

# SEROTONIN IS A FACILITATORY NEUROMODULATOR OF SYNAPTIC TRANSMISSION AND “REINFORCES” LONG-TERM POTENTIATION INDUCTION IN THE VERTICAL LOBE OF *OCTOPUS VULGARIS*

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**Abstract**—The modern cephalopod mollusks (coleoids) are considered the most behaviorally advanced invertebrate, yet little is known about the neurophysiological basis of their behaviors. Previous work suggested that the vertical lobe (VL) of cephalopods is a crucial site for the learning and memory components of these behaviors. We are therefore studying the neurophysiology of the VL in *Octopus vulgaris* and have discovered a robust activity-dependent long-term potentiation (LTP) of the synaptic input to the VL. Moreover, we have shown that the VL and its LTP are involved in behavioral long-term memory acquisition. To advance our understanding of the VL as a learning neural network we explore the possible involvement of neuromodulation in VL function. Here we examine whether the well studied serotonergic modulation in simple models of learning in gastropods mollusks is conserved in the octopus VL. We demonstrate histochemically that the VL is innervated by afferent terminals containing 5-HT immunoreactivity (5-HT-IR). Physiologically, 5-HT has a robust facilitatory effect on synaptic transmission and activity-dependent LTP induction. These results suggest that serotonergic neuromodulation is a part of a reinforcing/reward signaling system conserved in both simple and complex learning systems of mollusks. However, there are notable functional differences. First, the effective concentration of 5-HT in the VL is rather high (100  $\mu$ M); secondly, only neuropilar regions but not cell bodies in the VL are innervated by terminals containing 5-HT-IR. Thirdly, repetitive or long exposures to 5-HT do not lead to a clear long-term facilitation. We propose that in the octopus VL, while the basic facilitatory properties of molluscan 5-HT system are conserved, the system has adapted to convey signals from other brain areas to reinforce the activity-dependent associations at specific sites in the large connections matrix in the VL. © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

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**Abbreviations:** ASW, artificial seawater; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; DAB, 3, 3'-diaminobenzidine tetrachloride; DMSO, dimethyl sulfoxide; EA, egg albumin; EM, electron microscopy; fPSP, synaptic field potential; HF, high frequency; IBMX, 3-isobutyl-1-methylxanthine; LM, light microscopy; LTF, long-term facilitation; LTP, long-term potentiation; MSF, median superior frontal lobe; NGS, normal goat serum; NMDA, N-methyl-D-aspartic acid; PPF, paired pulse facilitation; RT, room temperature; TP, tract potential; VL, vertical lobe; 5-HT-IR, 5-HT, immunoreactivity.

**Key words:** learning and memory, synaptic plasticity, reward signal, mollusk, cephalopods.

Octopuses possess the largest nervous system of all invertebrates, containing ~500 million neurons, a number similar to that in advanced mammals (e.g. dogs, cats, see Hochner, 2008). This highly developed nervous system supports the complex behavioral learning and memory repertoire of the octopus that has been extensively described and studied for over a century (Sanders, 1975; Wells, 1978; Fiorito and Scotto, 1992; Hanlon and Messenger, 1996; Hochner et al., 2006; Hochner, 2008). Yet the organization of this large nervous system is relatively simple, both anatomically and functionally (Budelman, 1995; Flash and Hochner, 2005). Octopuses and other modern cephalopods are therefore favorable animals for investigating the neural organization and cellular processes involved in the function and evolution of “complex brains.” Recent progress in our laboratory has allowed us, for the first time, to use electrophysiological approaches to study the structures related to learning and memory in the octopus brain.

Based on behavioral, anatomical and lesion experiments, mainly in *Octopus vulgaris*, J.Z. Young, M.J. Wells and their colleagues suggested that several lobes in the supraesophageal ganglia of the octopus brain are dedicated to learning and memory (Sanders, 1975; Wells, 1978; Young, 1995). The median superior frontal (MSF) and vertical (VL) lobes are mainly involved in visual and tactile learning (Young, 1991) and show a rare matrix-like organization of the connections between the input (MSF) and output (VL) lobes (Young, 1971). This matrix of exceptionally large *en passant* connections (1.8 million input axons from the MSF onto 25 million VL amacrine interneurons; Young, 1971) appears analogous to vertebrate brain structures involved in learning and memory, like the hippocampus and cerebellum, as well as to the insect mushroom bodies (Young and Boycott, 1955; Young, 1991; Hochner et al., 2006; Hochner, 2010).

Using our MSF-VL slice and isolated brain preparations we have demonstrated a robust long-term potentiation (LTP) of the glutamatergic synaptic inputs from the MSF to the amacrine interneurons. Although this LTP is most likely N-methyl-D-aspartic acid (NMDA)-independent, at least some of these connections possess the Hebbian characteristics considered important for associative learning (Hochner et al., 2003, 2006). Importantly, experiments combining physiological and behavioral techniques have

shown that the VL can modulate short-term learning occurring outside the VL and that long-term acquisition of new memories depends on the VL and its LTP mechanism (Shomrat et al., 2008).

To advance our understanding of the VL as a learning neural network we have started to explore the role of neuromodulators in the function of the VL system. In addition to activity-dependent processes, neuromodulation is thought to be a fundamental and crucial mechanism in learning and memory networks. Here we have examined the possible involvement of serotonin (5-HT) in short- and long-term modulation of synaptic connections in the VL system. 5-HT is of special interest because it has been intensively researched in *Aplysia* and other gastropods and its functions as a neuromodulator in mollusks have been thoroughly documented. Especially in *Aplysia*, there is good evidence that 5-HT plays a role in short- and long-term neural modulation involved in the simple forms of short- and long-term memory that this animal demonstrates behaviorally (Byrne and Kandel, 1996; Kandel, 2001; Cai et al., 2008). There are also indications for the presence of 5-HT in the cephalopod brain, including in the octopus VL (Juorio, 1971; Tansey, 1979; Capasso et al., 1991; Messenger, 1996). In addition, biochemical experiments suggest cAMP as second messenger in the biochemical cascade mediated by 5-HT in the octopus optic lobes (Capasso et al., 1991). Here we investigate whether the forms of 5-HT-dependent modulation found in simple forms of molluscan learning and memory have been conserved during evolution of the advanced cephalopod brain and whether these play a role in areas subserving the complex octopus behaviors.

We show here that the VL system is innervated by fibers staining immunopositive for 5-HT. Similar to its effects on the sensory-motor synapses in *Aplysia* we show that 5-HT is a powerful excitatory neuromodulator of the synapses in the VL that undergo activity-dependent LTP. However, the effective 5-HT concentration is higher than at the *Aplysia* sensory-motor synapses, and repeated or long exposures to serotonin do not lead to a clearly measurable long-term facilitation as in *Aplysia*. Instead, 5-HT facilitates induction of activity-dependent LTP and thus may play a role in transmitting reinforcement signals to the neural structures involved in the acquisition of memory.

## EXPERIMENTAL PROCEDURES

### Animals

Specimens of *Octopus vulgaris* collected by local fishermen were held in individual aquaria in synthetic seawater (Aquarium Systems) as previously reported (Matzner et al., 2000). There are no specific ethical regulations relating to experimental work with octopuses in Israel. Our research using octopuses conforms to the ethical principles of Replacement, Reduction, Refinement and minimization of suffering.

### Slice preparation

The animals were deeply anesthetized in synthetic seawater (Instant Ocean) supplemented with 55 mM  $MgCl_2$  and 1% ethanol (Shomrat et al., 2008). The suprasophgeal mass was removed through a dorsal opening made in the cranium (Young, 1971). The brain was glued to a vibratome stage with acrylic glue and supported underneath and at the back by agar blocks, and the tough fibrous sheath was removed. The vibratome bath was filled with a 70:30% mixture of artificial seawater (ASW) and isotonic solution of  $MgCl_2$ , respectively, at 4–8 °C. Sagittal slices (400  $\mu m$ ) were cut and maintained at 15 °C in culture medium (L15 Gibco with L-glutamine, adjusted to seawater salt concentration). We used the slices for no longer than 3 days after preparation. During this time no substantial differences in their function could be detected. ASW composition was (in mM): NaCl, 460; KCL, 10;  $MgCl_2$ , 55;  $CaCl_2$ , 11; glucose, 10; HEPES, 10; pH 7.6. Physiological experiments started 1.5 h after slice preparation. A similar procedure, without slicing, was employed in experiments on the isolated suprasophgeal part of the brain (see Shomrat et al., 2008).

