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Serotonin facilitates long-term depression induction in prefrontal cortex via p38 MAPK/Rab5-mediated enhancement of AMPA receptor internalization

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The serotonin system in prefrontal cortex (PFC) is critically involved in the regulation of cognition and emotion. To understand the cellular mechanisms underlying its physiological actions, we investigated the role of serotonin in regulating synaptic plasticity in PFC circuits. We found that tetanic stimuli coupled to bath application of serotonin induced long-term depression (LTD) at excitatory synapses of PFC pyramidal neurons. This effect was mediated by 5-$HT_{2A/C}$ receptors and was independent of NMDA receptor activation. A group I metabotropic glutamate receptor (mGluR) antagonist blocked the LTD induction by serotonin + tetani, and co-application of a group I mGluR agonist and serotonin, but not application of either drug alone, induced LTD without tetani. The effect of serotonin on LTD was blocked by selective inhibitors of p38 mitogen-activated protein kinase (MAPK), but not p42/44 MAPK. Biochemical evidence also indicated that serotonin and a group I mGluR agonist synergistically activated p38 MAPK in PFC slices. The serotonin-facilitated LTD induction was prevented by blocking the activation of the small GTPase Rab5, as well as by blocking the clathrin-dependent internalization of AMPA receptors with postsynaptic injection of a dynamin inhibitory peptide, while it was unaffected by manipulating the cytoskeleton. Interestingly, in animals exposed to acute stress, the LTD induction by serotonin + tetani was significantly impaired. Taken together, these results suggest that serotonin, by cooperating with mGluRs, regulates synaptic plasticity through a mechanism dependent on p38 MAPK/Rab5-mediated enhancement of AMPA receptor internalization in a clathrin/dynamin-dependent manner. It provides a potential mechanism underlying the role of serotonin in controlling emotional and cognitive processes that are mediated by synaptic plasticity in PFC neurons.

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Prefrontal cortex (PFC) is a brain region critical for many high-level, 'executive' processes, such as working memory, attention, inhibition of distraction, novelty seeking, emotional control, decision making and encoding of context (Stuss & Knight, 2002). One of the most important neuromodulators that powerfully influence PFC functions is serotonin (Davidson *et al.* 2000; Williams *et al.* 2002; Yan, 2002). Aberrant serotonergic neurotransmission has long been implicated in the pathogenesis of neuropsychiatric disorders that are associated with PFC dysfunction, including schizophrenia, depression and anxiety (Breier, 1995; Dubovsky & Thomas, 1995; Abi-Dargham *et al.* 1997; Buhot, 1997; Stockmeier, 1997; Gross & Hen, 2004). Because of the complexity of the 5-HT receptor subtypes (Martin *et al.* 1998) distributed within the neuronal circuits of PFC (Goldman-Rakic *et al.* 1990; Feng *et al.* 2001), relatively little is known about the functional role of serotonin in PFC. The two most abundant 5-HT receptor subtypes in PFC, 5-HT_{1A} and 5-HT_{2A}, are enriched in postsynaptic dendritic shafts and dendritic spines of pyramidal neurons (Kia *et al.* 1996; Jakab & Goldman-Rakic, 1998) where glutamate receptors are concentrated, raising the possibility that serotonin may exert some of its functions by modulating glutamatergic synapses (Aghajanian & Marek, 1997; Cai *et al.* 2002; Yuen *et al.* 2005).

In both invertebrate and vertebrate nervous systems, glutamatergic synaptic transmission can undergo long-term changes in efficacy, a phenomenon called synaptic plasticity (Collingridge & Singer, 1990; Siegelbaum & Kandel, 1991; Malenka & Nicoll, 1999). The two most widely known examples of activity-dependent

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synaptic plasticity of excitatory transmission, long-term potentiation (LTP) and long-term depression (LTD), are leading synaptic models for experience-induced modification of brain function, such as learning and memory (Malenka & Bear, 2004). It has been found that the gating and the polarity of synaptic plasticity in cortex can be controlled by neuromodulators (Otani *et al.* 1998; Matsuda *et al.* 2006; Seol *et al.* 2007). Serotonin can affect the induction of LTP and LTD in a complicated manner, depending on the different 5-HT receptor subtypes, brain regions and developmental stages (Kojic *et al.* 1997; Edagawa *et al.* 2000, 2001; Kemp & Manahan-Vaughan, 2004). Administration of selective serotonin reuptake inhibitors also gives variable effects on synaptic plasticity, with the LTP induction in CA1 hippocampus being blocked (Shakesby *et al.* 2002), and LTP in the hippocampo-medial PFC pathway being significantly augmented (Ohashi *et al.* 2002). Moreover, it has been found that serotonin promotes the probability of LTP in 5-HT_{2C} receptor-rich zones and facilitates LTD induction in 5-HT_{2C} receptor-poor zones of visual cortex (Kojic *et al.* 2000), suggesting that serotonin may control not only whether plasticity occurs, but also where a given input is strengthened or weakened (Kirkwood, 2000).

In this study, we examined the impact of serotonin on synaptic plasticity of glutamatergic transmission in PFC pyramidal neurons, which could provide a potential cellular mechanism underlying the serotonergic regulation of cognitive processes associated with normal mental function and neuropsychiatric disorders.

Methods

Electrophysiological recordings in slices

Pyramidal neurons located in deep layers (V–VI) of the PFC of Sprague–Dawley rats (3–5 weeks postnatal) were recorded. All experiments were carried out with the approval of the State University of New York at Buffalo Animal Care Committee. Slice preparation procedures were similar to what was described before (Zhong *et al.* 2003; Tan *et al.* 2004; Yuen *et al.* 2005). In brief, animals were anaesthetized by inhaling 2-bromo-2-chloro-1,1,1-trifluoroethane (1 ml (100 g)$^{-1}$, Sigma) and decapitated. Brains were quickly removed and sliced (300–400 μm) with a Leica VP1000S Vibrotome while bathed in a Hepes-buffered salt solution. Slices were then incubated for 1–5 h at room temperature (22–24°C) in a $NaHCO_3$-buffered saline bubbled with 95% O_2–5% CO_2.

To measure excitatory postsynaptic currents in PFC slices, the whole-cell voltage-clamp recording technique was used (Zhong *et al.* 2003; Zhong & Yan, 2004). Electrodes (5–9 MΩ) were filled with the following internal solution (in mM): 130 caesium methanesulphonate, 10 CsCl, 4 NaCl, 10 Hepes, 1 $MgCl_2$, 5 EGTA, 2.2 QX-314, 12 phosphocreatine, 5 MgATP, 0.2 Na_3GTP, 0.1 leupeptin; pH 7.2–7.3; 265–270 mosmol l^{-1}. The slice (300 μm) was placed in a perfusion chamber attached to the fixed stage of an upright microscope (Olympus) and submerged in continuously flowing oxygenated artificial cerebrospinal fluid (ACSF, 130 mM NaCl, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 5 mM $MgCl_2$, 3 mM KCl, 10 mM glucose, 1.25 mM NaH_2PO_4). Cells were visualized with a ×40 water-immersion lens and illuminated with near infrared (IR) light and the image was detected with an IR-sensitive CCD camera. A Multiclamp 700A amplifier was used for these recordings. Tight seals (2–10 GΩ) from visualized pyramidal neurons were obtained by applying negative pressure. Recordings were done at room temperature. The membrane was disrupted with additional suction and the whole cell configuration was obtained. The access resistances ranged from 13 to 18 MΩ and were compensated 50–70%. For the recording of AMPAR-mediated eEPSCs, cells (voltage-clamped at −70 mV) were bathed in ACSF containing bicuculline (2 μM) to block $GABA_A$ receptors. A bipolar stimulating electrode (FHC) was positioned ∼100 μm from the neuron being recorded. Evoked currents were generated with a 50 μs test pulse from a stimulation isolation unit controlled by a S48 pulse generator (Astro-Medical). LTD-inducing tetanic stimuli consisted of four trains of 50 Hz stimuli (100 pulses per train), delivered at 0.1 Hz. The 0.033 Hz test stimuli (1 pulse (30 s)$^{-1}$) were resumed 30 s after tetanic stimulation. The averaged responses from the 10 min period just before tetani–drug application and the 50–60 min period after tetani–drug application were compared to express changes of the eEPSC amplitude.

