the time between initiation of withdrawal and initiation of relaxation of the siphon. Sensitization training by electrical stimulation consisted of four 10 sec blocks of 10 shocks (500 msec, 60 mA shocks delivered at 1 Hz) with 30 min intervals between blocks. Electrical stimulation was applied to the surface of the skin on one side of the animal. Post-training measurements of siphon withdrawal duration were made 24, 48, and 72 hr after the end of training for each animal. Averages of five post-training measurements of siphon withdrawal were obtained for each animal (*n* = 1). The person responsible for measuring siphon withdrawal durations was blind regarding which side of the animal was trained.

Glutamate and glutamine uptake in synaptosomes. To measure the increase in glutamate uptake induced by sensitization training, animals received sensitization training on one side of the body as described above. The untrained side of the animal was used as a control. Animals were anesthetized with an injection of isotonic $MgCl_2$, and pleural–pedal ganglia were removed at 24, 48, or 72 hr after the end of training. Pleural–pedal ganglia were trimmed of excess connective tissue in 50% buffered filtered seawater [BFSW; Instant Ocean seawater with 30 mM HEPES, pH 7.65, 100 U/ml penicillin, and 100 μg/ml streptomycin, sterilized using a 0.22 μM Millipore (Bedford, MA) filter] and 50% isotonic $MgCl_2$. Uptake of glutamate and glutamine was measured from synaptosomes derived from pleural–pedal ganglia as previously described (Levenson et al., 2000b). In brief, synaptosomes were prepared from pleural–pedal ganglia using sucrose gradient centrifugation (Chin et al., 1989). Synaptosomes were isolated from the synaptic fraction by centrifugation at 16,000 × g for 10 min at 4°C. Glutamate and glutamine uptake were measured by incubation of 10–30 μg of synaptosomal protein for 20 min in glutamate (10 μM [^{14}C >250 mCi/mmol] and 990 μM cold) and glutamine (1 μM [^{3}H >200 Ci/mmol] and 999 μM cold) in Ca^{2+}-free seawater (460 mM NaCl, 10 mM KCl, 55 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, and 0.1% glucose). Experiments were terminated by dilution of the synaptosomes with 10 volumes of ice-cold Ca^{2+}-free seawater. Then, synaptosomes were centrifuged (16,000 × g) for 5 min, and the pellet was rinsed three times with Ca^{2+}-free seawater and dissolved in scintillation fluid to determine total glutamate and glutamine uptake. Uptake was normalized to total synaptosomal protein.

To measure the change in glutamate uptake produced by treatments of ganglia *in vitro*, pleural–pedal ganglia (8–10 per group) were isolated and trimmed in a mixture of BFSW and isotonic $MgCl_2$ (1:1). Then, ganglia were incubated in culture media containing two parts isotonic L15 media, one part hemolymph, and one part BFSW for 2 hr before drug treatments. Experimental groups were treated for 1.5 hr with one of the following: 50 μM 5-HT, 2 mM 8-bt-cAMP and IBMX (0.5 mM), or 100 μM 7-deacetyl-6-[N-acetylgylcyl]-forskolin. In the MAPK experiments, experimental groups were treated for 2.5 hr with 20 μM U0126 or 30 μM PD98059 (1 hr before the addition of 5-HT and 1.5 hr during the 5-HT treatment). Treatments were given by mixing the drugs in culture media. After treatment, ganglia were rinsed six times with isotonic L15 and maintained in culture media at 15°C. Glutamate and glutamine uptake were measured 24 hr after treatments.

Uptake in cultured sensory neurons. Two experimental preparations consisting of either isolated pleural–pedal ganglia or cultured sensory neurons were used in these studies. The initial experiments of this study used isolated ganglia. Because isolated ganglia from 10–20 animals were required for each experiment but ganglia from only a few animals were required for each experiment using cultured neurons, we switched to using cultures of sensory neurons in most experiments. A number of results we have obtained indicate that the long-term regulation of glutamate uptake is the same in both types of preparations. For example, the time course of changes in glutamate uptake is the same in the two systems (Khabour et al., 2003). Inhibition of protein synthesis during induction blocks the long-term increase in glutamate uptake in both ganglia and cultures of neurons (data not shown). Finally, PDBu had the same effects on ganglia as it did on cultured neurons, as presented in Results.

Cultures of *Aplysia* sensory neurons were prepared at a density of 20–80 sensory neurons per dish as previously described (Schacher and

Proshansky, 1983; Chin et al., 1999; Levenson et al., 2000b). Neurons were grown for 5–6 d in cell culture medium consisting of equal parts isotonic L15 and hemolymph. Before treatment, the culture media was replaced with a 1:1 mixture of isotonic L15 and BFSW. After treatment, cultures were rinsed six times and incubated in culture media for 24 hr at 18°C. Glutamate uptake was measured as previously described (Levenson et al., 2000b). Cultures were rinsed six times with artificial seawater (ASW; in mM: 395 NaCl, 28 Na$_2$SO$_4$, 10 KCl, 50 MgCl$_2$, 10 CaCl$_2$, and 30 HEPES, pH 7.65). Glutamate uptake was measured by incubation of cultures in glutamate (10 μM [^{14}C >250 mCi/mmol]) in ASW for 30 min. Uptake was terminated by rinsing cultures with ice-cold ASW (six times). Cells were removed from plates using scintillation fluid, and uptake was normalized to the number of cells on each plate.

Protein kinase assay. PKA activity was measured using a Promega (Madison, WI) SignaTECT cAMP-dependent protein kinase assay system. Paired pleural–pedal ganglia were removed from the animal, and one ganglion served as a matched control for the other ganglion of the same animal. The ventral-caudal sensory neuron cluster of the pleural ganglion was surgically exposed in a solution of 50:50 MgCl$_2$ and BFSW. Ganglia were transferred to BFSW to equilibrate for 2 hr at room temperature before use. Experimental ganglia were treated with 5-HT (50 μM) for 10 min at room temperature. After treatments, sensory neuron clusters were removed and then immediately frozen in liquid nitrogen. A 5 μl aliquot of the sensory neuron cluster homogenate was added to the PKA assay buffer, which also contained [γ-^{32}P]ATP (~10,000 cpm/25 μl) and the PKA biotinylated peptide substrate, and then incubated for 20 min at room temperature. In experiments measuring total PKA activity, 25 μM cAMP was added to the mixture. The reactions were terminated and spotted on SAM^2 membrane squares (Promega), and the membranes were immersed in scintillation fluid. To obtain the dose–response curve for inhibition of PKA by KT5720, homogenized clusters of sensory neurons were treated with different concentrations of KT5720 (1, 10, 100, and 300 μM), and PKA activity was measured as discussed above.

Radioimmunoassay. To determine concentrations of cAMP in clusters of sensory neurons, radioimmunoassay was used as described (Hasegawa and Cahill, 1998). Pleural–pedal ganglia were dissected, and sensory neuron clusters were treated as described above. Treatments of desheathed ganglia with 5-HT (50 μM, 6 min) and 7-deacetyl-6-[*N*-acetylgylcyl]-forskolin (100 μM, 6 min) were performed in the presence of IBMX (0.5 mM, 6 min) at 15°C. After treatment, sensory clusters were removed and frozen in liquid N$_2$. Five clusters of sensory neurons were used per group. cAMP values were normalized to total protein.

Data analysis. Glutamate and glutamine uptake measurements are expressed as percentage of changes of the control, and LTS measurements are expressed as percentage of changes of pretraining measurements. Results are presented as means ± SEM. Data of Figures 1 and 2 were analyzed by *t* tests, and *p* values were adjusted for multiple comparisons using the modified Bonferroni procedure (Jaccard and Wan, 1996). Data of levels of cAMP and Figures 3*C* and 4–6 were analyzed using ANOVA followed by Tukey tests to measure specific differences among experimental groups. In experiments involving one control and one experimental group (Figs. 2, 3*A*) or when comparing one group to another, Student's *t* tests were used to measure significant differences; *p* < 0.05 was considered significant.