### Physiological experiments

The experimental chamber was continuously perfused with oxygenated ASW at room temperature (RT) at a rate of ~3.5 ml/min (~1 vol change/min). Drugs and experimental solutions were introduced via the perfusion system and took ~1–3 min to reach the recording site. The MSF tract was stimulated with Teflon insulated 0.08 mm diameter bipolar stainless steel electrodes or with ASW filled glass microelectrodes. MSF tract stimulation consisted of 0.2 ms monopolar pulses generated by an isolation unit. DC-coupled extracellular field potentials were recorded by glass microelectrodes filled with ASW (2–3 M $\Omega$ ) and signals were low-pass filtered at 10K Hz.

### Drugs

Stock solution of 10 mM 5-HT (serotonin creatinine sulfate complex, all drugs are from Sigma unless otherwise indicated) in double distilled water was freshly made before each experiment. Stock solution of 3-isobutyl-1-methylxanthine (IBMX) was freshly prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the experimental solution was 0.1% and was found to have no physiological effects.

### Immunohistochemistry for light (LM) and electron microscopy (EM)

Vertical lobes were fixed by immersion overnight in 0.25% glutaraldehyde and 4% formaldehyde in ASW pH 7.4 at 4 °C. They were sectioned sagittally at 100  $\mu m$  on the vibratome. To remove endogenous peroxidases the sections were incubated for 20 min in 0.015%  $H_2O_2$  (in PBS) for EM and in 0.15%  $H_2O_2$  for LM. The sections were first cryoprotected in a solution of 15% sucrose and 5% glycerol in PBS for 20 min at RT and were then placed overnight in a solution of 30% sucrose and 10% glycerol in PBS at 4 °C. For freeze and thaw treatment the sections were first soaked in liquid nitrogen-cooled isopentane and then in liquid nitrogen 1 min each and thawed in PBS. Next, the sections were incubated for 1 h at RT in a blocking solution containing 5% normal goat serum (NGS), 1% egg albumin (EA), 0.5% glycine, 0.5% lysine, 0.13% sodium azide in PBS (for EM 0.03% and for LM 0.3% triton X-100 was added). They were then incubated overnight at RT in the primary antibody solution (rabbit anti 5-HT; 1:10,000–1:30,000; DiaSorin, Stillwater, MN, USA), 1% NGS, 0.5% BSA (for EM 0.03% and for LM 0.3% triton X-100 was added). After rinsing in PBS the sections were incubated for 3 h at RT in biotinylated

secondary antibody (1:600 goat anti rabbit, Vector Laboratories, USA). The sections were placed in avidin biotin peroxidase complex (ABC Elite, Vector Laboratories, USA) for 2.5 h. To stain the labeling, the sections were incubated in 3, 3'-diaminobenzidine tetrachloride (DAB) and 0.006%  $H_2O_2$  for 10 min. The reaction was stopped by rinsing with PBS. In several experiments the reaction product was intensified and substituted with silver/gold particles as described by Livneh et al. (Livneh et al., 2009). Control vibratome sections were processed as described above, except that the primary antibody was omitted, resulting in no specific staining.

For LM the sections were collected on SuperFrost Plus slides (Menzel Glaser, Germany). For EM, the sections were post-fixed with 1% osmic acid and 1.5% potassium ferricyanide in 0.1 M cacodylate buffer pH 7.4 for 1 h at RT, washed in buffer and then dehydrated in a graded ethanol series (30–100%) and embedded in increasing concentrations of Agar 100 resin (Agar Scientific, Essex, England). After curing in a 60 °C oven for 48 h, ultrathin sections were cut on an LKB-3 ultramicrotome, collected on thin bar 200 mesh cuprum grids and stained with saturated aqueous uranyl acetate and lead citrate solutions.

Ultrathin sections were observed with a Tecnai 12 TEM (Philips, Eindhoven, the Netherlands) equipped with MegaView II CCD camera and AnalySIS® version 3.0 software (SoftImaging System GmbH, Münster, Germany).

## RESULTS

### Immunohistochemistry

Previous studies have indicated the presence of 5-HT immunoreactivity (5-HT-IR) in the cephalopod VL (Boyer et al., 2007). Using light and electron microscopy we searched the VL and neighboring lobes for 5-HT-IR in cell bodies, fibers and synaptic terminals. Fig. 1A shows that areas rich in 5-HT-IR granules are the neuropile region in the VL below the MSF tract in the lower part of the cell body layer (cf. Fig. 1A1,4), and the MSF neuropile region (cf. Fig. 1A2,5). No 5-HT-IR was visible in cell bodies in the VL, in the MSF, nor in the outer cortex of cell bodies of the large and amacrine cells. Instead, 5-HT-IR was seen in cell bodies outside the MSF and VL, e.g., in the subvertical lobe (cf. Fig. 1A3,6).

Electron microscopy revealed relatively sparse but clear positive 5-HT-IR in neural terminals and synaptic varicosities. That is, some presynaptic terminals in the VL neuropile (Fig. 1B5,6) and MSF tract region (Fig. 1B3,4) contain 5-HT-IR. Fig. 1B1,2 show positive reactivity in the area of amacrine cells' neurites but not close to the region of the cell bodies. These findings suggest that the MSF-VL system is innervated by a widespread afferent system, typical for neuromodulatory innervation. Light microscopy has revealed similar findings in cuttlefish (Boyer et al., 2007).

### 5-HT has excitatory modulatory effects on VL synaptic field potential

Fig. 2A shows the effect of 5-HT on the field potential evoked by twin pulse stimulation of the MSF tract (see experimental arrangement in Fig. 2 inset). Rather commonly a synaptic field potential (fPSP) is not apparent after a single stimulus and it becomes visible only after twin (or even triple) pulse facilitation (e.g. Fig. 2A control). The