Serotonin receptor ligands 5-HT, α-Me-5-HT, (–)-2, 5-dimethoxy-4-iodoamphetamine (DOI), ketanserin, cyanopindolol, and actin/microtubule agents phalloidin and taxol (Sigma), as well as second messenger reagents SB203580, PD98059 and U0126 (Calbiochem) were made up as concentrated stock solutions in water or DMSO and stored at −20°C. Stocks were thawed and diluted immediately prior to use. The amino acid sequence for the dynamin inhibitory peptide (Tocris) is: QVPSRPNRAP. The polyclonal anti-Rab5 antibody (Santa Cruz) was raised against the full-length human Rab5A. Wild-type and mutant (S34N and Q79L) Rab5 proteins were generated as previous described (Chen *et al.* 2007). Data analyses were performed with Clampfit (Axon Instruments), Origin 6 (OriginLab) and Kaleidagraph (Albeck Software). ANOVA tests were performed to compare groups subjected to different treatment.

Western blot analysis

PFC slices were prepared as previously described (Gu *et al.* 2003; Gu & Yan, 2004). After drug treatment, equal

amounts of protein from slice homogenates were separated on 7.5% acrylamide gels and transferred to nitrocellulose membranes. The blots were blocked with 5% non-fat dried milk for 1 h at room temperature. Then the blots were incubated with the phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibody (1 : 1000, Cell Signaling) for 1 h at room temperature. After being rinsed, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit antibodies (Amersham, 1 : 2000) for 1 h at room temperature. Following three washes, the blots were exposed to the enhanced chemiluminescence substrate. Then the blots were stripped for 1 h at 50°C followed by saturation in 5% non-fat dried milk and incubated with the p38 MAPK antibody (1 : 1000, Cell Signaling). Quantification was obtained from densitometric measurements of immunoreactive bands on films.

Stress protocol

Two stress protocols (elevated platform test and forced swim test) as previously described (Xu *et al.* 1997; Roche *et al.* 2003; Tan *et al.* 2004) were used in our studies. In the elevated platform tests, rats were placed on an elevated platform (20 cm × 20 cm) for 20 min in a brightly lit room. The animal showed behavioural 'freezing' (i.e. immobility) for up to 10 min. In the swim tests, rats were placed in a cylindrical glass tank (24.5 cm high × 18.5 cm diameter) filled with water to a depth of 20 cm. Rats were forced to swim in warm water (24–26°C) for 20 min. About 30 min after the stress procedures, rats were anaesthetized and killed. Experimental groups were matched so that a stressed rat and a control rat were perfused on the same day and tissue was processed in parallel.

Results

Serotonin, by activating 5-$HT_{2A/2C}$ receptors, facilitates LTD induction in prefrontal cortex

To test the role of serotonin in the regulation of synaptic plasticity, we examined the effect of 5-HT on glutamatergic responses in PFC pyramidal neurons. As shown in Fig. 1*A*, stable AMPA receptor-mediated EPSCs were evoked by short test pulses (delivered in 30 s intervals) throughout the recording ($n = 10$). However, tetanic stimuli (100 pulses at 50 Hz, repeated four times in 10 s intervals) delivered at the end of a 10 min bath application of 5-HT (40 μM) induced long-term depression (LTD) of the EPSC amplitude. 5-HT alone only transiently depressed the EPSC (Fig. 1*B*). Tetanic stimulation alone also failed to induce long-term changes of the synaptic response (Fig. 1*C*). As summarized in Fig. 1*D*, a combination of serotonin application and the 50 Hz tetani induced $26.1 \pm 1.1\%$ ($n = 18$) reduction of the EPSC amplitude (measured at 50–60 min after washout), while serotonin alone or tetani alone had little long-term effect (5-HT: $8.3 \pm 1.6\%$, $n = 12$; tetani: $3.2 \pm 1.9\%$, $n = 6$).

Our previous study has shown that 5-HT_{1A} and 5-HT_{2A} receptors are the most predominant serotonin receptor subtypes expressed in PFC pyramidal neurons (Feng *et al.* 2001), thus we examined the role of these receptors in serotonergic facilitation of LTD. Co-application of the specific 5-$HT_{2A/2C}$ agonist α-Me-5-HT (20 μM) and 50 Hz tetani caused a sustained reduction in the EPSC amplitude (Fig. 2*A*; $32.0 \pm 3.1\%$, $n = 5$; Fig. 2*E*), mimicking the LTD induction by 5-HT + tetani. Another 5-$HT_{2A/2C}$ agonist (–)DOI (50 μM) induced similar LTD when coupled with tetani ($29.6 \pm 3.2\%$, $n = 3$). In the presence of ketanserin (20 μM), a selective 5-$HT_{2A/2C}$ antagonist, LTD failed to be induced by α-Me-5-HT + tetani (Fig. 2*B*; $8.2 \pm 2.9\%$, $n = 3$; Fig. 2*E*) or 5-HT + tetani (Fig. 2*C*; $8.9 \pm 3.0\%$, $n = 5$; Fig. 2*E*). However, the 5-HT_1-class antagonist cyanopindolol (10 μM) was ineffective in blocking LTD induction by 5-HT + tetani (Fig. 2*D*; $26.7 \pm 2.3\%$, $n = 5$; Fig. 2*E*). The pharmacological data thus suggest that serotonin released on PFC pyramidal neurons could modulate synaptic plasticity via the activation of 5-$HT_{2A/2C}$ receptors.

The serotonergic facilitation of LTD induction involves group I mGluR activation

We next tested whether the LTD induction by serotonin and tetanic stimulation requires the activation of specific glutamate receptors. As shown in Fig. 3*A*, in the presence of the NMDAR antagonist APV (50 μM), the LTD induction by 5-HT and tetani was intact ($25.5 \pm 1.8\%$, $n = 9$; Fig. 3*F*), suggesting the lack of involvement of NMDA receptors in this synaptic plasticity. In contrast, when group I mGluRs were blocked by their antagonists MPEP (10 μM) and CPCCOEt (50 μM), co-application of 5-HT and tetani failed to induce LTD (Fig. 3*B*; $9.2 \pm 2.4\%$, $n = 6$; Fig. 3*F*). A combined application of 5-HT and glutamate (50 μM) induced LTD without tetani (Fig. 3*C*; $22.3 \pm 1.7\%$, $n = 5$; Fig. 3*F*), while co-application of 5-HT and NMDA (20 μM) was ineffective in inducing significant LTD (Fig. 3*D*; $10.4 \pm 1.5\%$, $n = 6$; Fig. 3*F*). Moreover, a combined 10 min application of 5-HT and the group I mGluR agonist DHPG (100 μM) induced LTD without tetani (Fig. 3*E*; $28.1 \pm 4.0\%$, $n = 5$; Fig. 3*F*). Compared to the LTD induced by 5-HT + DHPG or by 5-HT + tetani, when 5-HT is coupled with both DHPG and tetani, no additive LTD was induced (Fig. 3*E*; $30.8 \pm 1.8\%$, $n = 3$, Fig. 3*F*), suggesting that DHPG occluded tetani in 5-HT-facilitated LTD induction. Glutamate, NMDA or DHPG itself was incapable of inducing sustained changes of the EPSC amplitude (Fig. 3*F*). These data suggest that

glutamate released from tetanic stimulation activates the group I mGluR, which is necessary and sufficient for the LTD induction in the presence of serotonin.

p38 MAP kinase mediates the serotonin effect on LTD induction

In the following set of experiments, we sought to find out the mechanisms underlying serotonin-facilitated LTD induction in PFC. To examine the pre- *versus* post-synaptic origin of this synaptic plasticity, we measured the ratio of paired-pulse facilitation (PPR), an index of presynaptic processes, before and after LTD induction. No significant change was observed in PPR (before LTD: 2.05 ± 0.13; after LTD: 2.11 ± 0.14, $n = 4$, $P > 0.5$, ANOVA), suggesting that the LTD induction is probably through a postsynaptic mechanism.