Results

Time course of LTS and glutamate uptake

Behavioral training and 5-HT treatment induce LTS in *Aplysia* (Alberini et al., 1994; Hegde et al., 1997; Levenson et al., 2000b; Fernandez et al., 2003) and also produce long-term increases in glutamate uptake (Levenson et al., 2000b). To examine further the parallel between LTS and the increase in glutamate uptake, the time course of the increase in glutamate uptake was compared with the time course of LTS (Fig. 1). Measurements of siphon withdrawal and glutamate uptake were made 24, 48, and 72 hr after animals received LTS training. Five siphon withdrawal measurements were taken from an animal at each time point. As previously reported, LTS persisted for at least 48 hr but was not
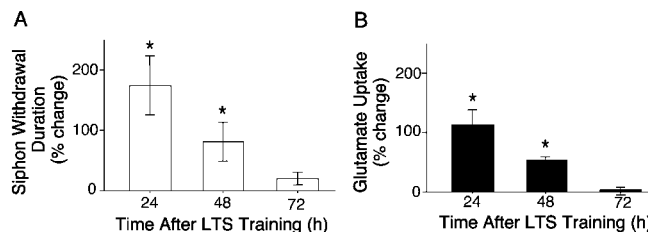

Figure 1. The time course of changes in glutamate uptake parallels the time course of LTS. Glutamate uptake and siphon withdrawal were significantly increased for at least 48 hr but were not significantly changed 72 hr after training. *A*, The duration of siphon withdrawal was measured 24, 48, and 72 hr after LTS training. The percentage of change in siphon withdrawal is the ratio of the duration of siphon withdrawal at each time tested to the pretraining siphon withdrawal duration. *B*, Glutamate uptake was measured in synaptosomes prepared from pleural–pedal ganglia 24, 48, or 72 hr after LTS training. Synaptosomes obtained from pleural–pedal ganglia on the untrained side of the animal were used as a control. *Significant percentage of change (*p* < 0.05) from control.

apparent 72 hr after training (24 hr, 164 ± 47%; *t* = 3.48; *n* = 11; *p* < 0.01; 48 hr, 81 ± 34%; *t* = 2.41; *n* = 11; *p* < 0.05; 72 hr, 19 ± 12%; *t* = 1.55; *n* = 8; *p* = 0.16) (Fig. 1*A*). Similarly, glutamate uptake measured in synaptosomes prepared from pleural–pedal ganglia was increased 48 hr but not 72 hr after LTS training (24 hr, 110 ± 23%; *t* = 4.83; *n* = 9; *p* < 0.01; 48 hr, 59 ± 5.4%; *t* = 20; *n* = 4; *p* < 0.01; 72 hr, 4 ± 4.3%; *t* = 0.73; *n* = 4; *p* = 0.51) (Fig. 1*B*). Therefore, the time courses of LTS appears to parallel the long-term increase in glutamate uptake.

Role of cAMP in the regulation of glutamate and glutamine uptake

The induction of LTS involves an increase in intracellular levels of cAMP and activation of PKA (for review, see Kandel, 2001). Extensive evidence demonstrates that 5-HT mediates the effects of LTS training (Montarolo et al., 1986; Glanzman et al., 1989; Clark and Kandel, 1993; Zhang et al., 1997; Mauelshagen et al., 1998; Levenson et al., 1999; Marinesco and Carew, 2002; Barbas et al., 2003). Moreover, treatments with 5-HT mimic the effects of LTS training on the long-term increase in glutamate uptake (Levenson et al., 2000b). Thus, we hypothesized that the cAMP–PKA signaling pathway mediates the effect of 5-HT on glutamate uptake. To test this hypothesis, we first confirmed previous findings that 5-HT induced an increase in cAMP levels in sensory neurons (Bernier et al., 1982; Ocorr and Byrne, 1985), an important site responsible for the plasticity that leads to LTS. Treatment of pleural–pedal ganglia with 5-HT (50 μM, 6 min) plus IBMX (0.5 mM) produced a twofold increase in the levels of cAMP in clusters of sensory neurons measured at the end of the treatment (5.24 ± 0.95 nmol of cAMP/μg of protein in the 5-HT-treated group vs 2.80 ± 0.47 of nmol cAMP/μg of protein in the IBMX-treated group; *n* = 16). IBMX alone did not affect basal levels of cAMP (2.80 ± 0.47 nmol of cAMP/μg of protein in the IBMX-treated group vs 2.61 ± 0.30 nmol of cAMP/μg of protein in the control group: *n* = 4). ANOVA revealed significant differences among groups ($F_{(2,26)}$ = 4.48; *p* < 0.025). A Tukey multiple-comparisons test revealed significant differences between the 5-HT group and both the control group (*p* < 0.025) and the IBMX-treated group (*p* < 0.05). No significant difference was detected between the IBMX-treated group and the control group (*p* > 0.5).

Treatment of isolated pleural–pedal ganglia with 5-HT significantly increased glutamate and glutamine uptake measured in synaptosomes 24 hr later (Levenson et al., 2000b). To investigate
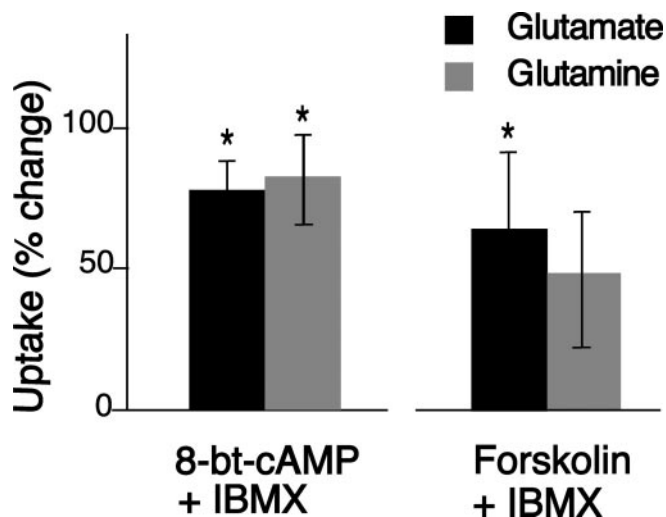
Figure 2. Elevation of cAMP increased glutamate and glutamine uptake. Treatments of isolated ganglia with 8-bt-cAMP (2 mm) plus IBMX (0.5 mm) or D-forskolin (100 μM) plus IBMX (0.5 mm) for 1.5 hr significantly increased glutamate and glutamine uptake in synaptosomes prepared from ganglia 24 hr after treatments. *Significant percentage of change ($p < 0.05$) from the IBMX (0.5 mm) control in the 8-bt-cAMP experiment and from the DMSO (0.025%) control in the D-forskolin experiment.

whether increases in cAMP are sufficient to mimic the effect of 5-HT on the long-term increases in glutamate and glutamine uptake, pleural–pedal ganglia were treated with the cAMP analog 8-bt-cAMP (2 mm, 1.5 hr) in the presence of IBMX (0.5 mm). Similar treatments with 8-bt-cAMP plus IBMX have been used previously in *Aplysia* (Schacher et al., 1988; Zwartjes and Eskin, 1990). 8-bt-cAMP induced a significant increase in glutamate ($75 \pm 10\%$; $t = 3.37$; $n = 4$; $p < 0.03$) and glutamine ($81 \pm 13\%$; $t = 4.22$; $n = 4$; $p < 0.04$) uptake 24 hr after treatment (Fig. 2). An additional way to investigate the role of an increase in cAMP is to use the adenylyl cyclase activator 7-deacetyl-6-[*N*-acetylgylcyl]-forskolin (D-forskolin) to increase intracellular cAMP. Similar treatments of D-forskolin have been used to increase the intracellular level of cAMP in *Aplysia* (Walsh and Byrne, 1984; Baxter and Byrne, 1990; Bacskai et al., 1993; Fox and Lloyd, 2000). Similar to 5-HT, treatment of pleural–pedal ganglia with D-forskolin (100 μM, 6 min) plus IBMX (0.5 mm) increased the levels of cAMP in clusters of sensory neurons (4.68 nmol of cAMP/μg of protein in the D-forskolin-treated group vs 2.57 nmol of cAMP/μg of protein in the IBMX-treated group; $n = 2$). Treatment of pleural–pedal ganglia with D-forskolin (100 μM, 1.5 hr) significantly increased glutamate uptake ($63 \pm 26\%$; $t = 2.8$; $n = 4$; $p < 0.01$) (Fig. 2). D-Forskolin also produced long-term substantial increases in glutamine uptake in pleural–pedal ganglia, but the increase was not statistically significant ($48 \pm 23\%$; $t = 2.31$; $n = 4$; $p = 0.084$) (Fig. 2). Therefore, elevating levels of cAMP in two different ways mimicked the effect of sensitization training and 5-HT on the long-term increase in glutamate uptake.