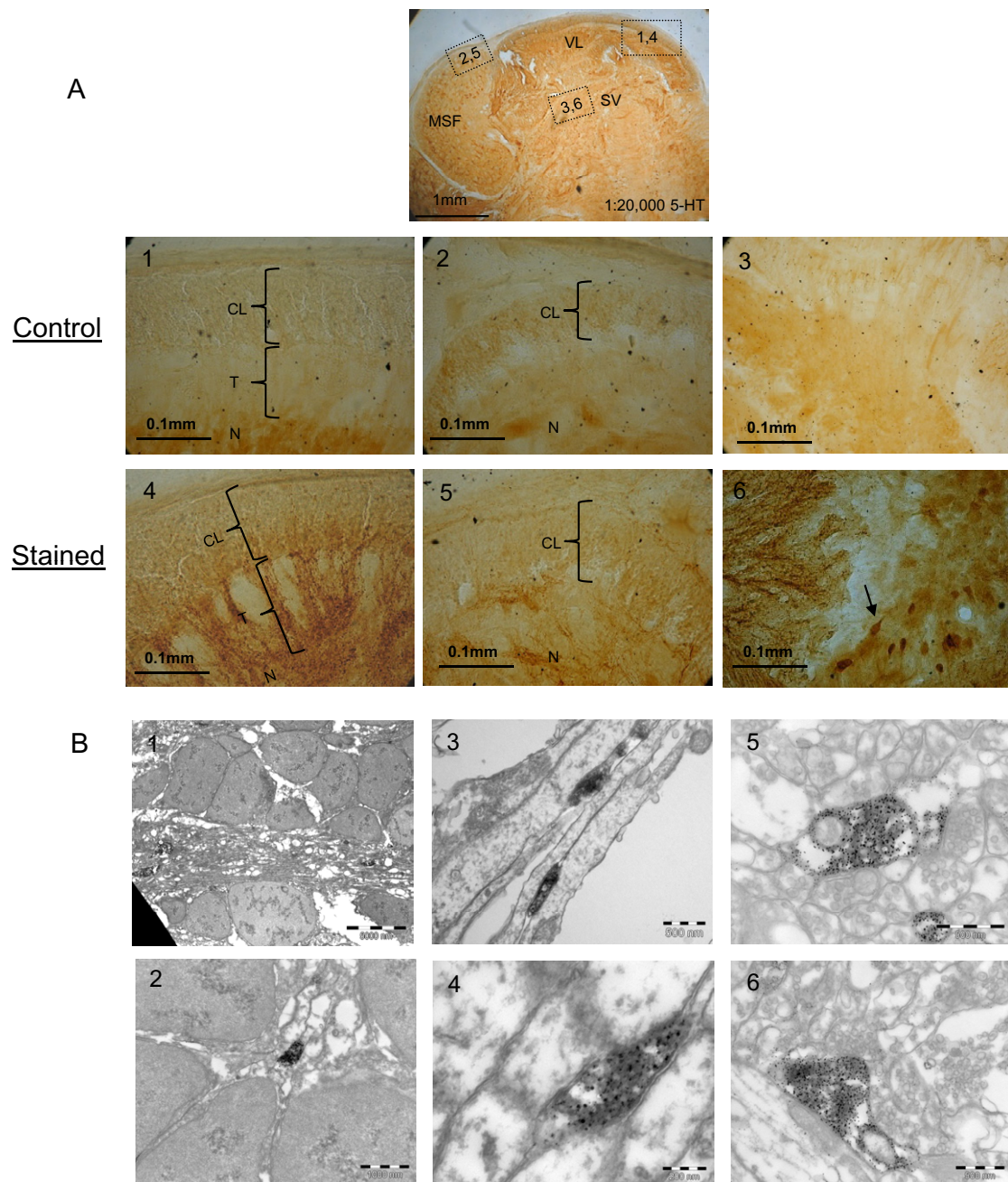
amplitude of the fPSP evoked by the first stimulus (even if zero) was measured every 10 or 20 s (Fig. 2B). Because the fPSP amplitude is almost linearly related to the tract potential (Hochner et al., 2003), the fPSP amplitude was normalized to the MSF tract potential (TP) to compensate for small changes in TP amplitude during the experiment and to reduce variability between experiments. Adding 100  $\mu$ M 5-HT after a 10 min control period (in which a constant average response was obtained in this set of experiments) caused a gradual increase in the fPSP amplitude, which reached an average steady-state increase of 3.57 times the control amplitude in about 15 min (Fig. 2B). On rinsing out the 5-HT, the fPSP amplitude slowly declined to a steady level significantly above the control amplitude (1.62-fold increase,  $P=0.002$ ). This latter increase suggests that either 5-HT has a persistent facilitatory effect on fPSP amplitude and/or that 5-HT enhances the activity-dependent induction of LTP by the test pulses via a short-term synaptic facilitation (STF). We differentiate between these two possibilities in the following experiments (Figs. 3 and 4).

The TP amplitude did not show consistent and reversible changes during the various stages of the experiment, also suggesting that stimulation efficacy was constant throughout the experiment. As there is an almost linear relationship between TP and fPSP amplitudes (Hochner et al., 2003), the 5-HT effect was not mediated by recruiting more MSF fibers due to changes in excitability. Moreover, preliminary work with enzymatically dissociated MSF neurons did not reveal significant 5-HT induced changes in the membrane properties of the MSF cells (not shown here). These whole-cell recording experiments employed cells that were isolated with part of their neurite (Hochner et al., 2006).

The threshold concentration of 5-HT for enhancing the fPSP in the *in vitro* slice preparation was 10–20  $\mu$ M 5-HT (Fig. 6). The maximum effect was obtained at 100  $\mu$ M 5-HT; 200  $\mu$ M 5-HT caused no further augmentation in several experiments. These doses are about one order of magnitude higher than those required in *Aplysia*, explaining our previous failure to detect significant effects of 5-HT with doses as for *Aplysia* (Hochner et al., 2003). We believe that these are the actual effective concentrations of 5-HT in the octopus VL and that this concentration does not cause nonspecific activation of other receptors. For example, responses to dopamine or octopamine require similar concentrations ranges (unpublished results and Greenwood et al., 2009). Moreover, *in vitro* experiments showed that 100  $\mu$ M octopamine or dopamine are needed to activate maximal production of cAMP in octopus optic lobe preparations (Capasso et al., 1991).

The records in Fig. 2A show that with paired pulse stimulation 5-HT facilitated both the first fPSP (absent in the first pulse in control) and the second fPSP. Nevertheless, the level of paired pulse facilitation (PPF), measured by the ratio of the second fPSP amplitude to the first, was reduced during the course of 5-HT-induced facilitation, increasing again on washout. (Note this limits PPF measurements to experiments where both the

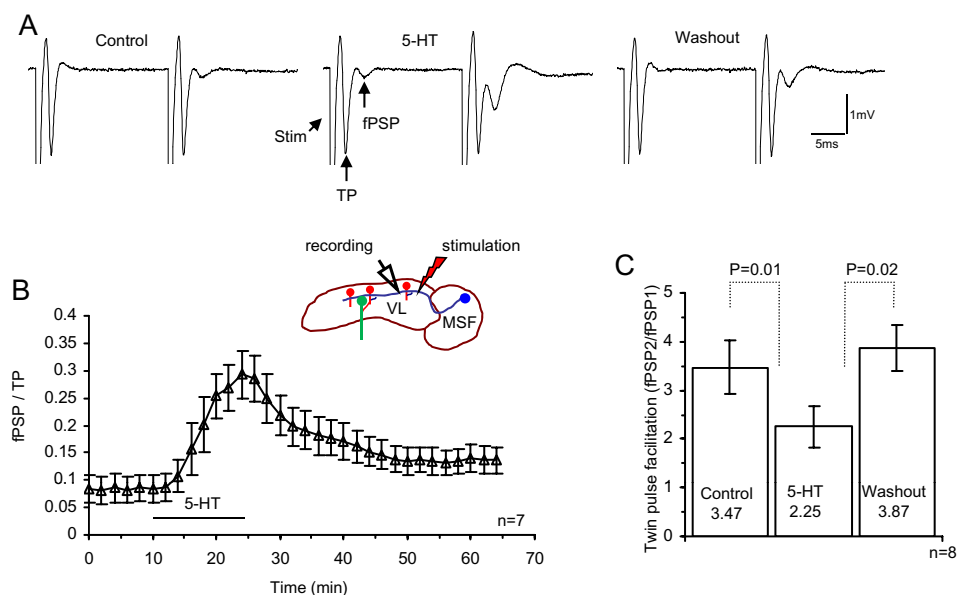




**Fig. 1.** 5-HT immunoreactivity (IR) in processes but not in cell bodies of the MSF and VL lobes. Inset. Low magnification of a sagittal section of the VL complex showing where the sections in Fig. A1–6 were taken. SV subvertical lobe. (A) Light micrographs of control (1–3) and stained (4–6) Sections 1 and 4, in VL area; 2 and 5 in the MSF area; 3 and 6, in the subvertical area. Note positively stained cell bodies (arrow) in six. CL-cell bodies layer; T-MSF tract; N-neuropile (B). Electron micrographs showing processes and terminals containing 5-HT-IR in an amacrine neurite bundle (cut longitudinally in (1) and transversally in (2)) but not close to cell bodies, in the MSF tracts (3, 4) and VL neuropile (5, 6).

first and second stimuli evoked an fPSP.) The histogram in Fig. 2C, summarizing 8 such measurements, shows that the reduction was significant and reversible. Such modifications suggest that at least some of the 5-HT effects are mediated by a presynaptic mechanism (Hochner et al., 1991). However, these modifications do not rule out additional postsynaptic mechanisms like those in the *Aplysia* sensory-motor synapse (Cai et al.,

2008). The fact that 5-HT, like LTP (Hochner et al., 2003), also modulates presynaptic facilitation implies that only the first evoked fPSP should be considered for the accurate quantification of the direct effects on synaptic transmission. Therefore, the results here are usually displayed as averages of the fPSP/TP ratio (including zeros) rather than the more conventional normalization relative to the control fPSP amplitude.