Previous studies have found that MAP kinases are involved in various forms of synaptic plasticity (Bolshakov *et al.* 2000; Zhu *et al.* 2002; Rush *et al.* 2002; Guan *et al.* 2003). Thus, we applied selective MAP kinase inhibitors and examined the LTD induction by 5-HT and tetani. As shown in Fig. 4*A*, postsynaptic injection of the specific p38 MAPK inhibitor SB203580 (Lee *et al.* 1999; 20 μM) prevented the LTD induction ($8.7 \pm 1.1\%$, $n = 15$; Fig. 4*F*). Moreover, dialysis with an affinity-purified antibody raised against activated (Thr^{180}/Tyr^{182}-phosphorylated) p38 MAPK (1 : 100) also

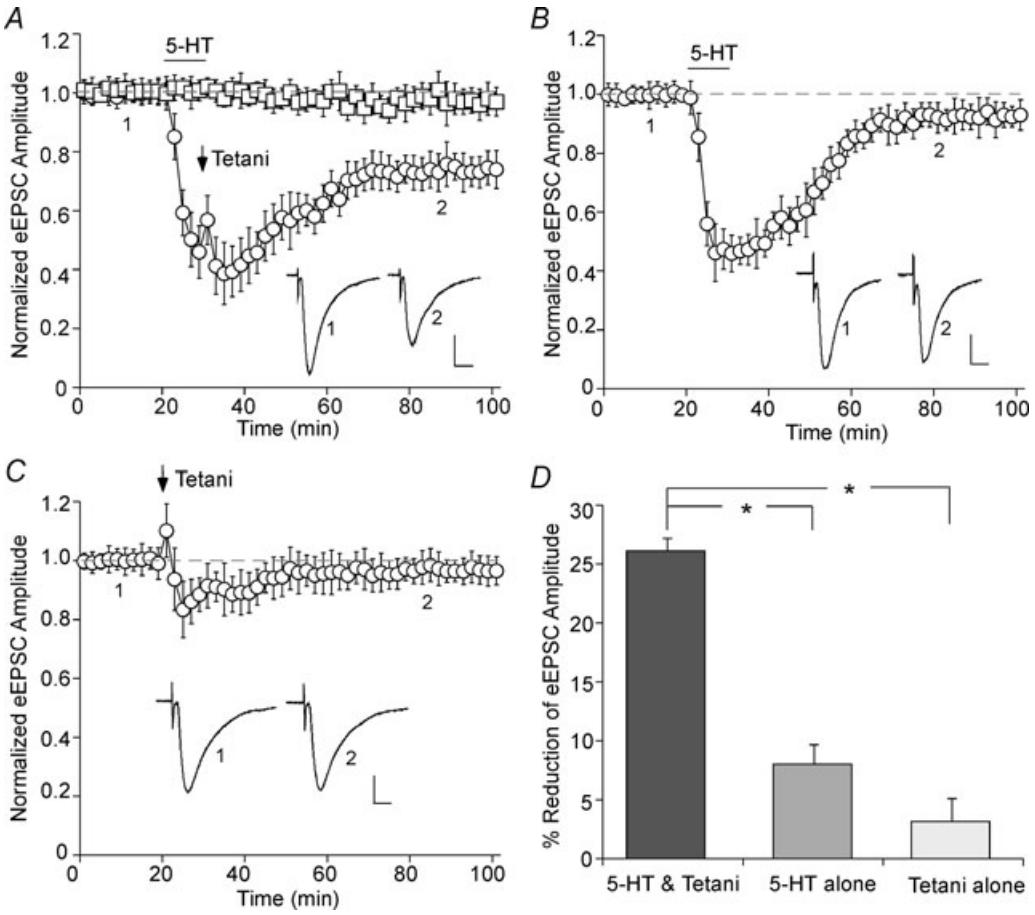

Figure 1. Serotonin facilitates LTD induction in PFC pyramidal neurons
A, plot of normalized eEPSC amplitude in PFC neurons with or without 5-HT (40 μM, 10 min) application coupled to 50 Hz tetanic stimuli (added 9 min after the start of 5-HT application). Each point is the average (mean ± S.E.M.) of four responses. Note the induction of LTD by 5-HT + tetani. *B*, plot of normalized eEPSC amplitude in PFC neurons subjected to 5-HT (40 μM, 10 min) application. Note that the synaptic responses were acutely suppressed by bath application of 5-HT, but they fully recovered within 40 min after 5-HT washout. *C*, plot of normalized eEPSC amplitude in PFC neurons subjected to 50 Hz tetani application. Note that the synaptic responses were largely unchanged by tetanic stimuli. Inset (*A*–*C*): averaged synaptic responses taken from the indicated time points in neurons subjected to 5-HT + tetani application (*A*), 5-HT alone (*B*), or tetanic stimuli alone (*C*). Scale bars: 50 pA, 10 ms. *D*, cumulative data (mean ± S.E.M.) showing the percentage reduction of eEPSC by different treatments (measured at 50–60 min after washout). $^*P < 0.005$, ANOVA.

blocked the LTD induction (Fig. 4*B*; 8.9 ± 2.2%, $n = 5$; Fig. 4*F*). On the other hand, postsynaptic injection of PD98059 (40 μM), a selective inhibitor of MEK, the p42/44 MAPK-activating enzyme (Dudley *et al.* 1995), did not affect the LTD induction (Fig. 4*C*; 24.9 ± 1.9%, $n = 10$; Fig. 4*F*). Another structurally different selective p42/44 MAPK kinase inhibitor U0126 (20 μM) gave similar results, i.e. failed to block the LTD induction