Role of PKA in the regulation of glutamate uptake
One of the consequences of increases in intracellular levels of cAMP is activation of PKA. We therefore investigated whether activation of PKA occurred during the induction of the long-term increase in glutamate uptake. As previously reported, 5-HT induced an increase in PKA activity in the sensory neurons (Muller and Carew, 1998). PKA activity was measured in homogenates of clusters of sensory neurons at the end of treatments (see Materials and Methods). Total PKA was assayed by adding a saturat-
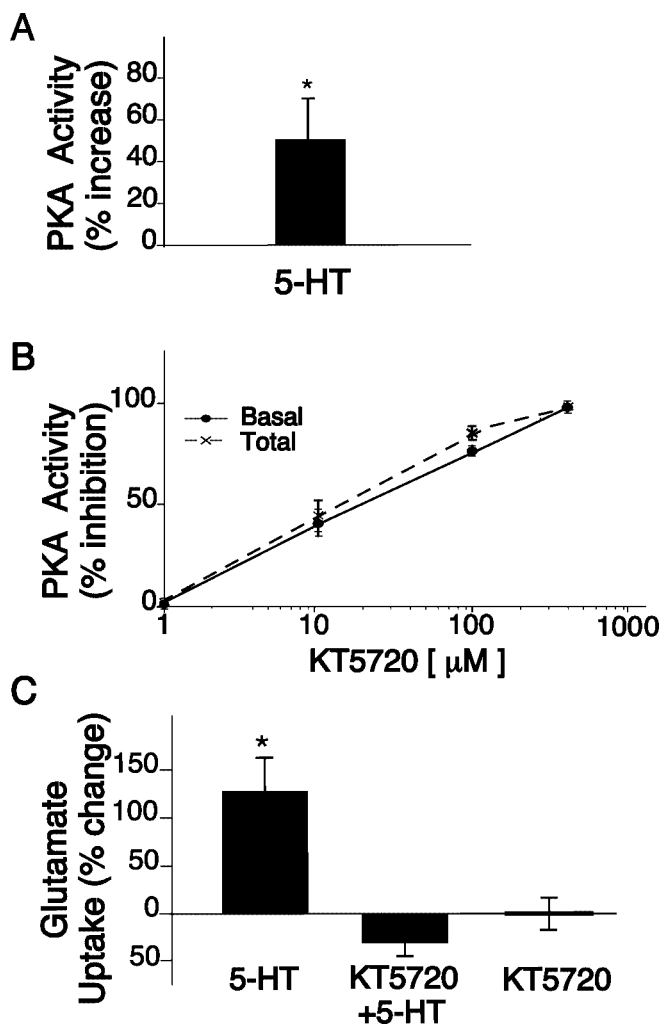

Figure 3. Inhibition of PKA blocked the 5-HT-induced increase in glutamate uptake. *A*, 5-HT (50 μM, 10 min) increased PKA activity at the end of 5-HT treatment in sensory neuron clusters. *B*, Homogenized clusters of sensory neurons were exposed to different concentrations of KT5720. KT5720 inhibited both basal and total PKA activity in sensory neuron clusters, with an IC$_{50}$ value of $\sim$20 μM. *C*, 5-HT (50 μM, 1.5 hr) increased glutamate uptake in cultures of sensory neurons 24 hr after treatment. KT5720 (10 μM, given 1 hr before and during 1.5 hr 5-HT) blocked the long-term increase in glutamate uptake induced by 5-HT. KT5720 (10 μM, 2.5 hr) alone did not affect basal levels of glutamate uptake. *Significant difference ($p < 0.05$) from both the KT5720 group and the KT5720 + 5-HT group.

ing amount of cAMP (25 μM) to the PKA assay reaction mixture. 5-HT (50 μM, 10 min) significantly increased PKA activity ($52 \pm 22\%$; $t = 2.43$; $n = 7$; $p < 0.03$) in sensory neuron clusters (Fig. 3*A*). 5-HT did not change the total amount of PKA activity ($-2 \pm 5\%$; $t = 0.182$; $n = 7$; $p > 0.56$), which reflects the lack of change in the amount of PKA.

If an increase in PKA activity is necessary for 5-HT to induce the long-term increase in glutamate uptake, then inhibition of PKA should block the effect of 5-HT on glutamate uptake. The PKA inhibitor KT5720 was used to investigate the role of PKA activation in the increase in glutamate uptake. KT5720 has been shown to inhibit PKA activity in different systems (Wicher, 2001; Mize and Alper, 2002), and it has been used previously to block PKA activity in *Aplysia* (Sutton et al., 2001; Angers et al., 2002). The ability of KT5720 to inhibit PKA activity in *Aplysia* sensory neurons was determined. Both basal activity and total PKA activity (stimulated by 25 μM cAMP for 20 min) was inhibited by KT5720 in a dose-dependent manner, with an IC$_{50}$ value close to
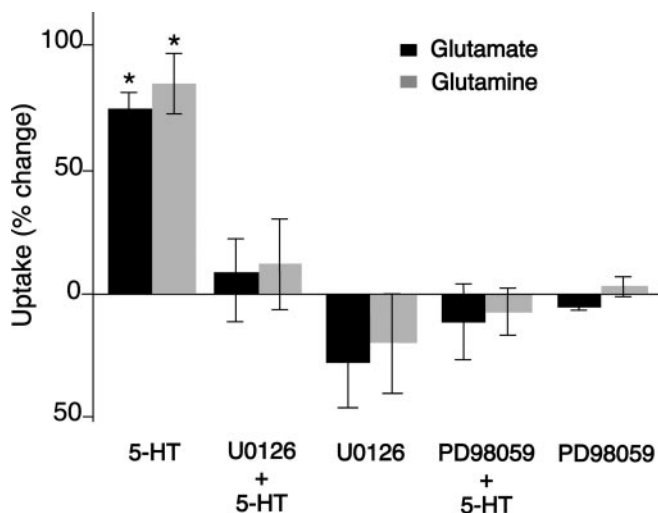
Figure 4. Inhibition of MAPK blocked the increase in glutamate and glutamine uptake produced by 5-HT. Treatment of pleural–pedal ganglia with the MAPK inhibitors U0126 (20 μM) and PD98059 (30 μM) for 2.0 hr (0.5 hr before and during 1.5 hr 50 μM 5-HT) significantly blocked the increase in both glutamate and glutamine uptake produced by 5-HT. Treatment with U0126 or PD98059 for 2.0 hr did not significantly affect basal uptake of glutamate and glutamine measured 24 hr after treatment. *Significant difference ($p < 0.05$) from both U0126 and PD98059 groups and the U0126 + 5-HT and PD98059 + 5-HT groups.

20 μM (Fig. 3B). Glutamate uptake in cultures of sensory neurons was increased 24 hr after treatments with 5-HT ($137 \pm 30\%$; $n = 7$) (Fig. 3C) as previously observed (Levenson et al., 2000b). This long-term increase in glutamate uptake produced by 5-HT was blocked by KT5720 ($-32 \pm 15\%$; $n = 7$) (Fig. 3C). KT5720 (10 μM, 2.5 hr) alone did not affect basal levels of glutamate uptake ($1 \pm 30\%$; $n = 7$) when measured 24 hr after treatment (Fig. 3C). ANOVA revealed significant differences among groups ($F_{(2,16)} = 5.7$; $p < 0.02$). A Tukey multiple-comparisons test revealed significant differences between the 5-HT group and both the 5-HT and KT5720 group ($p < 0.005$) and the KT5720-alone group ($p < 0.01$). No significant difference was detected between the 5-HT and KT5720 group and the KT5720-alone group ($p > 0.2$). Taken together, these results (5-HT increased the levels of cAMP and PKA activity; elevation of cAMP increased glutamate uptake; and inhibition of PKA activity blocked the increase in glutamate uptake produced by 5-HT) establish the involvement of the cAMP–PKA signaling pathway in mediating the long-term effects of 5-HT on glutamate uptake.