**Fig. 2.** 5-HT induced short-term synaptic facilitation. Inset: Scheme showing the slice and the experimental arrangement. Blue - MSF cell; red - amacrine interneuron; green - large efferent cell. (A) Records from one experiment. Responses to stimulation by twin pulses. Stim. stimulation artifact; TP tract potential; fPSP postsynaptic field potential. (B) Summary of seven experiments demonstrating the facilitatory effect of 100–200  $\mu$ M 5-HT and its reversal on washout. Test stimuli were applied every 10 or 20 s (C) 5-HT reversibly reduced the level of paired-pulse facilitation measured as the ratio of the second to the first fPSP amplitudes. Unlike the example shown in (A), the histogram in (C) includes only experiments in which the first fPSP was apparent.

### 5-HT has mainly a short-term facilitatory effect, and does not induce clear long-term facilitation (LTF)

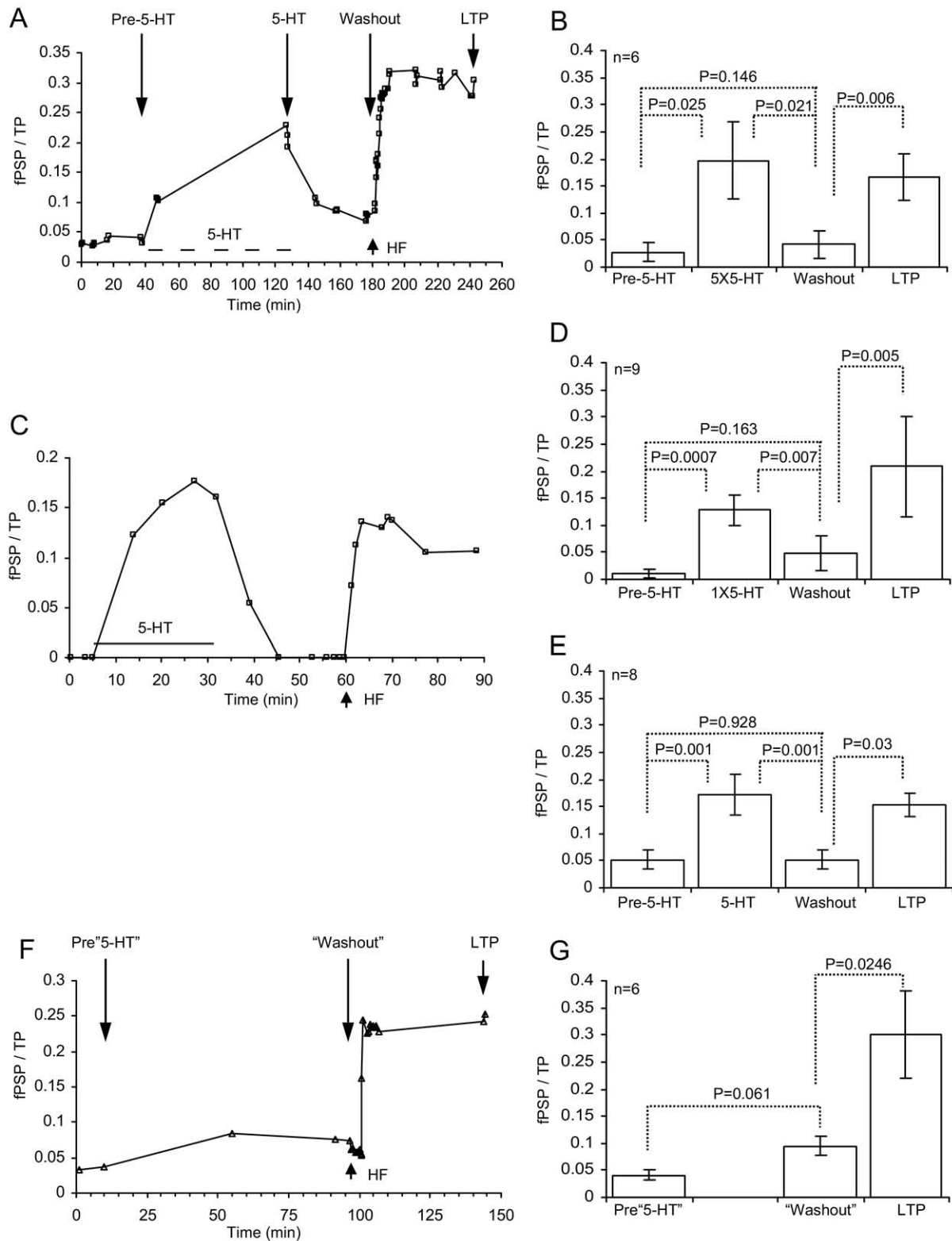
To examine whether the sustained facilitation shown in Fig 2B was the expression of a long-term 5-HT induced facilitation, as in *Aplysia* sensory-motor synapses, we used the *Aplysia* protocol for inducing LTF (five consecutive exposures of the VL slice to 5-HT (Montarolo et al., 1986; Mauelshagen et al., 1998). As shown in Fig. 3A, C, F, in each experiment we used a minimal number of test measurements to minimize the possible contribution of activity-dependent modulation. After a control period (Fig. 3A) the slices were exposed to 100  $\mu$ M 5-HT for five 10–15 min periods; separated by 15–20 min washouts. As seen in Fig. 3A, the fPSP was acutely facilitated by the 5-HT applications, but it declined during the long rinsing period. After rinsing, a high frequency (HF) stimulation protocol (four trains of 20 pulses at 50 Hz; 10 s inter-train interval) activated a robust LTP. Fig. 3B, summarizing six such experiments, shows that the average 7.19 times facilitation of the fPSP (relative to before (Pre) 5-HT application) obtained in the fifth exposure to 5-HT, declined to 1.53-fold after washout. As shown in the histogram (Fig. 3B), this average increase was not significantly different from the pre-5-HT level ( $P=0.146$ ), and indeed, HF stimulation given after 5-HT washout, induced the average facilitation 6.09-fold. Similar results were obtained after one long exposure to 5-HT (Fig. 3C, D).

Many of the MSF tract axons in the slice preparation may have lost their cell bodies. We thus repeated the experiment in the isolated brain preparation in case the 5-HT effect is mediated via transcriptional mechanisms