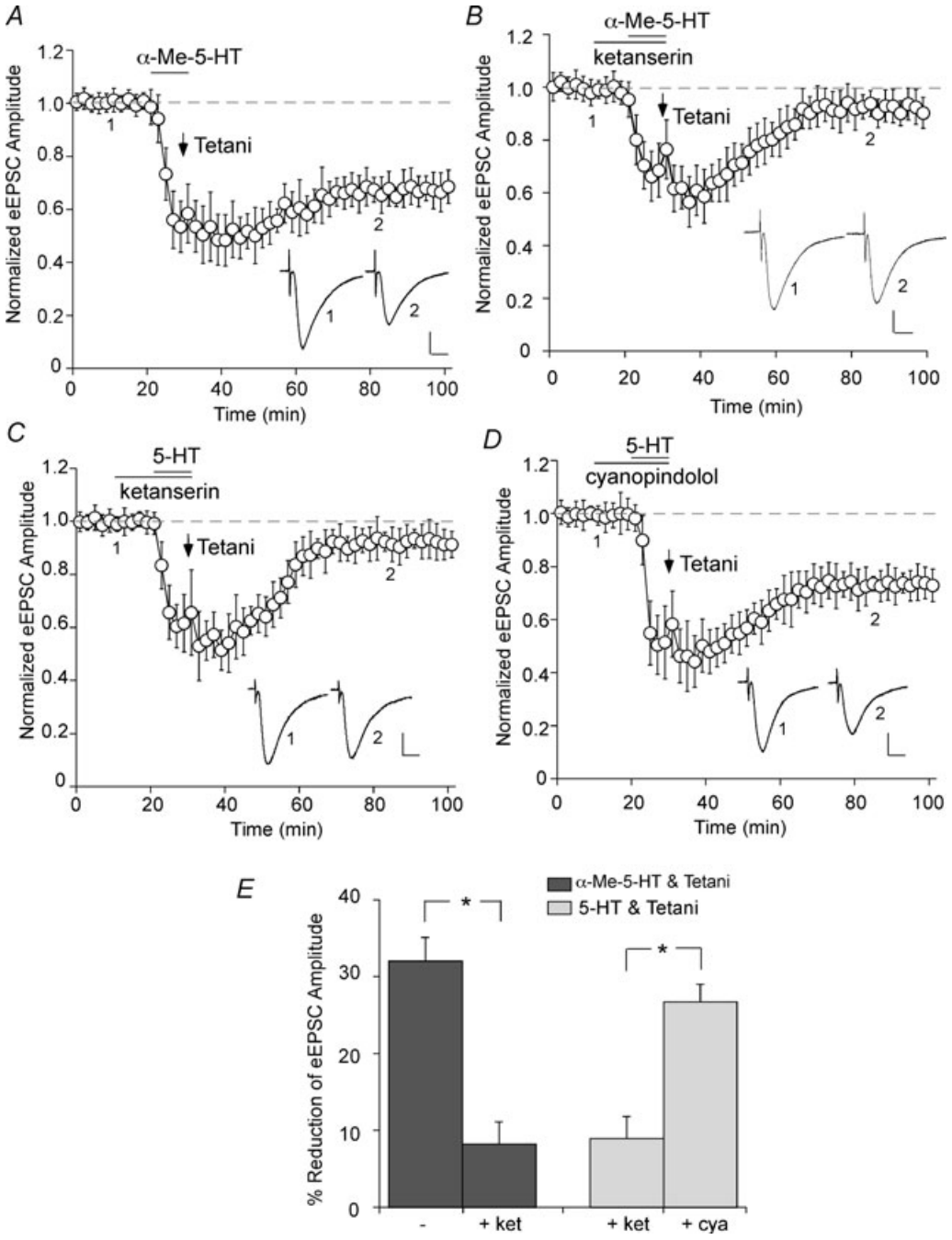

Figure 2. 5-HT$_{2A/2C}$ receptors mediate the effect of serotonin on LTD induction
A, plot of normalized eEPSC amplitude in PFC neurons subjected to co-application of the 5-HT$_{2A/2C}$ agonist α-Me-5-HT (20 μM, 10 min) and 50 Hz tetanic stimuli. Note the induction of LTD by α-Me-5-HT + tetani. *B* and *C*, plot of normalized eEPSC amplitude in PFC neurons pretreated with the 5-HT$_{2A/2C}$ antagonist ketanserin (20 μM) and subjected to α-Me-5-HT + tetani (*B*) or 5-HT + tetani (*C*) application. *D*, plot of normalized eEPSC amplitude in PFC neurons pretreated with the 5-HT$_1$ antagonist cyanopindolol (10 μM) and subjected to 5-HT + tetani application. Note that ketanserin, but not cyanopindolol, blocked the LTD induction. Inset (*A–D*): averaged synaptic responses taken from the indicated time points. Scale bars: 50 pA, 10 ms. *E*, cumulative data (mean ± S.E.M.) showing the percentage reduction of eEPSC by different treatments (measured at 50–60 min after washout). $^*P < 0.005$, ANOVA.

(27.0 ± 3.1%, $n = 6$; Fig. 4*F*). Moreover, dialysis with an antibody against active (Thr^{202}/Tyr^{204}-phosphorylated) p42/44 MAPK (1 : 100) was also ineffective in blocking the LTD induction (Fig. 4*D*; 24.3 ± 3.5%, $n = 3$; Fig. 4*F*). These results suggest that serotonergic facilitation of LTD induction in PFC requires activation of p38, but not p42/44, MAP kinase.

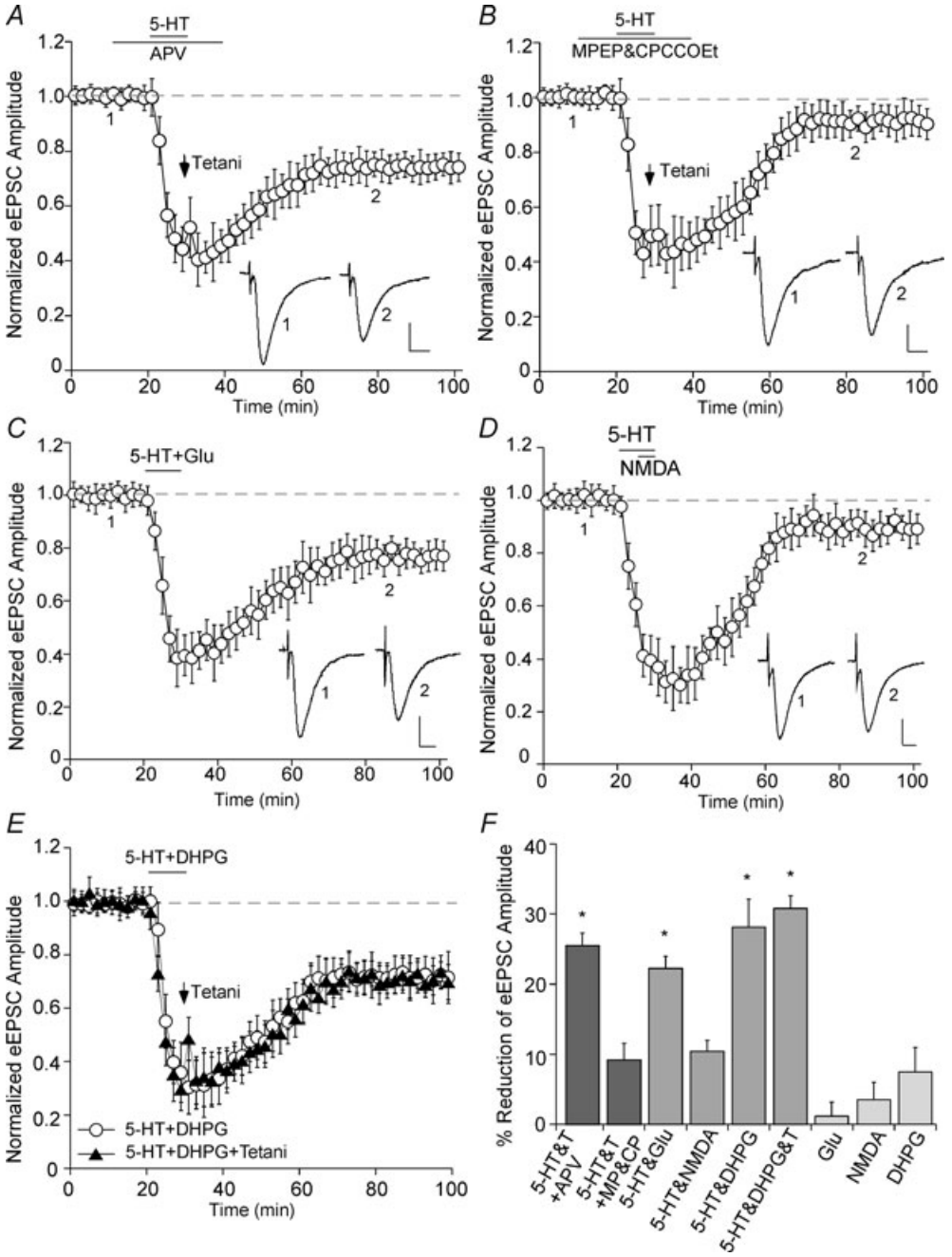

Figure 3. The LTD induction by serotonin and tetani is independent of NMDA receptors, but depends on group I mGluR activation

A and *B*, plot of normalized eEPSC amplitude in PFC neurons pretreated with the NMDAR antagonist APV (50 μM, *A*) or the group I mGluR antagonist MPEP (10 μM) plus CPCCOEt (50 μM) (*B*) and subjected to 5-HT + tetani application. Note that MPEP plus CPCCOEt, but not APV, blocked the LTD induction. *C* and *D*, plot of normalized eEPSC amplitude in PFC neurons subjected to co-application of 5-HT (40 μM) and glutamate (50 μM, *C*) or NMDA (20 μM, *D*). *E*, plot of normalized eEPSC amplitude in PFC neurons subjected to co-application of 5-HT (40 μM) and the group I mGluR agonist DHPG (100 μM) with or without coupling to tetani. Note the induction of LTD by 5-HT + glutamate or 5-HT + DHPG, but not 5-HT + NMDA. Inset (*A*–*E*): averaged synaptic responses taken from the indicated time points. Scale bars: 50 pA, 10 ms. *F*, cumulative data (mean ± S.E.M.) showing the percentage reduction of eEPSC by different treatments (measured at 50–60 min after washout). $^*P < 0.005$, ANOVA.

To further test the requirement of p38 MAP kinase, we blocked one of its upstream activators, the small GTPase Rap1 (Huang *et al.* 2004), and examined the LTD induction by 5-HT and tetani. PFC slices were pretreated with the Rap1 inhibitor GGTI-298 (20 μM) for more than 1 h, and neurons were recorded with GGTI-298 in the pipette. As shown in Fig. 4*E*, GGTI-298 largely blocked the LTD induction (11.4 ± 1.2%, $n = 3$; Fig. 4*F*).