Regulation of glutamate and glutamine uptake by the MAPK pathway

Activation of the MAPK signaling pathway also plays an important role in the induction of LTF and LTS (for review, see Sharma and Carew, 2004). 5-HT treatments capable of inducing LTS result in phosphorylation and activation of MAPK and its translocation to the nucleus of sensory neurons (Martin et al., 1997; Michael et al., 1998; Sharma et al., 2003). Furthermore, inhibition of MAPK signaling with U0126 and PD98059 blocks the induction of LTF by 5-HT (Martin et al., 1997; Sharma et al., 2003). Therefore, we investigated the effects of inhibition of the MAPK signaling pathway on the ability of 5-HT to increase glutamate and glutamine uptake. Treatment of isolated pleural–pedal ganglia with 5-HT (50 μM) for 1.5 hr significantly increased glutamate uptake ($74 \pm 9\%$; $n = 3$) and glutamine uptake ($83 \pm 17\%$; $n = 3$) 24 hr later (Fig. 4). U0126 (20 μM (applied 1 hr before and during the 1.5 hr 5-HT treatment) blocked the long-
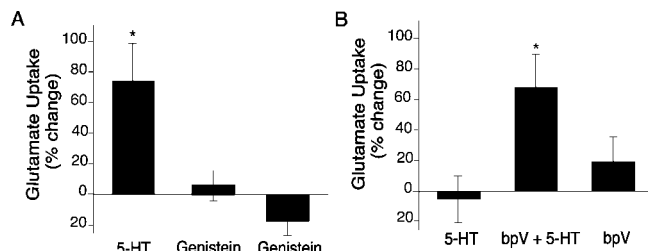

Figure 5. Requirement for tyrosine kinase activity in the long-term increase in glutamate uptake. *A*, Inhibition of tyrosine kinase blocked the increase in glutamate uptake produced by 5-HT. 5-HT (1.5 hr, 50 μM) increased glutamate uptake in cultures of sensory neurons 24 hr after treatment. Genistein (100 μM, given 0.5 hr before and during 1.5 hr 5-HT treatment) blocked the long-term increase in glutamate uptake induced by 5-HT. Genistein alone did not affect basal levels of glutamate uptake. *Significant difference ($p < 0.05$) from both the Genistein group and the Genistein + 5-HT. *B*, Enhancement of tyrosine kinase activity facilitated the induction of the long-term increase in glutamate uptake by 5-HT. Treatment of sensory neuron cultures with 15 min 5-HT or 45 min bpV (tyrosine phosphatase inhibitor) did not produce a significant change in glutamate uptake measured 24 hr later. Treatment of cultures with 15 min 5-HT added during the last 15 min of 45 min bpV treatment produced a significant increase in glutamate uptake. *Significant difference ($p < 0.05$) from both the bpV group and the bpV + 5-HT group.

term increase in both glutamate ($15 \pm 15\%$; $n = 4$) and glutamine ($28 \pm 24\%$; $n = 4$) uptake produced in pleural–pedal ganglia by treatment with 5-HT (Fig. 4). U0126 (2.5 hr treatments) had no significant effects on basal glutamate uptake ($-28 \pm 24\%$; $n = 4$) and glutamine uptake ($-25 \pm 23\%$; $n = 4$) when measured 24 hr later (Fig. 4). To confirm these results, a second MAPK signaling inhibitor, PD98059, was also used to investigate the role of MAPK in regulating the long-term increase in glutamate uptake. Similar to U0126, PD98059 (30 μM, applied 1 hr before and during the 1.5 hr 5-HT treatment) significantly blocked the long-term increase in both glutamate ($-11 \pm 21\%$; $n = 3$) and glutamine ($-21 \pm 26\%$; $n = 3$) uptake (Fig. 4). PD98059 by itself (2.5 hr treatment) had no effects on basal glutamate ($-6 \pm 5\%$; $n = 3$) and glutamine ($2 \pm 6\%$; $n = 3$) uptake when measured 24 hr later (Fig. 4). ANOVA indicated significant differences among groups (glutamate uptake, $F_{(4,13)} = 3.78$; $p < 0.03$; glutamine uptake, $F_{(4,13)} = 3.34$; $p < 0.05$). Tukey *post hoc* tests showed significant differences between the 5-HT-treated group and all other treated groups (both glutamate and glutamine, $p < 0.05$). These results, together with those of others (Martin et al., 1997; Michael et al., 1998; Sharma et al., 2003) showing that 5-HT activates MAPK in sensory neurons, indicate that the MAPK signaling pathway is also involved in the long-term regulation of glutamate and glutamine uptake.

Regulation of glutamate uptake by the tyrosine kinase pathway

Recent evidence indicates that tyrosine kinase, in addition to PKA and MAPK, is involved in the signaling cascade responsible for induction of LTF. Inhibition of tyrosine kinase activity with genistein (100 μM) blocks the induction of LTF and the increase in MAPK phosphorylation produced by 5-HT (Purcell et al., 2003). Furthermore, enhancement of tyrosine kinase activity using bpV (100 μM), a phosphatase inhibitor, facilitates the induction of LTF and the phosphorylation of MAPK produced by below-threshold concentrations of 5-HT (Purcell et al., 2003). Therefore, we investigated the effects of inhibition and enhancement of tyrosine kinase activity on 5-HT-induced increases in glutamate uptake. Treatment of cultures of sensory neurons with 1.5 hr 5-HT (50 μM) produced a significant increase in glutamate

uptake (74 ± 18%; $n = 8$) 24 hr later (Fig. 5A). Genistein (100 μM, applied 0.5 hr before and 1.5 hr during 5-HT treatment) blocked the long-term increase in glutamate uptake (6 ± 3%; $n = 8$) (Fig. 5A). Genistein alone had no significant effects on basal glutamate uptake (−18 ± 9%; $n = 8$) as measured 24 hr after treatment (Fig. 5A). ANOVA revealed a significant difference between groups ($F_{(2,21)} = 15.1$; $p < 0.001$). A Tukey multiple-comparisons test showed significant differences between the 5-HT-treated group and both the 5-HT and genistein groups ($p < 0.005$) and the genistein-alone group ($p < 0.005$). No significant difference was detected between the genistein-alone group and the 5-HT and genistein group ($p > 0.1$). Thus, inhibition of tyrosine kinase activity with genistein blocked the long-term increase in glutamate uptake produced by 5-HT.

To investigate whether enhancement of tyrosine kinase activity can facilitate the induction of the long-term increase in glutamate uptake, the tyrosine phosphatase inhibitor bpV was used. Cultures of sensory neurons were treated with bpV (100 μM) for 45 min, either alone or in conjunction with 15 min 5-HT (50 μM). A short treatment of 5-HT (15 min) did not produce a significant change in glutamate uptake measured 24 hr after treatment (−6 ± 16%; $n = 5$) (Fig. 5B). However, when 5-HT was applied during the last 15 min of the 45 min bpV treatment, a significant increase in glutamate uptake was produced (68 ± 22%; $n = 6$) (Fig. 5B). BpV alone did not produce a significant change in glutamate uptake (20 ± 16.5%; $n = 6$) (Fig. 5B). ANOVA revealed a significant difference between groups ($F_{(2,14)} = 3.94$; $p < 0.04$). A Tukey multiple-comparisons test showed significant differences between the 5-HT and bpV group and both the 5-HT group ($p < 0.01$) and the bpV group ($p < 0.05$). No significant difference was detected between the bpV-alone group and the 5-HT-alone group. Thus, enhancement of tyrosine kinase activity appears to facilitate the effect of 5-HT on glutamate uptake. Our results, together with those of Purcell et al. (2003), who showed the effects of genistein and bpV on LTF and MAPK phosphorylation, indicate that tyrosine kinases are involved in the long-term increase in glutamate uptake as they are in the induction of LTF.