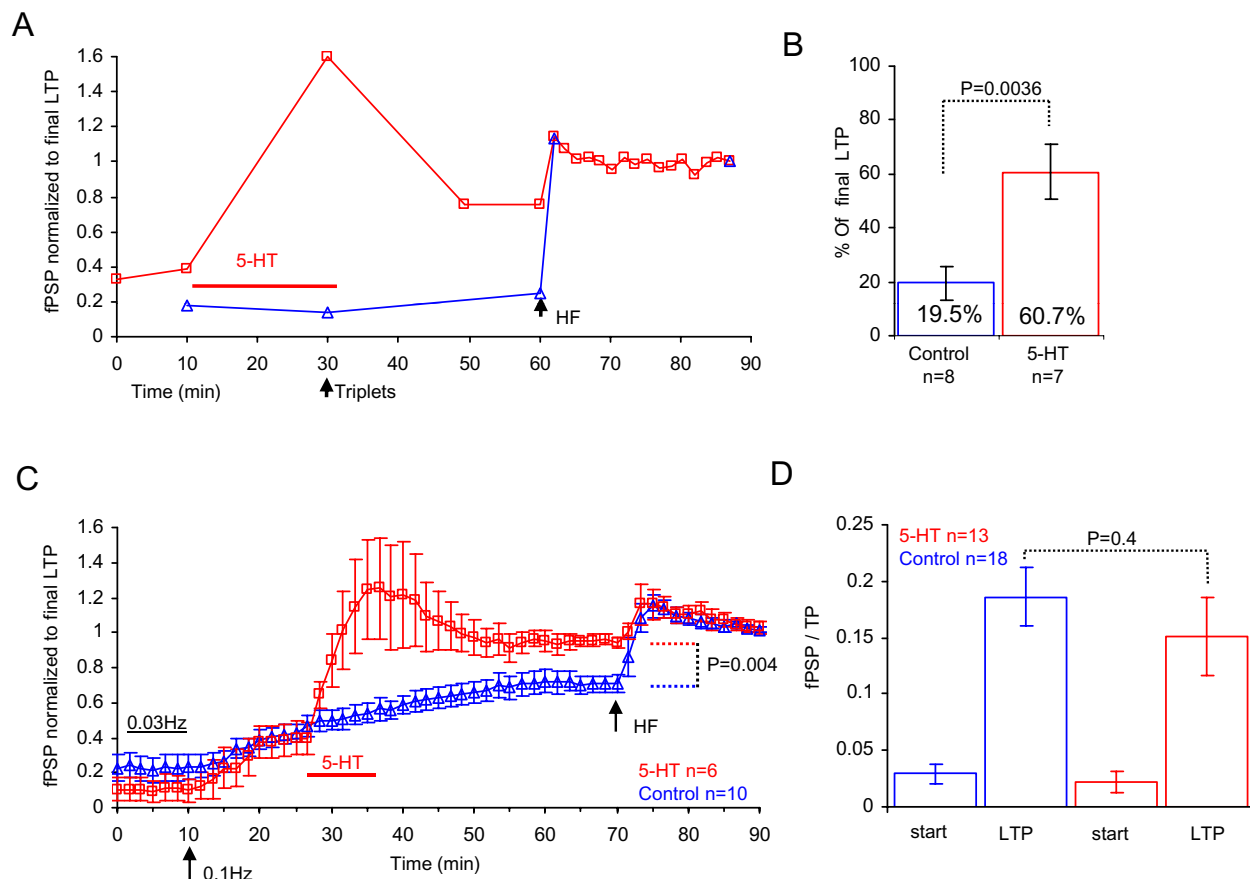
requiring the cell bodies of the MSF neurons. The results (Fig. 3E) again suggest that five short or one long 5-HT exposure do not activate long-term facilitation even in the intact MSF-VL system.

The facilitation seen after washing out 5-HT, although not statistically significant in the individual experiments (Fig. 3B, D, E, G; Pre-5-HT vs. Washout), was significant when they were pooled ( $n=29$ ,  $P=0.001$ ). We believe that this facilitation is mainly due to activity-dependent facilitation induced by the test pulses. This “unavoidable” long-term slow facilitation is elicited by even the minimal number of test stimuli used in the control experiments without 5-HT (Fig. 3F, G). That the facilitation after washout (“washout” in Fig. 3G) was not due to a long-term effect of 5-HT is demonstrated by the lack of significant difference between the “washout” level ( $\text{fPSP/TP}=0.094\pm0.0172$ ) in the control experiments (Fig. 3G) and that after 5-HT washouts (Fig. 3B, D, E,  $P=0.11, 0.29, 0.11$ , respectively). Thus, in these experiments, and in contrast to those shown in Fig. 4 below, 5-HT showed no significant long-term effect. Nevertheless, this “unavoidable” long-term low facilitation may possibly mask small 5-HT dependent long-term modulations.

More than one independent recording/stimulating site could be tested in a single slice or intact brain preparations during the same or consecutive days. These sites all showed a normal level of LTP or 5-HT facilitation after the preparation had been exposed to 5-HT five times or for one long exposure. This provides additional and independent evidence that 5-HT does not elicit long-term effects.



**Fig. 3.** Long-term exposure to 5-HT had a mainly short-term facilitatory effect on fPSP amplitude. (A) An experiment testing the effect of five short exposures to 5-HT (10–15 min) and subsequent susceptibility to LTP induction (HF). The points where measurements were taken for the histogram in B are indicated. (B) Summary of six experiments as in A. (C, D) are similar to (A, B), respectively, but with one long exposure to 5-HT (30–60 min). (E) Summary of similar experiments on isolated brain preparations. (F, G) Control for 5-HT-independent long-term effects. (F, G) similar to (A, B), respectively, but without exposure to 5-HT. The points where measurements were taken for the histogram in (G) are indicated in (F).



**Fig. 4.** 5-HT reinforced activity-dependent LTP induction. (A) Partial LTP was induced by the triplet pulse stimulation protocol 5-HT (red trace, open squares) caused large short-term facilitation (measuring the first fPSP in the triplet at ~30 min). After 30 min washout (60 min), high frequency (HF) stimulation revealed the residual LTP. HF stimulation gave rise to greater facilitation of the fPSPs in the control experiments (blue trace, open triangles), indicating less recruitment of LTP than in the presence of 5-HT. (B) Summary histogram showing the percentage of the final LTP measured at the end of the experiments exemplified in (A). LTP was induced by the triplet stimulation protocol with and without 5-HT. (C) Activity-dependent gradual development of LTP ("slow facilitation") was accelerated by 5-HT. In the control experiments (blue triangles), increasing the rate of test pulses from 0.03 to 0.1 Hz (at 10 min) induced gradual facilitation. At 70 min, the HF protocol revealed the residual LTP and the slow facilitation disappeared. Exposure to 5-HT (red squares) accelerated the slow facilitation and a saturated level of LTP was attained; there was no further LTP following the HF protocol. (D) Histogram summarizing the experiments in (B, C), showing that there were no significant differences between the control and 5-HT groups, neither in the amplitudes (normalized to the tract potential) of the start fPSPs, nor in the fPSPs amplitudes after LTP induction.

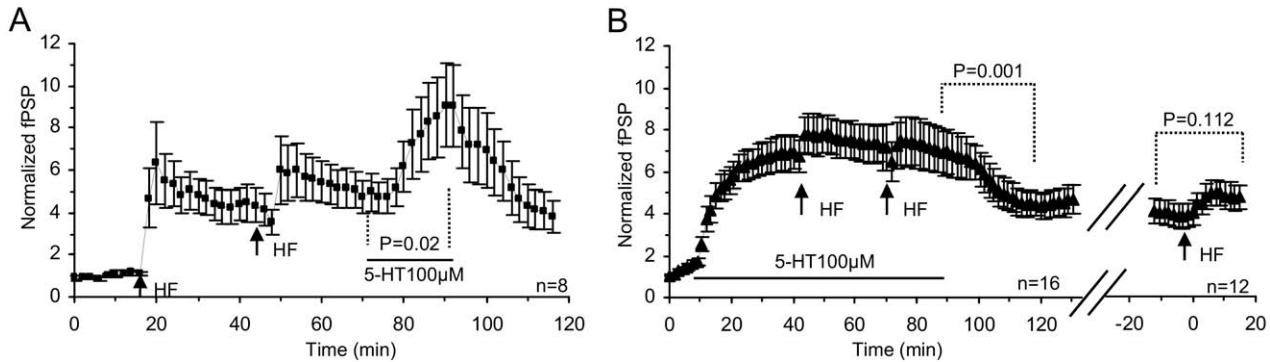
### 5-HT reinforces activity dependent LTP induction