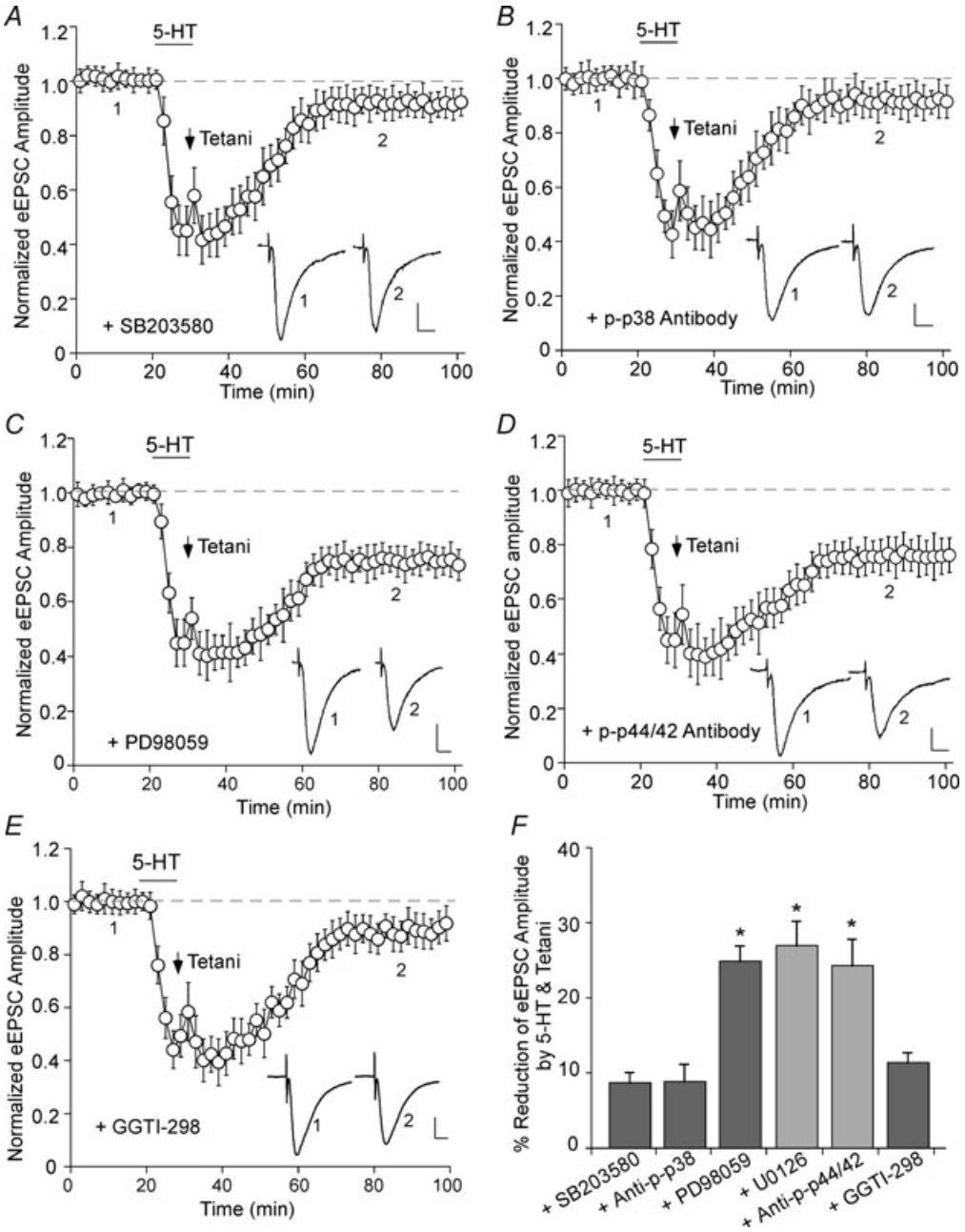

Figure 4. The serotonergic facilitation of LTD induction requires activation of p38 MAP kinase, but not p42/44 MAP kinases

A and *B*, plot of normalized eEPSC amplitude in PFC neurons dialysed with the selective p38 MAPK inhibitor SB203580 (20 μM, *A*) or an antibody against active (Thr^{180}/Tyr^{182}-phosphorylated) p38 MAPK (1 : 100, *B*) and subjected to 5-HT + tetani application. Note the block of LTD induction with these p38 MAPK inhibitors. *C* and *D*, plot of normalized eEPSC amplitude in PFC neurons dialysed with the selective p42/44 MAPK inhibitor PD98059 (40 μM, *C*) or an antibody against active (Thr^{202}/Tyr^{204}-phosphorylated) p42/44 MAPK (1 : 100, *D*) and subjected to 5-HT + tetani application. Note that these p42/44 MAPK inhibitors failed to block LTD induction. *E*, plot of normalized eEPSC amplitude in PFC neurons dialysed with the Rap1 inhibitor GGTI-298 (20 μM) and subjected to 5-HT + tetani application. Inset (*A*–*E*): averaged synaptic responses taken from the indicated time points. Scale bars: 50 pA, 10 ms. *F*, cumulative data (mean ± S.E.M.) showing the percentage reduction of eEPSC by 5-HT + tetani (measured at 50–60 min after washout) in the presence of different agents. $^*P < 0.005$, ANOVA.

To provide more evidence on the involvement of p38 MAPK in the serotonin-faciliated LTD induction, we measured p38 MAPK activation in PFC slices by protocols that can or cannot induce LTD. Activation of p38 MAPK occurs through a dual phosphorylation at threonine 180 and tyrosine 182 (Raingeaud *et al.* 1995), thus a phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibody was used to detect activated p38 MAPK. As shown in Fig. 5*A* and *B*, treatment of PFC slices with 5-HT (20 μM, 10 min) alone failed to induce p38 MAPK activation (1.2 ± 0.2 fold, $n = 4$). Glutamate (50 μM, 10 min) alone had a small effect on p38 activation (1.6 ± 0.3 fold, $n = 4$). However, a combined application of 5-HT and glutamate induced a strong activation of p38 MAPK (2.7 ± 0.6 fold, $n = 4$, $P < 0.005$, ANOVA). Similarly, co-application of 5-HT (20 μM, 10 min) with the group I mGluR agonist DHPG (100 μM, 10 min) strongly activated p38 MAPK (2.2 ± 0.5 fold, $n = 4$, $P < 0.005$, ANOVA), despite the minimal effect of DHPG itself (1.4 ± 0.3 fold, $n = 4$). The level of total p38 MAPK was not changed by any of these treatments. These data indicate that the LTD induction by 5-HT and tetani (or group I mGluR) involves a synergistic activation of p38 MAPK.

The serotonergic facilitation of LTD induction requires the Rab5-mediated internalization of AMPA receptors in a clathrin/dynamin-dependent manner

Next, we searched for the signalling pathway downstream of p38 MAPK to induce synaptic depression. Recent evidence has shown that p38 MAPK is capable of regulating endocytic trafficking via activation of the small GTPase Rab5 (Cavalli *et al.* 2001; Huang *et al.* 2004), a key mediator of protein transport from plasma membrane to early endosomes during clathrin-dependent endocytosis (Bucci *et al.* 1992; de Hoop *et al.* 1994). To test the role of Rab5 in serotonin-facilitated LTD induction, we dialysed postsynaptic neurons with an antibody against Rab5 that can cause inactivation of endogenous, membrane-associated Rab5 (Gorvel *et al.* 1991). As shown in Fig. 6*A*, the LTD induction by 5-HT + tetani was blocked in neurons loaded with the Rab5 antibody ($8.8 \pm 2.5\%$, $n = 6$; Fig. 6*D*). In contrast, the heat-inactivated Rab5 antibody, which does not bind to Rab5 and has lost the competence to inhibit Rab5, failed to affect the LTD induction (Fig. 6*B*; $23.4 \pm 4.1\%$, $n = 3$; Fig. 6*D*). To further examine the role of Rab5, we injected neurons with the purified dominant-negative (dn) mutant form of Rab5 protein (Rab5S34N), which inhibits clathrin-mediated endocytosis (Stenmark *et al.* 1994). As shown in Fig. 6*C*, the serotonin-facilitated induction of LTD in PFC was significantly diminished in dnRab5-loaded cells ($8.9 \pm 2.1\%$, $n = 6$; Fig. 6*D*). Moreover, we injected neurons with the purified constitutively active (ca) variant of Rab5 protein (Rab5Q79L), which accelerates endocytosis (Stenmark *et al.* 1994). We found that caRab5 caused a gradual depression of eEPSC amplitude ($21.0 \pm 2.1\%$, $n = 4$), and largely occluded the effect of serotonin + tetani application ($9.3 \pm 2.5\%$, $n = 5$; data not shown). These results suggest that Rab5 activity is required for the serotonin-facilitated PFC synaptic plasticity.