PKC is not involved in the long-term increase in glutamate uptake
The previous findings indicate that several signaling pathways (cAMP–PKA, MAPK, and tyrosine kinase) involved in the induction of LTS and LTF are also involved in the induction of long-term increases in glutamate uptake. To further test our coregulation hypothesis, we investigated the specificity of kinase signaling by determining the role of PKC in the long-term regulation of glutamate uptake. Although PKC is involved in short-term facilitation and intermediate-term facilitation (for review, see Byrne and Kandel, 1996), no direct role for PKC signaling has been shown for LTF. Two isoforms of PKC are found in the *Aplysia* nervous system, the Ca^{2+}-activated Apl I and the Ca^{2+}-independent Apl II (Sacktor and Schwartz, 1990; Kruger et al., 1991; Sossin and Schwartz, 1992; Braha et al., 1993). Both isoforms are activated by 5-HT and phorbol ester and inhibited by chelerythrine (Che) (Kruger et al., 1991; Sossin and Schwartz, 1992; Sossin et al., 1994; Sossin, 1997; Pepio et al., 1998).

To investigate whether PKC activation is required for 5-HT-induced long-term increases in glutamate uptake, we blocked PKC activity with Che. Treatment of cultures of sensory neurons with 1.5 hr 5-HT (50 μM) produced a significant increase in glutamate uptake 24 hr after treatment (96 ± 19%; $n = 4$) (Fig. 6A). Che (10 μM, applied 0.5 hr before and 1.5 hr during 5-HT treatment) had no effect on the long-term increase in glutamate
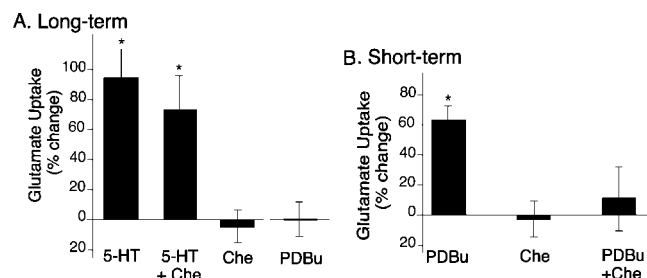

Figure 6. PKC is not involved in the long-term increase in glutamate uptake induced by 5-HT. *A*, 5-HT (1.5 hr, 50 μM) increased glutamate uptake in cultures of sensory neurons 24 hr after treatment. Che (10 μM), an inhibitor of PKC (given 0.5 hr before and during 1.5 hr 5-HT treatment), did not affect the long-term increase in glutamate uptake produced by 5-HT. Che alone did not affect basal levels of glutamate uptake measured 24 hr after treatment. Activation of PKC with PDBu (1.5 hr, 0.25 μM) had no effects on glutamate uptake in cultures of sensory neurons measured 24 hr after treatment. *Significant difference ($p < 0.05$) from the Che group. *B*, Activation of PKC has intermediate-term effects on glutamate uptake. Treatment with PDBu (0.25 μM) for 1.5 hr increased glutamate uptake in cultures of sensory neurons directly after treatment. Che (10 μM, given 0.5 hr before and during 1.5 hr treatment of PDBu) blocked the increase in glutamate uptake produced by PDBu. Che alone did not affect basal levels of glutamate uptake measured directly after treatment. *Significant difference ($p < 0.05$) from both the Che group and the PDBu + Che group.

uptake (74 ± 23%; $n = 4$) produced by 5-HT (Fig. 6A). Che alone had no effect (−6 ± 11%; $n = 3$) on basal glutamate uptake as measured 24 hr after treatment (Fig. 6A). ANOVA revealed a significant difference between groups ($F_{(2,8)} = 6.6$; $p < 0.02$). A Tukey multiple-comparisons test showed no significant difference between the 5-HT-alone group and the 5-HT and Che group ($p > 0.5$). A significant difference was detected between the 5-HT and Che group and the Che-alone group ($p < 0.01$) and between the 5-HT-alone group and the Che-alone group ($p < 0.01$). Thus, activation of PKC is not necessary for the 5-HT induced long-term increase in glutamate uptake.

To investigate whether activation of PKC is sufficient to produce the long-term increase in glutamate uptake, PDBu was used to activate PKC. PDBu has been used previously in *Aplysia* to activate PKC (Sacktor and Schwartz, 1990; Critz and Byrne, 1992; Wu et al., 1995; Sasaki et al., 1997; Manseau et al., 1998) and has been shown to induce long-term increases in sensory neuron excitability in *Aplysia* (Manseau et al., 1998). Treatment of cultures with 1.5 hr PDBu (0.25 μM) produced no change in glutamate uptake (1 ± 14%; $t = 0.08$; $n = 8$; $p > 0.9$) measured 24 hr after treatment (Fig. 6A). Similar results were obtained in pleural–pedal ganglia treated with 1.5 hr PDBu (18 ± 15%; $t = 1.26$; $n = 6$; $p > 0.26$). Thus, activation of PKC is not sufficient to produce long-term changes in glutamate uptake in either sensory neuron cultures or isolated ganglia.

Because activation of PKC is involved in short-term facilitation and intermediate-term facilitation, we investigated whether PKC activation could produce intermediate-term effects on glutamate uptake in *Aplysia*. Sensory neuron cultures were treated with PDBu (0.25 μM) for 1.5 hr, and glutamate uptake was measured directly after treatment. PDBu produced a significant increase in glutamate uptake (64 ± 10%; $n = 9$) (Fig. 6B). A similar result was obtained when isolated pleural–pedal ganglia were treated with 1.5 hr PDBu (57 ± 16%; $n = 5$). To test whether the intermediate-term effect of PDBu on glutamate uptake was mediated by PKC, Che (10 μM) was used to block PKC activity in cultures of sensory neurons. Che (applied 0.5 hr before and 1.5 hr during PDBu treatment) blocked the increase in glutamate uptake (13 ± 21%; $n = 6$) (Fig. 6B). Che alone had no effect on basal

glutamate uptake ($-4 \pm 13\%$; $n = 5$) measured directly after treatment (Fig. 6B). ANOVA revealed a significant difference between groups ($F_{(3,24)} = 6.05$; $p < 0.005$). A Tukey multiple-comparisons test showed a significant difference between the PDBu group and both the PDBu and Che group ($p < 0.01$) and the Che-alone group ($p < 0.01$). No significant difference was detected between the Che-alone group and the PDBu and Che group ($p > 0.2$). Thus, PKC activation is not involved in LTF or the long-term effects of 5-HT on glutamate uptake, but PKC may play a role in short- and intermediate-term effects on glutamate uptake as it does on short- and intermediate-term forms of facilitation.

Discussion

Because an increase in transmitter release at the sensorimotor synapse is responsible to a large extent for LTF (Dale et al., 1988), we hypothesized that increases in glutamate uptake would accompany increases in transmitter release. Consistent with this hypothesis, we found that two different *in vivo* treatments (electrical stimulation and 5-HT) that produced LTS also produced large increases in glutamate uptake 24 hr after treatment (Levenson et al., 2000b). This long-term increase of glutamate uptake has displayed several types of synapse specificity. Because the behavioral change associated with LTS is unilateral, only synaptosomes prepared from ganglia on the treated side show the long-term increase in glutamate uptake (Fig. 1) (Levenson et al., 2000b). In addition, the long-term increase in glutamate uptake is present in synaptosomal fractions but not cellular and glial fractions obtained from ganglia (Levenson et al., 2000b). In support of this cellular specificity, cultures of sensory neurons without glia also exhibit long-term increases in glutamate uptake after treatment with 5-HT (Figs. 3C, 5, 6A) (Levenson et al., 2000b).