Although 5-HT did not show robust long-term modulatory effects on synaptic connections, it could still modulate LTP induction, for example, indirectly via its short-term facilitatory effect, especially if LTP induction depends on the magnitude of the fPSP (Hochner et al., 2003). We therefore devised a protocol to induce partial LTP to test whether 5-HT could amplify LTP induction. The partial LTP was induced using four trains of three pulses (triplets at 50 Hz) instead of the 20 pulses in the normal protocol (Fig. 4A). As in the experiments in Fig 3, we used only a few test pulses in order to minimize long-term effects (Fig. 4A). Triplet stimulation was applied at the end of a 20 min exposure to 5-HT (Fig. 4A; red, open squares) and was compared to controls without 5-HT (Fig. 4A; blue, open triangles). Changes in test fPSP amplitude were expressed relative to the final LTP (measured at 90 min) that was induced by standard HF stimulation after 30 min washout of 5-HT (at 60 min). By using this normalization

method it was possible to include and quantify experiments in which the fPSP evoked by the first stimulus was undetectable at the beginning and to quantify the triplet-induced LTP as a fraction of the final LTP. The level of stable enhancement attained by the triplet protocol with and without 5-HT was checked after a 30 min washout of 5-HT (at 60 min).

fPSP amplitude after the four triplet stimuli in 5-HT and the 30 min washout showed a higher level of long-term facilitation than in the control (Fig. 4A). A summary of eight control and 7 5-HT experiments is shown in the histogram in Fig. 4B. While the control triplets induced only  $19.5 \pm 6.2\%$  of the final LTP, triplets given in the presence of 5-HT induced  $60.7 \pm 10.2\%$  of the total LTP facilitation. The significant differences ( $P=0.0036$ ) demonstrate that the partial LTP induced by the triplet stimulation protocol was highly facilitated by 5-HT, most likely because of the robust short-term facilitation of the triplet fPSPs (Fig. 4A).





**Fig. 5.** 5-HT and LTP do not share common facilitation mechanisms. (A) Saturated level of LTP was induced by two consecutive HF protocols. Adding 100  $\mu$ M 5-HT caused additional and reversible facilitation. (B) 5-HT together with the test pulses caused maximal facilitation. The two HF stimulations show LTP saturation. Washout of 5-HT demonstrated the reversibility of its effect. A third HF showed only a marginal non-significant residual LTP (the experiments are aligned at the time of the last HF).

The fPSP amplitude could increase slowly during the test stimulation, especially when the fPSP was relatively large. As the fPSPs induced by a single stimulus were often very small or absent and became apparent only after twin or even triplet stimuli (see Fig. 2A), we developed a stimulation protocol to induce this slow facilitation by facilitating the fPSPs (Fig. 4C). A slow and asymptotic increase in fPSP amplitude was evident when the frequency of the test pulses was increased (from 0.03 to 0.1 Hz, see Fig. 4C) and when twin pulses at 50 Hz were used instead of a single test pulse (or three pulses in the few cases where the fPSP was absent even after the second test pulse). This slow increase was due to a gradual induction of LTP, as HF-induced LTP summated with the slow facilitation to give the saturated level of LTP (Fig. 4C, blue open triangles). Moreover, slow facilitation disappeared following HF LTP induction. This result independently supports the suggestion that slow facilitation and LTP are both driven by the same activity-dependent processes.

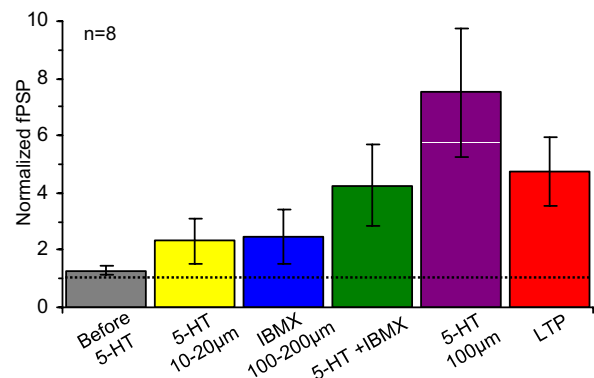
Fig. 4C shows the modulatory effect of 5-HT on the slow facilitation. As in Fig. 4A, B, the test fPSP amplitude was expressed relative to the final LTP obtained after HF stimulation at 70 min. Exposure of the preparations to 100  $\mu$ M 5-HT for 10 min enhanced the rate of slow facilitation (open red squares). This reached a steady-state level of LTP significantly higher than the control experiments (at 70 min,  $P=0.004$ ; open blue triangles). Moreover, after rinsing out 5-HT the LTP was saturated, as HF stimulation induced only a short-term facilitation. In contrast, HF in the control experiments induced an additional 1.5 times increase in the average fPSP (Fig. 4C). These results, together with the lack of a long-term effect of 5-HT (Fig. 3), suggest that the STF effect of 5-HT enhances the activity-dependent slow LTP development as it affects HF-induced LTP (Fig. 4A, B). Therefore our results (Figs. 3 and 4) indicate that the 5-HT-induced STF indirectly accelerates or reinforces the LTP induction. Thus, the sustained level of enhancement shown in Fig. 2B is explained by an STF of the test pulses during 5-HT exposure.

This reinforcement effect of 5-HT on LTP induction did not affect the final level of LTP. Fig. 4D summarizes the experiments in which 5-HT was present during LTP induc-

tion by triplet stimulation or slow facilitation (Fig. 4B, C). The histogram shows that there were no significant differences between the control and 5-HT groups, neither in the amplitudes of the initial fPSPs (normalized to the tract potential), nor in the fPSP amplitudes after LTP induction. The average final LTP of 7.029-fold in the 5-HT experiments did not differ from the control value (6.38-fold).

#### 5-HT and activity-dependent potentiation do not share a common mechanism for facilitation

To determine whether the facilitatory effects of 5-HT and activity-dependent LTP induction are based on the same mechanisms we tested whether LTP occluded the 5-HT effect and *vice versa*. To better detect small changes in fPSPs in this set of experiments (Figs. 5 and 6) we measured the amplitude of the first apparent fPSP (e.g. the second fPSP in Fig. 2A control). To check for occlusion we first induced a saturated level of LTP. This was shown by marginal additional long-term fPSP enhancement after a second series of HF that caused only short-term facilitation (Fig. 5A). Then, 100  $\mu$ M 5-HT was given and a further



**Fig. 6.** Phosphodiesterase inhibitor IBMX did not enhance 5-HT-induced facilitation. Summary of experiments testing the effect of low doses of 5-HT (10–20  $\mu$ M; yellow bar, n=7) and IBMX (100–200  $\mu$ M; blue bar), their additive effect (green bar), a high dose of 5-HT (100  $\mu$ M, purple bar) and HF-induced LTP at the end of experiment (red bar). In each of the eight experiments these tests were carried out in the same sequence separated by washouts.



facilitation was induced. This additional enhancement disappeared on washout with ASW. Fig. 5A summarizes eight such experiments. While the saturated LTP level was  $4.74 \pm 0.67$  times the control fPSP amplitude, addition of 5-HT induced additional facilitation reaching  $9.06 \pm 1.9$  times the control value. This result suggests that at least some of the processes involved in mediating 5-HT effects differ from those mediating LTP induction.

The complementary experiments (Fig. 5B) show a similar separation of processes. In these experiments the responses to test pulses given every 10 s together with 100  $\mu$ M 5-HT slowly reached saturated level, as two HF had no further effect ( $7.14 \pm 0.93$  times the control value). However when 5-HT was rinsed away, the facilitation declined to a level similar to that obtained by LTP alone ( $4.16 \pm 0.54$ , Fig. 5A) and the third HF protocol led to only small and insignificant enhancement (Fig. 5B).

Completely independent processes would be predicted to result in a multiplicative augmentation of the fPSP amplitude between 16 and 20-fold. However, a sub-linear summation of fPSP amplitude is to be expected in the extracellular recording configuration because of the asymptotic reduction in fPSP driving force, especially with such robust facilitation (Hochner et al., 2003). This biophysical constraint may attenuate the relative effect of 5-HT when superimposed on LTP and *vice versa* (Fig 5B vs. 5A).