Since Rab5 provides a potential link between p38 MAPK and AMPAR trafficking, we further tested the involvement of clathrin-dependent endocytosis of AMPA receptors in the serotonin-facilitated LTD induction. To do so, we dialysed neurons with a dynamin inhibitory peptide that interferes with the binding of amphiphysin with dynamin and therefore prevents clathrin-dependent endocytosis (Gout *et al.* 1993; Grabs *et al.* 1997; Marks & McMahon,

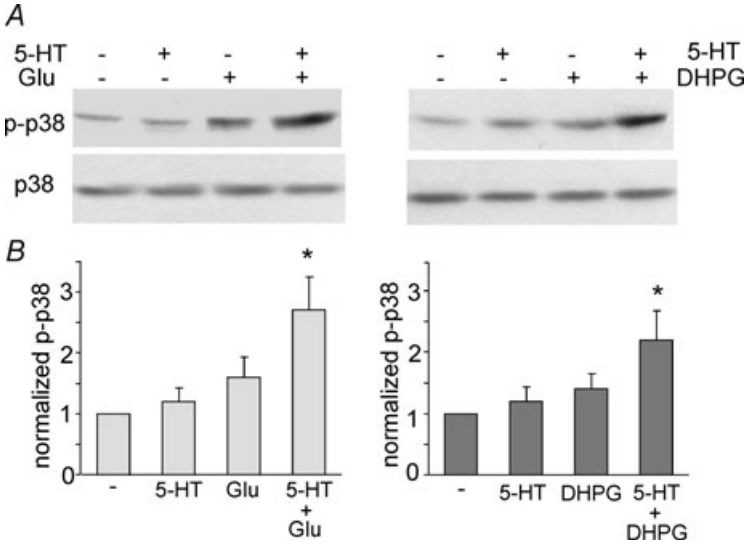

Figure 5. Co-activation of serotonin receptors and mGluRs synergistically activates p38 MAP kinase in PFC slices
A, Western blot analysis of activated (Thr^{180}/Tyr^{182}-phosphorylated) p38 MAPK (top) and total p38 MAPK (bottom) in lysates of PFC neurons treated without (–) or with 10 min of 5-HT (40 μM), glutamate/glycine (Glu, 50 μM/5 μM) or 5-HT + Glu (left). Alternatively, PFC neurons were treated without (–) or with 10 min of 5-HT (40 μM), DHPG (100 μM), or 5-HT + DHPG (right). Note the strong activation of p38 with 5-HT co-applied with Glu or DHPG. *B*, quantification of p38 activation with different treatments. Each bar represents mean ± S.E.M. of 4–5 independent experiments. $^*P < 0.005$, ANOVA.

1998). Previous studies have shown that this peptide can block AMPAR endocytosis and hippocampal LTD (Luscher *et al.* 1999; Morishita *et al.* 2005). As shown in Fig. 7*A*, the LTD induction by 5-HT and tetani was blocked in neurons loaded with the dynamin inhibitory peptide (100 μM, 9.7 ± 2.7%, $n = 6$; Fig. 7*E*), but not a scrambled control peptide (100 μM, Fig. 7*B* and 25.6 ± 4.1%, $n = 3$; Fig. 7*E*), suggesting that the clathrin-mediated AMPAR endocytosis is required for the LTD induction by 5-HT and tetani.

Because Rab5 activity has also been linked to actin remodelling (Lanzetti *et al.* 2004), and cytoskeleton integrity has been implicated in AMPA receptor trafficking (Zhou *et al.* 2001; Ives *et al.* 2004), we next tested the role of actin and microtubule networks in the serotonin-facilitated LTD induction. As shown in Fig. 7*C*, dialysis with phalloidin (4 μM), an actin-stabilizing compound, failed to affect the LTD induction by 5-HT and tetani (22.4 ± 3.0%, $n = 6$; Fig. 7*E*). Similarly, the tetani-induced LTD in the presence of 5-HT was also intact in neurons loaded with taxol (10 μM), a microtubule-stabilizing agent (Fig. 7*D*; 21.5 ± 2.6%, $n = 6$; Fig. 7*E*). These data suggest that the LTD induction by 5-HT and tetani is not dependent on the stability of actin filaments or microtubule assembly.

The serotonergic facilitation of LTD induction is impaired in stressed animals

To understand the potential implication of the serotonin-facilitated PFC synaptic plasticity in cognitive and emotional processes, we examined LTD induction by 5-HT and tetani in animals exposed to stress, since many mental illnesses are exacerbated by stress

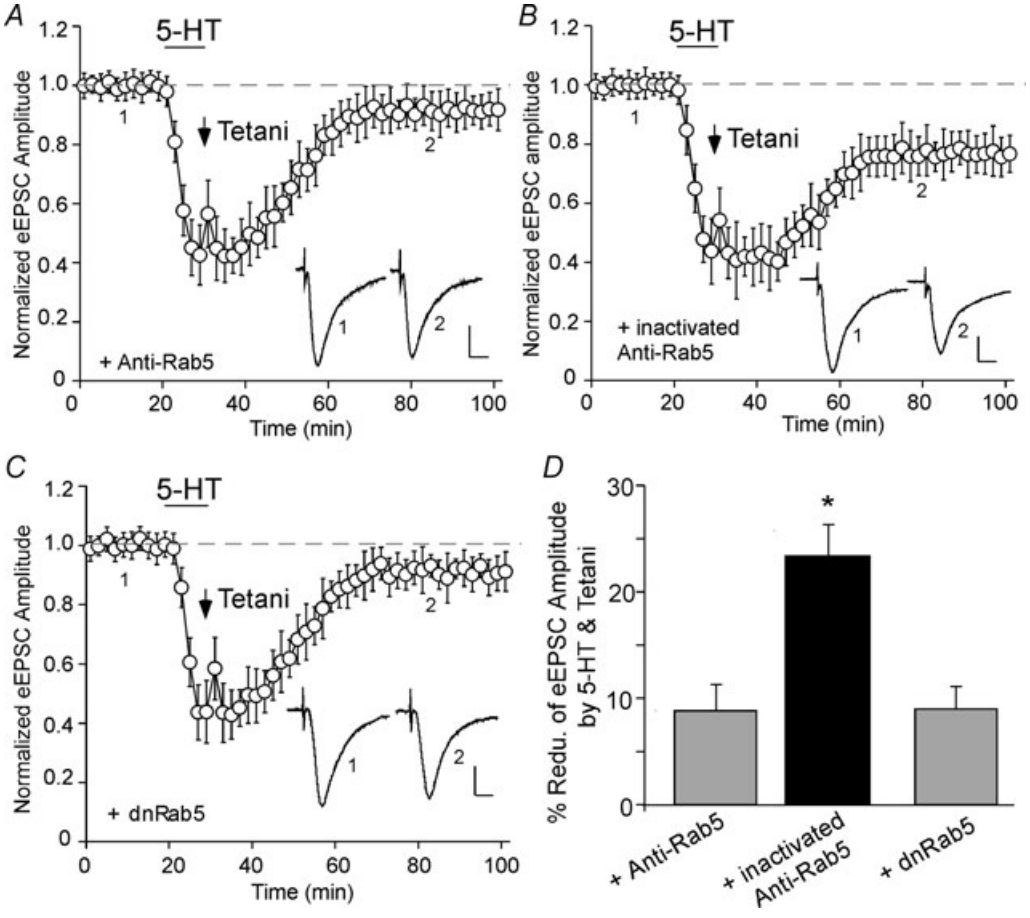

Figure 6. Activation of the small GTPase Rab5, a key mediator of receptor endocytosis and endosomal dynamics, is required for the LTD induction by serotonin and tetani
A–C, plot of normalized eEPSC amplitude in PFC neurons injected with an anti-Rab5 antibody (2 μg ml^{-1}, *A*), a heat-inactivated anti-Rab5 antibody (2 μg ml^{-1}, *B*) or the purified dominant negative Rab5 (dnRab5) protein (4 μg ml^{-1}, *C*) and subjected to 5-HT + tetani application. Note the block of LTD induction with anti-Rab5 or dnRab5. Inset (*A–C*): averaged synaptic responses taken from the indicated time points. Scale bars: 50 pA, 10 ms. *D*, cumulative data (mean ± S.E.M.) showing the percentage reduction of eEPSC by 5-HT + tetani (measured at 50–60 min after washout) in the presence of different agents. *$P < 0.005$, ANOVA.