In the current studies, we present additional data showing that long-term increases in glutamate uptake accompany increases in transmitter release, and coregulation is responsible for the long-term changes in transmitter release and glutamate uptake. The new results are as follows: (1) the time course of the long-term increase in glutamate uptake parallels the time course of LTS (Fig. 1); (2) the same constellation of second messengers and kinases (cAMP, PKA, MAPK, and tyrosine kinase) appears to be involved in the long-term regulation of glutamate uptake and LTS; and (3) one kinase (PKC) not involved in producing LTF also is not involved in producing the long-term increase in glutamate uptake (Fig. 6B). The similarities in the long-term regulation of LTS and LTF and glutamate uptake indicate that glutamate uptake is coordinated with glutamate release through coregulation of these two processes. Our previous result that inhibition of transcription and translation during the induction period blocks both LTS and the long-term increase in glutamate uptake (Levenson et al., 2000b) strengthens this conclusion. Coregulation of the long-term increase in glutamate uptake with LTS ensures that both processes occur together, suggesting that the increase in glutamate uptake plays an important role in the expression of LTS. Moreover, our results suggest that a large number of neuronal properties change in a coordinated manner to store a given type of memory.

At the sensorimotor synapse, postsynaptic as well as presynaptic changes may contribute to LTF (for review, see Roberts and Glanzman, 2003). For example, changes in the sensitivity of glutamate receptors have been observed during LTF. The induction mechanisms for the postsynaptic changes may be different from those mediating the presynaptic changes involved in LTF. The signaling pathways regulating the postsynaptic changes may also play a role in the presynaptic or possible postsynaptic regulation of glutamate uptake at the synapse. Thus far, our experiments

have focused only on the presynaptic regulation of glutamate uptake (Levenson et al., 2000b). It is unlikely that we have fully elaborated the signaling pathways involved in the long-term regulation of glutamate uptake at this synapse. However, what we have accomplished so far is to show that the signaling pathways involved in the presynaptic component of LTF and the long-term regulation of glutamate uptake are the same. Hence, the similarity in presynaptic signaling pathways is the basis for proposing coregulation (presynaptic) of glutamate release and glutamate uptake.

PKA, MAPK, and tyrosine kinase could regulate glutamate uptake by at least two different mechanisms. First, the kinases could regulate transcription of the glutamate transporter or some other protein that regulates the activity of the glutamate transporter. Inhibition of either transcription or translation blocks the induction of the increase in glutamate uptake as well as LTF and LTS (Montarolo et al., 1986; Levenson et al., 2000b). The requirement for transcription and translation for LTS and regulation of glutamate uptake could arise because the same mechanisms of induction are involved in LTF and the increase in glutamate uptake. Activation of PKA and MAPK and their nuclear translocation leads to activation of CREB1 (cAMP response element-binding protein 1), derepression of CREB2, and the subsequent induction of immediate early genes (for review, see Kandel, 2001). Phosphorylation of C/EBP (CCAAT enhancer binding protein) by MAPK stabilizes C/EBP and is essential for C/EBP binding to DNA (Yamamoto et al., 1999). Tyrosine kinase activity appears to be involved in the induction of LTS through activation of MAPK (Purcell et al., 2003). Therefore, as with LTF, changes in kinase activity might produce the long-term increase in glutamate uptake through the induction of immediate early genes, such as C/EBP, which would lead to increased transcription and translation of glutamate transporters. The long-term increase in glutamate transporters in synaptosomes prepared from pleural–pedal ganglia after LTS training indicates that synthesis of transporters may be involved in the increase in glutamate uptake (Collado et al., 2002). Another possibility is that synthesis of transporter-associated proteins is involved rather than synthesis of glutamate transporters (Jackson et al., 2001; Lin et al., 2001).

A second way in which kinases could regulate glutamate transporters is by covalent modifications of the transporters. Potentially, PKA, MAPK, and tyrosine kinase could directly phosphorylate glutamate transporters or associated proteins (Kalandadze et al., 2002; Boehmer et al., 2003), leading either to their activation in the plasma membrane or to maturation of glutamate transporters and their insertion in the plasma membrane. In either case, activity of one or more of the kinases would lead to an increase in glutamate uptake. Persistent phosphorylation of the transporter or an associated protein would then be responsible for the long-term increase in glutamate uptake. However, persistent phosphorylation is unlikely to account for the time ($\sim$48 hr) (Fig. 1) that glutamate uptake is increased. More likely, new transporters inserted into the plasma membrane have a long half-life and are responsible for the long-term increase in glutamate-uptake. Although regulation of glutamate uptake by direct phosphorylation could be involved, it is not consistent with the regulation by transcription and translation or with the observation that the number of transporters was increased by LTS training (Collado et al., 2002). Further research investigating the regulation of transcription and translation of glutamate transporters should help elucidate the specific roles of second messengers and kinases in the increase in glutamate uptake that accompanies synaptic facilitation.

Previously, Levenson et al. (2000b) showed that regulation of

glutamine uptake parallels the regulation of glutamate uptake, although uptake of glutamate and glutamine occurs through different transporters. The results in the present study further demonstrate that glutamate and glutamine uptake are tightly coupled. Treatments that induced an increase in glutamate uptake such as 8-bt-cAMP also induced an increase in glutamine uptake (Fig. 2). Likewise, inhibition of the 5-HT-induced increase in glutamate uptake by MAPK inhibitors also blocked 5-HT-induced increases in glutamine uptake (Fig. 4). The significance of the tight coupling of glutamate and glutamine transporters in *Aplysia* has yet to be determined. Potentially, increased glutamine uptake might function as a mechanism for increasing the amount of available glutamate for release, because in the mammalian brain, glutamate can be synthesized from glutamine (Hamberger et al., 1979). Alternatively, glutamine could be used for energy production and a source of nitrogen for the synthesis of nitrogen-containing compounds, as has been shown in birds (Rickard et al., 1998).

Coregulation of LTS and LTF and glutamate uptake ensures that both processes occur together, suggesting that the increase in glutamate uptake may play an important role in LTS. In general, glutamate uptake is the major mechanism for clearance of glutamate from the synaptic cleft (for review, see Danbolt, 2001). Specifically in *Aplysia*, glutamate uptake plays an important role in transmission at the sensorimotor synapse because inhibition of uptake alters synaptic transmission (Chin et al., 2002). Potentially, increased neurotransmitter release during LTS could lead to a buildup of glutamate in the synaptic cleft and the subsequent desensitization of postsynaptic glutamate receptors (Dudel et al., 1988; Raman and Trussell, 1995; Otis et al., 1996; Turecek and Trussell, 2000; Antzoulatos et al., 2003). Thus, the long-term increase in glutamate uptake that accompanies LTS might be necessary to maintain facilitation by preventing receptor desensitization. Increased glutamate uptake during LTS might also function to prevent extrasynaptic diffusion of glutamate and the subsequent activation of neighboring synapses, as observed in the mammalian brain (Asztely et al., 1997; Min et al., 1998; Diamond and Jahr, 2000). Finally, increased neuronal glutamate uptake might function to maintain steady levels of intracellular glutamate during periods of increased release of glutamate. Thus, transporters could serve to maintain the fidelity of excitatory glutamatergic neurotransmission within a single synapse or across multiple neighboring synapses.

The finding that the same constellation of kinase signaling pathways is involved in the long-term regulation of glutamate uptake and LTF and LTS confirms in a general way results of numerous studies outlining the kinases involved in the signaling pathways responsible for the induction of LTF. Future experiments studying the regulation of glutamate transporter synthesis, targeting, insertion, and turnover will aid in understanding the mechanisms that are involved in memory formation.

References

Alberini CM, Ghirardi M, Metz R, Kandel ER (1994) C/EBP is an immediate-early gene required for the consolidation of long- term facilitation in *Aplysia*. Cell 76:1099–1114.

Angers A, Fioravante D, Chin J, Cleary LJ, Bean AJ, Byrne JH (2002) Serotonin stimulates phosphorylation of *Aplysia* synapsin and alters its subcellular distribution in sensory neurons. J Neurosci 22:5412–5422.