#### Cyclic nucleotides are involved in mediation/regulation of LTP induction but not in the 5-HT facilitatory effect

The aim of the following experiments was not to decipher the cellular mechanisms of LTP induction and 5-HT effects but, rather, to determine whether the reinforcement interaction involves separate or shared cellular mechanisms. A common mechanism for mediation of the effects of 5-HT in mollusks is via the adenylyl cyclase—cAMP and phospholipase C (PLC) diacylglycerol pathways (Ghirardi et al., 1992; Kandel, 2001). Phosphodiesterase inhibitors such as IBMX are useful for testing the involvement of cyclic nucleotides like cAMP and cyclic guanosine monophosphate (cGMP). Inhibition of phosphodiesterase accelerates processes dependent on cyclic nucleotides, as phosphodiesterase slows cAMP or cGMP breakdown, thereby activating protein kinases regulated by cyclic nucleotides.

IBMX itself caused substantial facilitation in our preparation. The histogram in Fig. 6 summarizes the multiplicative effects of low doses of 5-HT and IBMX. 5-HT (10–20  $\mu$ M) caused moderate facilitation ( $2.32 \pm 0.78$ , yellow bar). The 5-HT effect was noticeable in only three of seven experiments where threshold values of 5-HT were tested (10–20  $\mu$ M). Concentrations of 100–200  $\mu$ M IBMX elicited a similar facilitation in four of eight experiments ( $2.47 \pm 0.95$ , blue bar). Giving the two drugs together led to a facilitation of only  $4.27 \pm 1.39$  (green bar) and thus no robust synergistic effect was evident. Postsynaptic saturation may have attenuated, but would not have masked, a synergistic interaction, as a higher dose of 100  $\mu$ M 5-HT led to  $7.51 \pm 2.23$  facilitation (Fig. 6 purple bar).

Forskolin, a direct cyclase activator in sensory cells of *Aplysia* (Bacskai et al., 1993), had no effect on the fPSP, yet we cannot rule out that the type of cyclase in the VL is forskolin-insensitive (Yan et al., 1998). Similarly, several cAMP activators had no effect on the VL field potentials (not shown). In contrast, phorbol ester (5  $\mu$ M PDBU), an activator of c-kinases, increased fPSP amplitude about 7-fold, similar to the combined effects of LTP and 5-HT (unpublished results). It therefore seems unlikely that cAMP is involved in the mediation of the facilitatory effect of 5-HT.

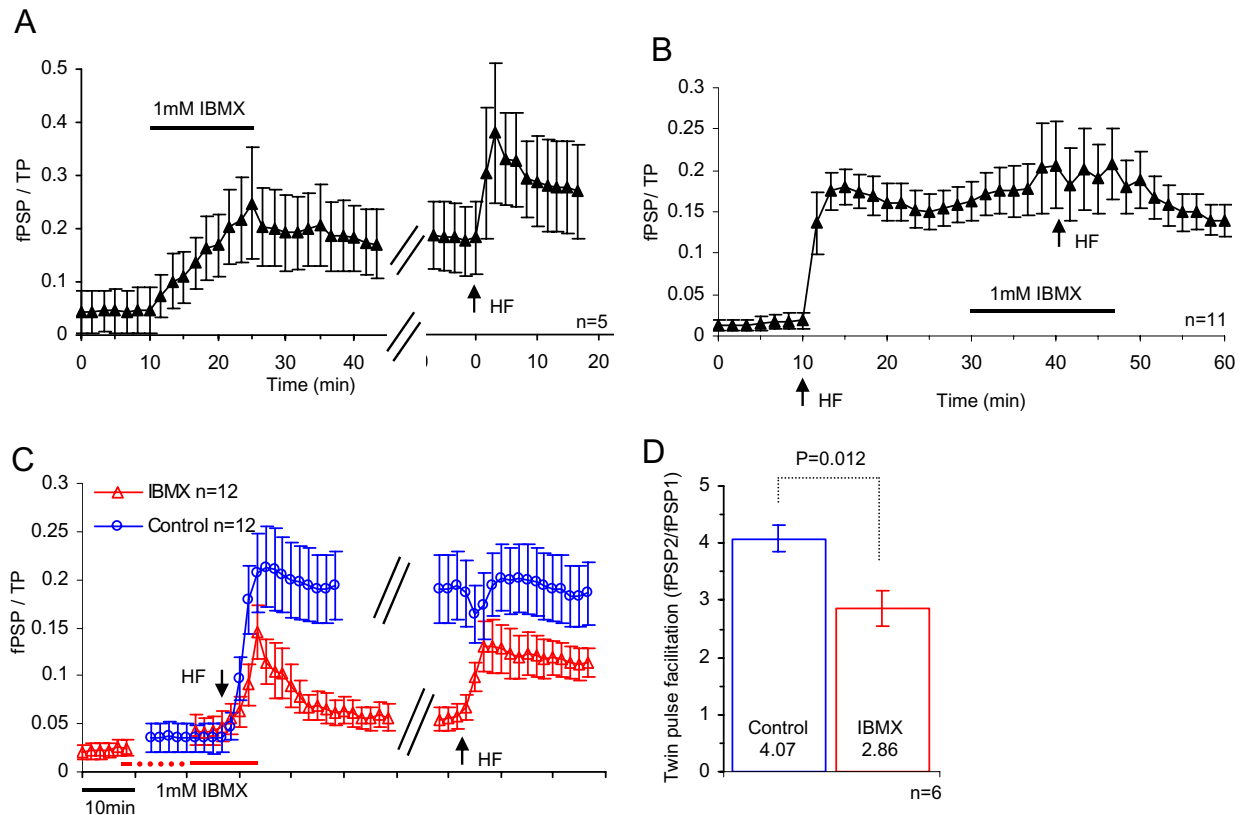
IBMX affected LTP induction mechanisms. Fig. 7A shows that 1 mM IBMX caused a gradual facilitation of the fPSP. This facilitation stopped after IBMX washout yet did not decline significantly. Instead, it remained at a steady level as typical for the activity-dependent LTP. Thus, IBMX may directly activate or facilitate the mechanisms inducing activity-dependent LTP. Indeed, the HF protocol given after the washout led to a transient facilitation that subsided to a small additional residual LTP (Fig. 7A). The experiments in Fig. 7B show that IBMX had a very little effect when added after induction of LTP. They thus support the conclusion that the facilitatory effect of IBMX is mediated by a process that also induces activity-dependent LTP. This lack of superimposition of IBMX effect and LTP contrasts sharply to 5-HT facilitation adding to LTP (Fig. 5A) and, thus, further indicates that different mechanisms are responsible for LTP and 5-HT facilitation.

Surprisingly, HF stimulation in the presence of IBMX led to only a transient facilitation that decreased to the pre-HF stimulation level on washout (Fig. 7C). Long washout of IBMX allowed recovery, at least partially, of the HF-induced LTP (Fig. 7C). Only the combination of HF stimulation with IBMX caused transient facilitation without LTP, while repetitive or stronger HF stimulation (with or without 5-HT) led to a saturated and stable LTP. This difference suggests a specific mechanism, in which the combination of intense activity-dependent processes with a higher level of cyclic nucleotides blocks LTP induction.

Unlike a classic LTD process, the combination of IBMX and HF stimulation did not cause “depotentialization” of already potentiated synapses. In Fig. 7B, 1 mM IBMX superimposed on LTP caused only marginal and insignificant facilitation. HF stimulation in the presence of IBMX (Fig. 7B) did not induce any consistent effect. Yet some reduction to pre-IBMX level is seen after IBMX washout. This result suggests that the combined effect of IBMX and activity is effective only during induction of LTP (Fig. 7C).

The bimodal effect of IBMX on activity-induced LTP—facilitation at a lower level of activity and suppression at HF—resembles the dual effect suggested for  $\text{Ca}^{2+}$  in mediating LTD and LTP depending on its intracellular concentration (Lisman, 1989). In the VL, too, the interaction of an activity-induced messenger (e.g.,  $\text{Ca}^{2+}$ ) with elevated levels of cyclic nucleotides may explain such bimodal effects.