(Mazure, 1995). The stress procedures we used entailed placing the rat on the elevated platform for 20 min (Xu *et al.* 1997), followed by forcing the rat to swim in deep water for 20 min (Roche *et al.* 2003; Tan *et al.* 2004). As shown in Fig. 8*A* and *B*, the LTD induction by 5-HT and tetani was substantially diminished in PFC neurons from stressed rats ($11.5 \pm 2.4\%$, $n = 6$), compared to PFC neurons from non-stressed control rats ($25.7 \pm 2.5\%$,

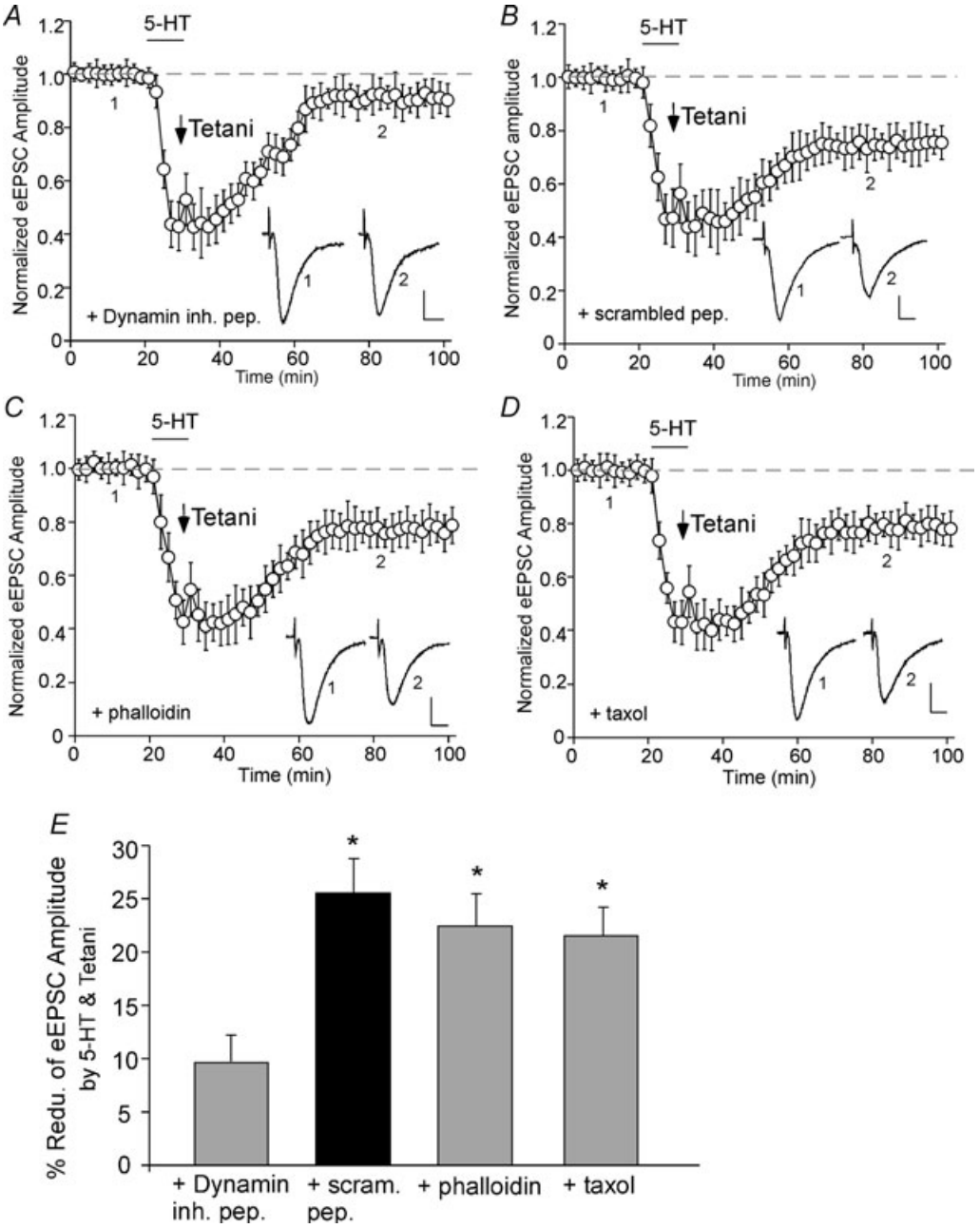

Figure 7. The serotonergic facilitation of LTD induction is mediated by AMPA receptor internalization in a clathrin/dynamin-dependent and cytoskeleton-independent manner
A and *B*, plot of normalized eEPSC amplitude in PFC neurons dialysed with a dynamin inhibitory peptide (100 μM, *A*) or a scrambled control peptide (100 μM, *B*) and subjected to 5-HT + tetani application. Note the block of LTD induction with the dynamin inhibitory peptide. *C* and *D*, plot of normalized eEPSC amplitude in PFC neurons dialysed with the actin stabilizer phalloidin (4 μM, *C*) or the microtubule stabilizer taxol (10 μM, *D*) and subjected to 5-HT + tetani application. Note that these cytoskeleton agents failed to block the LTD induction. Inset (*A–D*): averaged synaptic responses taken from the indicated time points. Scale bars: 50 pA, 10 ms. *E*, cumulative data (mean ± S.E.M.) showing the percentage reduction of eEPSC by 5-HT + tetani (measured at 50–60 min after washout) in the presence of different agents. $^*P < 0.005$, ANOVA.

$n = 6$). It suggests that the serotonin-regulated long-term plasticity is critically involved in mental processes subserved by PFC circuits.

Discussion

In this study, we have revealed that serotonin facilitates the induction of LTD of glutamatergic synaptic transmission in PFC pyramidal neurons. Previously it has been found that in visual cortex or hippocampus, the LTP or LTD induced by tetanic stimuli can be regulated by serotonin (Kojic *et al.* 1997, 2000; Edagawa *et al.* 2000, 2001; Shakesby *et al.* 2002). Tetanic stimuli (50 Hz, 100 pulses × 4 in 10 s intervals) delivered to layer II–III fibres of PFC did not induce significant changes of glutamatergic transmission in deep layers (V–VI) of PFC pyramidal neurons, similar to what has been found before (Otani *et al.* 1998). However, the same electrical stimuli coupled to 5-HT application induced a long-lasting depression of the excitatory transmission, while 5-HT alone only transiently depressed the glutamatergic response, indicating that serotonin plays an important role in modulating synaptic plasticity in the PFC network. This effect is primarily mediated by $5\text{-HT}_{2A/C}$ receptors. Consistently, these receptors have been implicated in the regulation of synaptic plasticity in visual cortex (Kojic *et al.* 1997, 2000; Edagawa *et al.* 2000) or hippocampus (Wang & Arvanov, 1998; Tecott *et al.* 1998).

Our results have shown that the serotonin-facilitated LTD in PFC neurons requires concurrent synaptic activation of group I metabotropic glutamate receptors during tetanus, but not NMDA receptors. Based on the amino acid sequences and signal transduction mechanisms, the eight mGluR subtypes are divided into three groups: group I mGluRs (mGluR1 and mGluR5) stimulate phospholipase C and phosphoinositide hydrolysis, group II (mGluR2 and mGluR3) and group III mGluRs (mGlu4, mGluR6–8) primarily couple to the inhibition of cAMP formation (Pin & Duvoisin, 1995). While group II and III mGluRs are largely regarded as presynaptic (Gereau & Conn, 1995; Petralia *et al.* 1996), group I mGluRs are abundantly expressed in postsynaptic elements throughout cortex (Romano *et al.* 1995), with mGluR5 localized on dendritic spines and shafts of cortical pyramidal neurons (Romano *et al.* 1995) and mGluR1 present in non-pyramidal cortical neurons (Fotuhi *et al.* 1993). Many forms of synaptic plasticity rely on mGluR-mediated signalling (Bashir *et al.* 1993; Cho & Bashir, 2002). Mice lacking mGluR subtypes show impaired learning and altered synaptic plasticity (Aiba *et al.* 1994; Yokoi *et al.* 1996; Lu *et al.* 1997). Thus, mGluRs have been implicated in regulating neuronal communication and signal processing underlying higher cognitive functions (Nakanishi, 1994; Conn & Pin, 1997). In this study, we found that the LTD induction by serotonin and tetanic stimulation was blocked by group I mGluR antagonists, and co-application of serotonin and the group I mGluR agonist, but neither drug alone induced LTD without tetani, suggesting that co-activation of 5-HT receptors and group I mGluRs is sufficient for LTD induction in PFC.