Antzoulatos EG, Cleary LJ, Eskin A, Baxter DA, Byrne JH (2003) Desensitization of postsynaptic glutamate receptors contributes to high-frequency homosynaptic depression of *Aplysia* sensorimotor connections. Learn Mem 10:309–313.

Armitage BA, Siegelbaum SA (1998) Presynaptic induction and expression of homosynaptic depression at *Aplysia* sensorimotor neuron synapses. J Neurosci 18:8770–8779.

Asztely F, Erdemli G, Kullmann DM (1997) Extrasynaptic glutamate spillover in the hippocampus: dependence on temperature and the role of active glutamate uptake. Neuron 18:281–293.

Bacskai BJ, Hochner B, Mahaut-Smith M, Adams SR, Kaang BK, Kandel ER, Tsien RY (1993) Spatially resolved dynamics of cAMP and protein kinase A subunits in *Aplysia* sensory neurons. Science 260:222–226.

Barbas D, DesGroseillers L, Castellucci VF, Carew TJ, Marinesco S (2003) Multiple serotonergic mechanisms contributing to sensitization in *Aplysia*: evidence of diverse serotonin receptor subtypes. Learn Mem 10:373–386.

Baxter DA, Byrne JH (1990) Reduction of voltage-activated K+ currents by forskolin is not mediated via cAMP in pleural sensory neurons of *Aplysia*. J Neurophysiol 64:1474–1483.

Bernier L, Castellucci VF, Kandel ER, Schwartz JH (1982) Facilitatory transmitter causes a selective and prolonged increase in adenosine 3′,5′-monophosphate in sensory neurons mediating the gill and siphon withdrawal reflex in *Aplysia*. J Neurosci 2:1682–1691.

Boehmer C, Henke G, Schniepp R, Palmada M, Rothstein JD, Broer S, Lang F (2003) Regulation of the glutamate transporter EAAT1 by the ubiquitin ligase Nedd4–2 and the serum and glucocorticoid-inducible kinase isoforms SGK1/3 and protein kinase B. J Neurochem 86:1181–1188.

Braha O, Edmonds B, Sacktor T, Kandel ER, Klein M (1993) The contributions of protein kinase A and protein kinase C to the actions of 5-HT on the L-type Ca^{2+} current of the sensory neurons in *Aplysia*. J Neurosci 13:1839–1851.

Byrne JH, Kandel ER (1996) Presynaptic facilitation revisited: state and time dependence. J Neurosci 16:425–435.

Chin GJ, Shapiro E, Vogel SS, Schwartz JH (1989) *Aplysia* synaptosomes. I. Preparation and biochemical and morphological characterization of subcellular membrane fractions. J Neurosci 9:38–48.

Chin J, Angers A, Cleary LJ, Eskin A, Byrne JH (1999) TGF-b1 in *Aplysia*: role in long-term changes in the excitability of sensory neurons and distribution of TbR-II-like immunoreactivity. Learn Mem 6:317–330.

Chin J, Burdohan JA, Eskin A, Byrne JH (2002) Inhibitor of glutamate transport alters synaptic transmission at sensorimotor synapses in *Aplysia*. J Neurophysiol 87:3165–3168.

Clark GA, Kandel ER (1993) Induction of long-term facilitation in *Aplysia* sensory neurons by local application of serotonin to remote synapses. Proc Natl Acad Sci USA 90:11411–11415.

Cleary LJ, Lee WL, Byrne JH (1998) Cellular correlates of long-term sensitization in *Aplysia*. J Neurosci 18:5988–5998.

Collado MS, Levenson J, Lyons LC, Nunez-Regueiro M, Eskin A (2002) Characterization and regulation of a glutamate transporter in *Aplysia*. Soc Neurosci Abstr 28:376.312.

Critz SD, Byrne JH (1992) Modulation of IK,Ca by phorbol ester-mediated activation of PKC in pleural sensory neurons of *Aplysia*. J Neurophysiol 68:1079–1086.

Dale N, Schacher S, Kandel ER (1988) Long-term facilitation in *Aplysia* involves increase in transmitter release. Science 239:282–285.

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1–105.

Diamond JS, Jahr CE (2000) Synaptically released glutamate does not overwhelm transporters on hippocampal astrocytes during high-frequency stimulation. J Neurophysiol 83:2835–2843.

Dudel J, Franke C, Hatt H, Ramsey RL, Usherwood PN (1988) Rapid activation and desensitization by glutamate of excitatory, cation-selective channels in locust muscle. Neurosci Lett 88:33–38.

Emptage NJ, Carew TJ (1993) Long-term synaptic facilitation in the absence of short-term facilitation in *Aplysia* neurons. Science 262:253–256.

Fernandez RI, Lyons LC, Levenson J, Khabour O, Eskin A (2003) Circadian modulation of long-term sensitization in *Aplysia*. Proc Natl Acad Sci USA 100:14415–144120.

Fox LE, Lloyd PE (2000) Role of cAMP in the short-term modulation of a neuromuscular system in *Aplysia*. J Neurophysiol 83:1567–1579.

Glanzman DL, Mackey SL, Hawkins RD, Dyke AM, Lloyd PE, Kandel ER (1989) Depletion of serotonin in the nervous system of *Aplysia* reduces the behavioral enhancement of gill withdrawal as well as the heterosynaptic facilitation produced by tail shock. J Neurosci 9:4200–4213.

Greenberg SM, Castellucci VF, Bayley H, Schwartz JH (1987) A molecular mechanism for long-term sensitization in *Aplysia*. Nature 329:62–65.

Hamberger A, Chiang GH, Sandoval E, Cotman CW (1979) Glutamate as a CNS transmitter. II. Regulation of synthesis in the releasable pool. Brain Res 168:531–541.

Hasegawa M, Cahill GM (1998) Cyclic AMP resets the circadian clock in cultured *Xenopus* retinal photoreceptor layers. J Neurochem 70:1523–1531.

Hegde AN, Inokuchi K, Pei W, Casadio A, Ghirardi M, Chain DG, Martin KC, Kan-

del ER, Schwartz JH (1997) Ubiquitin C-terminal hydrolase is an immediate-early gene essential for long-term facilitation in *Aplysia*. Cell 89:115–126.

Jaccard J, Wan CK (1996) LISREL approaches to interaction effects in multiple regression. Thousand Oaks, CA: Sage.

Jackson M, Song W, Liu MY, Jin L, Dykes-Hoberg M, Lin CI, Bowers WJ, Federoff HJ, Sternweis PC, Rothstein JD (2001) Modulation of the neuronal glutamate transporter EAAT4 by two interacting proteins. Nature 410:89–93.

Kalandadze A, Wu Y, Robinson MB (2002) Protein kinase C activation decreases cell surface expression of the GLT-1 subtype of glutamate transporter: requirement of a carboxyl-terminal domain and partial dependence on serine 486. J Biol Chem 277:45741–45750.

Kandel ER (2001) The molecular biology of memory storage: a dialog between genes and synapses. Biosci Rep 21:565–611.

Khabour O, Biby D, Nunez-Regueiro M, Lyons LC, Rawashdeh O, Byrne JH, Fioravantes D, Eskin A (2003) Time course of changes in glutamate uptake produced by serotonin and PDBU in *Aplysia*. Soc Neurosci Abstr 29:291.17.

Kruger KE, Sossin WS, Sacktor TC, Bergold PJ, Beushausen S, Schwartz JH (1991) Cloning and characterization of Ca(2+)-dependent and Ca(2+)-independent PKCs expressed in *Aplysia* sensory cells. J Neurosci 11:2303–2313.

Levenson J, Byrne JH, Eskin A (1999) Levels of serotonin in the hemolymph of *Aplysia* are modulated by light/dark cycles and sensitization training. J Neurosci 19:8094–8103.

Levenson J, Sherry DM, Dryer L, Chin J, Byrne JH, Eskin A (2000a) Localization of glutamate and glutamate transporters in the sensory neurons of *Aplysia*. J Comp Neurol 423:121–131.