Like 5-HT (Fig. 2C), the histogram in Fig. 7D, summarizing six experiments, shows that IBMX-induced enhancement was associated with a significant reduction in the paired-pulse facilitation ratio. Such modifications suggest



**Fig. 7.** Phosphodiesterase inhibitor IBMX facilitated slow facilitation but suppressed HF induction of LTP. (A) IBMX facilitated fPSP amplitude which remained enhanced after IBMX washout. A second HF exposed the residual LTP. (B) Effect of IBMX superimposed on LTP. The second HF in the presence of IBMX caused no consistent effect. After rinsing, the fPSP decreased to the level established by the first HF. (C) Exposure to IBMX for 10–20 min followed by an HF protocol induced synaptic facilitation that declined after IBMX washout. Note that in the presence of IBMX, HF caused only short-term facilitation. A second HF tested for residual LTP which was larger in the IBMX-treated preparations. The graphs are aligned at the times of HF. (D) IBMX reduced the level of paired-pulse facilitation. The histogram includes only experiments in which the first fPSP was apparent.

that like LTP (Hochner et al., 2003) and 5-HT (Fig. 2C), at least some of the IBMX effects are mediated by presynaptic mechanisms.

## DISCUSSION

Our findings show that the neuromodulator 5-HT reinforces activity-dependent long-term plasticity. This result supports both biological and theoretical findings emphasizing the importance of the mutual interactions between homosynaptic activity-dependent plasticity and heterosynaptic neuromodulation in both vertebrate and invertebrate learning systems (Bailey et al., 2000b; Swinehart and Abbott, 2005; Daw and Doya, 2006; Giurfa, 2006; Schroll et al., 2006; Unoki et al., 2006).

Even though serotonergic functions in molluscs may differ in the same group of neurons across different species (Marinesco et al., 2003), the basic overall effects of 5-HT in a learning neural network of octopus appear similar to its effects in learning in other mollusks, even in more fundamental learning systems such as withdrawal reflexes. Our results thus support evolutionary conservation of 5-HT-dependent plasticity in complex learning networks.

However, there are species differences in the details. An inter-species comparison suggests that the 5-HT sys-

tem in the octopus VL (as possibly in other systems) has acquired several properties, which may have important functional implications for the special function of the VL system as an advanced learning neural network.

The first functional difference shown here is that, in the octopus, 5-HT has mainly short-term modulatory effects, while in *Aplysia*, in addition to the short-term presynaptic facilitation, repeated exposures simulating a repeated noxious stimulus lead to long-term synaptic enhancement (Montarolo et al., 1986; Dash et al., 1990; Mauelshagen et al., 1998; Cai et al., 2008). This suggests that, in the octopus VL, 5-HT has either lost or has not achieved the capacity to directly induce long-term synaptic modifications. This finding is interesting in light of the immunohistochemical results demonstrating the absence of direct 5-HT innervation of cell bodies in contrast to *Aplysia* sensory neurons (Zhang et al., 1991), where 5-HT mediates long-term facilitation involving regulation of gene expression (Dash et al., 1990; Bacskai et al., 1993). It is possible that long-term plasticity in the whole cell is important for long-term adaptation of reflexive behaviors. In contrast, such global modifications may be less useful in the matrix-like organization of learning neural networks like that of the VL. Here, modifications of specific connections may be

important. Such branch specific effect have been demonstrated in cultured sensory-motor synapses, where local application of 5-HT paired with activity led to branch-specific long-term facilitation (Bailey et al., 2000a).

The inability of 5-HT to induce long-term synaptic facilitation also suggests that LTP in the VL is not induced solely via 5-HT release evoked by the HF stimulation. This does not rule out, however, that 5-HT released during HF stimulation participates in the activity-dependent induction of LTP. Increase in the extracellular concentration of modulators as a result of high frequency stimulation has been demonstrated in the hippocampus (Neugebauer et al., 2009). We have so far not succeeded in testing this idea in the octopus VL, because the inhibitors of 5-HT receptors in mollusks that we have tested failed to block the effect of 5-HT in the octopus (e.g. Methiothepin mesylate salt, Sigma; Methysergide maleate salt solid, Sigma, Cyproheptadine hydrochloride, Tocris). 5-HT antagonists in marine mollusks are not readily available; they differ from those of vertebrates and their efficiency varies in different molluscan preparations and species (Dumitriu et al., 2006). In addition, the relatively high effective concentrations of 5-HT needed in the octopus VL may call for higher doses of antagonists that are technically and economically difficult to achieve.

Bailey et al. (2000b) have suggested that both in mammals and mollusks homosynaptic processes alone cannot lead to a stable long-term modification in synaptic strength. In contrast, heterosynaptic modulatory processes have long-term effects. Thus, it is the co-activation of both homo- and heterosynaptic processes that leads to a stable and long-lasting strengthening of synaptic connections. The interaction found here in the octopus VL system shows such a mutually reinforcing interaction. As pointed out earlier in the Discussion, we cannot, however, rule out the possibility that 5-HT or other modulators participate in this process. Nevertheless, it is feasible that 5-HT reinforces LTP indirectly via its short-term facilitation of synaptic connections, similar to the mechanism tested in a theoretical study (Swinehart and Abbott, 2005). This simple mechanism, however, does not rule out a possible additional enhancing mechanism mediated by the interaction between the cellular processes activated by activity and 5-HT.

The second special characteristic of 5-HT in the octopus VL is the effective concentration, which is about one order of magnitude higher than in *Aplysia* (100 vs. 10  $\mu$ M). An even lower concentration of 5-HT affects the neuromuscular system of cephalopod chromatophore muscles ( $\sim 2.5$   $\mu$ M, (Florey and Kriebel, 1969). The reason for these differences is not yet clear. However, neuromodulators are typically effective at rather low concentrations (nM– $\mu$ M range), but when acting as fast neurotransmitters they are thought to gate postsynaptic ionotropic receptors in the mM concentration range (Giocomo and Hasselmo, 2007) (a low Kd together with high concentration ensure fast and localized responses). The intermediate 5-HT concentrations effective in the octopus VL ( $\sim 100$   $\mu$ M) may correlate with a specific function of 5-HT in the VL. For

example, the high 5-HT concentration could suggest a rather localized effect of the neuromodulator, allowing modulation of specific synaptic connections in the VL matrix. As discussed above, such a localized modulation may serve to induce a branch-specific associative plasticity as suggested by Bailey et al. (2000a). This suggestion is supported by the fact that 5-HT-IR in terminals is closely associated with synaptic connections (Fig. 1B).

The third special property relates to the indications here that the effects of 5-HT and LTP are mediated by different biochemical processes (Figs. 5–7). It is likely that to achieve an efficient reinforcing relationship between 5-HT modulation and activity-dependent long-term plasticity, each of the two processes is mediated by separate mechanisms. The robustness of facilitation evoked by 5-HT is comparable to that caused by the activity-dependent mechanism (about 4-fold enhancement). Nevertheless, only activity leads to a long-lasting potentiation, while 5-HT facilitation appears to be of a short duration (Fig. 3). In addition, although 5-HT, octopamine and dopamine have been shown to activate adenylyl cyclase in the octopus optic lobes (Capasso et al., 1991), our physiological results suggest that the enhancing effect of 5-HT on the glutamateric MSF-VL synapses is not mediated via a cAMP cascade (Fig. 6). In contrast, the IBMX experiments support involvement of cyclic nucleotides in the activity-dependent LTP and possibly also in an activity-dependent potentiation block (Fig. 7C).

## CONCLUSION

In summary, our immunohistochemical and physiological results support the hypothesis that 5-HT inputs convey modulatory input signals into the VL from other brain structures, as do the global dopamine, noradrenaline and acetylcholine fibers innervating the hippocampus (Matsuda et al., 2006) and modulatory inputs to the insect mushroom bodies (Fiala, 2007). The 5-HT afferents to the VL may thus be part of the reward/punishment system in the octopus VL that J. Z. Young postulated more than 50 years ago (see Altman, 1997). We suggest that the afferent modulatory inputs enable localized modulation of LTP at specific sites in the VL matrix. Such organization may permit rich diversification of branch-specific associative strengthening in the huge and highly divergent (1.8/25 million cells) learning matrix of the octopus VL.

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