To identify the signalling molecules mediating the serotonin-facilitated LTD induction in PFC, we have examined MAP kinases, a family of serine/threonine protein kinases including the extracellular signal-regulated kinases (p42/44 ERK1/2), p38 MAPK and the c-Jun N-terminal kinases (JNKs 1/2/3). Both p42/44 MAPK

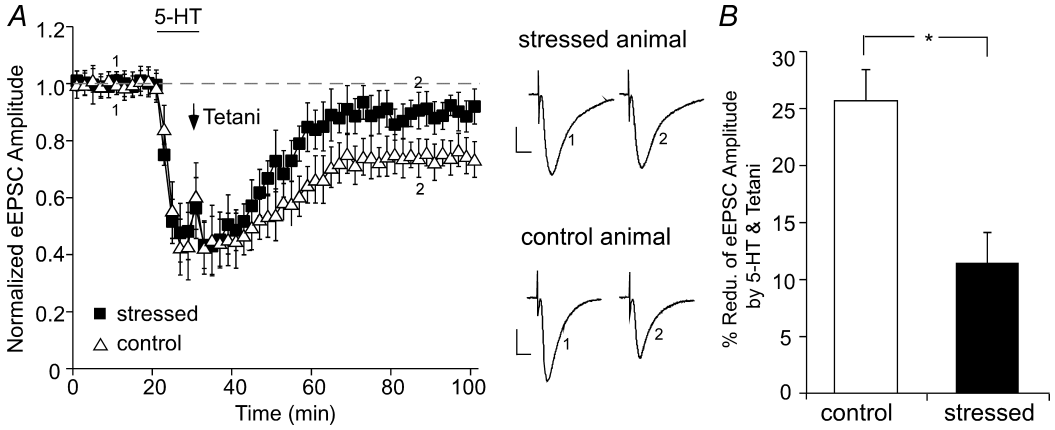

Figure 8. The serotonergic facilitation of LTD induction is impaired in animals exposed to stress
A, plot of normalized eEPSC amplitude subjected to 5-HT + tetani application in PFC neurons from stressed and control (non-stressed) rats. Note the diminished LTD induction in animals exposed to stress protocols (elevated platform and forced swim). Inset: averaged synaptic responses taken from the indicated time points. Scale bars: 50 pA, 10 ms. *B*, cumulative data (mean ± S.E.M.) showing the percentage reduction of eEPSC by 5-HT + tetani (measured at 50–60 min after washout) in PFC neurons from stressed and control rats. $^*P < 0.005$, ANOVA.

and p38 MAPK have been implicated in various forms of synaptic plasticity (Bolshakov *et al.* 2000; Zhu *et al.* 2002; Rush *et al.* 2002; Guan *et al.* 2003). The serotonin + tetanus-induced LTD in PFC neurons was blocked by postsynaptic injection of a specific p38 MAPK inhibitor but not p42/44 MAPK inhibitors, as well as by intracellular dialysis with the antibody against activated p38 MAPK but not activated p42/44 MAPK, suggesting that p38 MAPK mediates the serotonin-facilitated LTD in PFC. Moreover, our biochemical data have shown that combined activation of 5-HT receptors and group I mGluRs synergistically increased the level of active (dual phosphorylated) p38 MAPK, further indicating that both receptors may cooperate to induce LTD through converging activation of postsynaptic p38 MAPK. It is different from dopamine-facilitated prefrontal cortical LTD, which is mediated by p42/44 MAPK activation (Otani *et al.* 1999). Consistent with our finding on the involvement of p38 MAPK in LTD of glutamatergic synapses in PFC neurons, p38 MAPK pathway has been found to mediate the induction of mGluR-dependent LTD in hippocampus, while p42/p44 MAPK pathway mediates LTP (Bolshakov *et al.* 2000; Zhu *et al.* 2002). The mechanism for p38 activation in response to co-stimulation of 5-HT_2 receptors and group I mGluRs is not very clear. Presumably, the increase of $[Ca^{2+}]_i$ triggered by 5-HT + tetanus activates the small GTPase Rap1 via a PKA-dependent mechanism (Grewal *et al.* 2000), which leads to the activation of its downstream target p38 MAPK (Sawada *et al.* 2001; Zhu *et al.* 2002; Huang *et al.* 2004).

One of the important downstream targets of p38 MAPK that is potentially involved in LTD is Rab5 (Cavalli *et al.* 2001; Huang *et al.* 2004), a member of the Rab family of small GTPases that function as specific regulators of vesicle transport between organelles (Zerial & McBride, 2001; Pfeffer, 2001). Rab5 is a key coordinator of early endocytic trafficking events including early endosome fusion (Gorvel *et al.* 1991), internalization (Bucci *et al.* 1992), clathrin-coated vesicle formation (McLauchlan *et al.* 1998), and motility of early endosomes on microtubules (Nielsen *et al.* 1999). In addition to cycling between inactive (GDP-bound) and active (GTP-bound) states, Rab5 also cycles between a membrane-bound and a cytosolic state depending on the guanyl-nucleotide dissociation inhibitor (GDI, Sasaki *et al.* 1990). Activation of p38 MAPK stimulates formation of the GDI : Rab5 complex (Cavalli *et al.* 2001; Huang *et al.* 2004), which leads to the delivery of Rab5 to the plasma membrane where it is activated and triggers endocytosis by facilitating the formation of clathrin-coated pits and sorting of membrane proteins into endosomes (Bucci *et al.* 1992; McLauchlan *et al.* 1998). Using antibody-mediated inactivation of membrane-associated Rab5 (Gorvel *et al.* 1991) or injection with the dominant negative Rab5 (Stenmark *et al.* 1994), the serotonin-facilitated LTD induction was blocked, suggesting that activation of Rab5 is required for the synaptic plasticity in PFC.

Given the key role of Rab5 in regulating protein transport from plasma membrane to early endosomes (Bucci *et al.* 1992; de Hoop *et al.* 1994), one possibility underlying the serotonin-facilitated depression of glutamatergic transmission is the Rab5-mediated AMPAR internalization (Huang *et al.* 2004; Brown *et al.* 2005). AMPAR endocytosis plays a critical role in hippocampal LTD (Beattie *et al.* 2000; Malinow & Malenka, 2002). Removal of synaptic AMPARs is mediated by clathrin-dependent endocytosis (Carroll *et al.* 1999; Man *et al.* 2000) through the interaction between the AMPA receptor GluR2 subunit and the clathrin adaptor protein AP2 (Lee *et al.* 2002). In the clathrin-mediated transport pathway, the GTPase dynamin is thought to be involved in 'pinching off' endocytic vesicles from the plasma membrane (Schmid *et al.* 1998). Injection of a dynamin inhibitory peptide, which interferes with the binding of amphiphysin with dynamin and therefore prevents endocytosis through clathrin-coated pits (Gout *et al.* 1993; Grabs *et al.* 1997), blocked the serotonin-facilitated LTD induction, suggesting that clathrin-dependent AMPAR endocytosis mediates the synaptic plasticity in PFC neurons.

Finally, we examined the functional implication of the serotonin-regulated LTD in PFC. We found that the serotonin-facilitated long-term plasticity of glutamatergic transmission in PFC is impaired in animals exposed to stress. Moreover, our previous study has shown that acute stress alters the serotonergic regulation of GABA transmission in PFC pyramidal neurons (Tan *et al.* 2004). These results provide a framework for understanding the interactions between monoamines, glutamate and GABA in normal mental functions and neuropsychiatric disorders (Carlsson *et al.* 2001).

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