Levenson J, Endo S, Kategaya LS, Fernandez RI, Brabham DG, Chin J, Byrne JH, Eskin A (2000b) Long-term regulation of neuronal high-affinity glutamate and glutamine uptake in *Aplysia*. Proc Natl Acad Sci USA 97:12858–12863.

Levenson J, Weeber E, Selcher JC, Kategaya LS, Sweatt JD, Eskin A (2002) Long-term potentiation and contextual fear conditioning increase neuronal glutamate uptake. Nat Neurosci 5:155–161.

Lin CI, Orlov I, Ruggiero AM, Dykes-Hoberg M, Lee A, Jackson M, Rothstein JD (2001) Modulation of the neuronal glutamate transporter EAAC1 by the interacting protein GTRAP3-18. Nature 410:84–88.

Lin XY, Glanzman DL (1994) Hebbian induction of long-term potentiation of *Aplysia* sensorimotor synapses: partial requirement for activation of an NMDA-related receptor. Proc R Soc Lond B Biol Sci 255:215–221.

Manseau F, Sossin WS, Castellucci VF (1998) Long-term changes in excitability induced by protein kinase C activation in *Aplysia* sensory neurons. J Neurophysiol 79:1210–1218.

Marinesco S, Carew TJ (2002) Serotonin release evoked by tail nerve stimulation in the CNS of *Aplysia*: characterization and relationship to heterosynaptic plasticity. J Neurosci 22:2299–2312.

Martin KC, Michael D, Rose JC, Barad M, Casadio A, Zhu H, Kandel ER (1997) MAP kinase translocates into the nucleus of the presynaptic cell and is required for long-term facilitation in *Aplysia*. Neuron 18:899–912.

Mauelshagen J, Sherff CM, Carew TJ (1998) Differential induction of long-term synaptic facilitation by spaced and massed applications of serotonin at sensory neuron synapses of *Aplysia californica*. Learn Mem 5:246–256.

Michael D, Martin KC, Seger R, Ning MM, Baston R, Kandel ER (1998) Repeated pulses of serotonin required for long-term facilitation activate mitogen-activated protein kinase in sensory neurons of *Aplysia*. Proc Natl Acad Sci USA 95:1864–1869.

Min MY, Rusakov DA, Kullmann DM (1998) Activation of AMPA, kainate, and metabotropic receptors at hippocampal mossy fiber synapses: role of glutamate diffusion. Neuron 21:561–570.

Mize AL, Alper RH (2002) Rapid uncoupling of serotonin-1A receptors in rat hippocampus by 17beta-estradiol in vitro requires protein kinases A and C. Neuroendocrinology 76:339–347.

Montarolo PG, Goelet P, Castellucci VF, Morgan J, Kandel ER, Schacher S (1986) A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. Science 234:1249–1254.

Muller U, Carew TJ (1998) Serotonin induces temporally and mechanistically distinct phases of persistent PKA activity in *Aplysia* sensory neurons. Neuron 21:1423–1434.

Ocorr KA, Byrne JH (1985) Membrane responses and changes in cAMP levels in *Aplysia* sensory neurons produced by serotonin, tryptamine, FMRFamide and small cardioactive peptideB (SCPB). Neurosci Lett 55:113–118.

Ocorr KA, Tabata M, Byrne JH (1986) Stimuli that produce sensitization lead to elevation of cyclic AMP levels in tail sensory neurons of *Aplysia*. Brain Res 371:190–192.

Otis TS, Wu YC, Trussell LO (1996) Delayed clearance of transmitter and the role of glutamate transporters at synapses with multiple release sites. J Neurosci 16:1634–1644.

Pepio AM, Fan X, Sossin WS (1998) The role of C2 domains in Ca2+-activated and Ca2+-independent protein kinase Cs in *Aplysia*. J Biol Chem 273:19040–19048.

Purcell AL, Sharma SK, Bagnall MW, Sutton MA, Carew TJ (2003) Activation of a tyrosine kinase-MAPK cascade enhances the induction of long-term synaptic facilitation and long-term memory in *Aplysia*. Neuron 37:473–484.

Raman IM, Trussell LO (1995) The mechanism of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptor desensitization after removal of glutamate. Biophys J 68:137–146.

Rickard NS, Ng KT, Gibbs ME (1998) Further support for nitric oxide-dependent memory processing in the day- old chick. Neurobiol Learn Mem 69:79–86.

Roberts AC, Glanzman DL (2003) Learning in *Aplysia*: looking at synaptic plasticity from both sides. Trends Neurosci 26:662–670.

Sacktor TC, Schwartz JH (1990) Sensitizing stimuli cause translocation of protein kinase C in *Aplysia* sensory neurons. Proc Natl Acad Sci USA 87:2036–2039.

Sasaki K, Kawasaki S, Kimura S, Fujita R, Takashima K, Matsumoto M, Sato M (1997) Functional uncoupling between the receptor and G-protein as the result of PKC activation, observed in *Aplysia* neurons. Jpn J Physiol 47:241–249.

Schacher S, Proshansky E (1983) Neurite regeneration by *Aplysia* neurons in dissociated cell culture: modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. J Neurosci 3:2403–2413.

Schacher S, Castellucci VF, Kandel ER (1988) cAMP evokes long-term facilitation in *Aplysia* sensory neurons that requires new protein synthesis. Science 240:1667–1669.

Scholz KP, Byrne JH (1987) Long-term sensitization in *Aplysia*: biophysical correlates in tail sensory neurons. Science 235:685–687.

Sharma SK, Carew TJ (2004) The role of MAPK cascades in synaptic plasticity and memory in *Aplysia*: facilitatory effects and inhibitory constraints. Learn Mem 11:373–378.

Sharma SK, Sherff CM, Shobe J, Bagnall MW, Sutton MA, Carew TJ (2003) Differential role of mitogen-activated protein kinase in three distinct phases of memory for sensitization in *Aplysia*. J Neurosci 23:3899–3907.

Sossin WS (1997) An autonomous kinase generated during long-term facilitation in *Aplysia* is related to the Ca(2+)-independent protein kinase C Apl II. Learn Mem 3:389–401.

Sossin WS, Schwartz JH (1992) Selective activation of Ca(2+)-activated PKCs in *Aplysia* neurons by 5-HT. J Neurosci 12:1160–1168.

Sossin WS, Sacktor TC, Schwartz JH (1994) Persistent activation of protein kinase C during the development of long-term facilitation in *Aplysia*. Learn Mem 1:189–202.

Sutton MA, Masters SE, Bagnall MW, Carew TJ (2001) Molecular mechanisms underlying a unique intermediate phase of memory in *Aplysia*. Neuron 31:143–154.

Turecek R, Trussell LO (2000) Control of synaptic depression by glutamate transporters. J Neurosci 20:2054–2063.

Walsh JP, Byrne JH (1984) Forskolin mimics and blocks a serotonin-sensitive decreased K+ conductance in tail sensory neurons of *Aplysia*. Neurosci Lett 52:7–11.

Wicher D (2001) Peptidergic modulation of an insect Na(+) current: role of protein kinase A and protein kinase C. J Neurophysiol 85:374–383.

Wu F, Friedman L, Schacher S (1995) Transient versus persistent functional and structural changes associated with facilitation of *Aplysia* sensorimotor synapses are second messenger dependent. J Neurosci 15:7517–7527.

Yamamoto N, Hegde AN, Chain DG, Schwartz JH (1999) Activation and degradation of the transcription factor C/EBP during long-term facilitation in *Aplysia*. J Neurochem 73:2415–2423.

Zhang F, Endo S, Cleary LJ, Eskin A, Byrne JH (1997) Role of transforming growth factor-beta in long-term synaptic facilitation in *Aplysia*. Science 275:1318–1320.

Zhu H, Wu F, Schacher S (1997) Site-specific and sensory neuron-dependent increases in postsynaptic glutamate sensitivity accompany serotonin-induced long-term facilitation at *Aplysia* sensorimotor synapses. J Neurosci 17:4976–4986.

Zwartjes RE, Eskin A (1990) Changes in protein phosphorylation in the eye of *Aplysia* associated with circadian rhythm regulation by serotonin. J Neurobiol 21:376–383.