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Research report

Serotonin, via 5-HT_{2A} receptors, increases EPSCs in layer V pyramidal cells of prefrontal cortex by an asynchronous mode of glutamate release

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Abstract

Previously, serotonin (5-HT) was found to induce a marked increase in glutamatergic spontaneous excitatory postsynaptic currents (EPSCs) in apical dendrites of layer V pyramidal cells of prefrontal cortex; this effect was mediated by 5-HT_{2A} receptors, a proposed site of action of hallucinogenic and atypical antipsychotic drugs. Unexpectedly, although the effect of 5-HT was Ca^{2+}-dependent and tetrodotoxin-sensitive, it did not appear to involve the activation of excitatory afferent impulse flow. This paradox prompted us to investigate (in rat brain slices) whether 5-HT was acting through an atypical mode of excitatory transmitter release. We found that the frequency of 5-HT-induced *spontaneous* EPSCs was fully supported by Sr^{2+} in the absence of added Ca^{2+}, implicating the mechanism of asynchronous transmitter release which has been linked to the high-affinity Ca^{2+}-sensor synaptotagmin III. Although the early, synchronous component of *electrically evoked* EPSCs was reduced while 5-HT was being applied, late, nonsynchronous components were enhanced during 5-HT washout and also by the 5-HT_2 partial agonist 1-(2,5-dimethoxy-4-iodophenyl-2-aminopropane (DOI); the effect of DOI was blocked by a selective 5-HT_{2A} antagonist (MDL 100,907). This late, nonsynchronous component was distinct from conventional polysynaptic EPSCs evoked in the presence of the $GABA_A$ antagonist bicuculline, but resembled asynchronous glutamatergic excitatory postsynaptic potentials (EPSPs) evoked in the presence of Sr^{2+}. An enhancement of asynchronous EPSCs by a specific neurotransmitter receptor has not been reported previously. The possible role of excessive asynchronous transmission in the cerebral cortex in mediating the hallucinogenic effects of 5-HT_{2A} agonists such as DOI is discussed.

Keywords: Apical dendrite; Brain slice; Hallucinogen; Sr^{2+}; Synaptotagmin III; Whole-cell recording

1. Introduction

Previously, by whole-cell recording in rat brain slices, we found that serotonin (5-HT) induces a rapid and dramatic increase in the frequency and amplitude of spontaneous (nonelectrically evoked), glutamatergic excitatory postsynaptic potentials/currents (EPSPs/EPSCs) in layer V pyramidal cells of transitional and neocortex [1]. The 5-HT-induced increase in spontaneous EPSCs was blocked by selective antagonists of 5-HT_{2A} (e.g., MDL 100907) but not 5-HT_{2C} receptors [1,19] and was seen most prominently in medial prefrontal cortex and other frontal areas [1] where 5-HT_{2A} receptors are enriched in pyramidal cell apical dendrites [10,12,33]. The increase in EPSCs in neocortex was surprising because previous studies in paleocortical regions (e.g., piriform cortex, dentate gyrus, and hippocampus) had shown predominantly an activation of inhibitory postsynaptic potentials (IPSPs) by 5-HT_{2A} receptor stimulation [15,21,25,26]. The induction of EPSCs by 5-HT appears to occur through a novel, tetrodotoxin (TTX)-sensitive, Ca^{2+}-dependent mechanism which, paradoxically, does not involve the activation of afferent impulse flow [1]. Instead, as shown by 5-HT-responsive 'hot spots' on the apical but not basilar dendrites of layer V pyramidal cells, there was a focal mechanism for the increase in spontaneous EPSCs [1].

The present study investigated possible mechanisms by which 5-HT could induce a focal, TTX/Ca^{2+}-sensitive

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release of glutamate in the absence of an increase in afferent impulse flow. Studies in cortical synaptosomal preparations have shown that mildly depolarizing agents such as the K^+ channel blocker 4-aminopyridine (4-AP) can induce a TTX-sensitive release of glutamate from isolated cortical synaptosomes (in contrast to the TTX-insensitive release produced by strongly depolarizing, high concentrations of KCl) [29]. Interestingly, 4-AP preferentially enhances the 'slow' rather then 'fast' component of glutamate release and is supported by Sr^{2+} in the absence of Ca^{2+}, suggesting the involvement of the 'asynchronous' mode of release [11]. Sr^{2+} substitutes for Ca^{2+} at the high-affinity Ca^{2+} sensor synaptotagmin III, which is believed to be responsible for slow, asynchronous transmitter release, while Sr^{2+} is ineffective at the low-affinity Ca^{2+} sensor synaptotagmin I which is thought to be responsible for fast, synchronous transmitter release [7,9,14]. In various regions of brain including cerebral cortex [2,25,28], the effects of 5-HT_{2A}-receptor activation resemble those of 4-AP in producing a slow depolarization through a reduction in K^+ channel conductance. Therefore, by analogy with 4-AP, it is possible that 5-HT induces a focal, TTX/Ca^{2+}-sensitive, impulse-flow independent increase in spontaneous EPSCs through the asynchronous mode of glutamate release. To test this possibility, we have examined the ability of Sr^{2+} to substitute for Ca^{2+} in supporting 5-HT-induced *spontaneous* EPSCs. In addition, we have examined the effects of 5-HT and 1-(2,5-dimethoxy-4-iodophenyl-2-aminopropane (DOI), a selective 5-HT_2 agonist [30], on a late, nonsynchronous component of electrically-evoked EPSCs.

2. Materials and methods

2.1. Preparation of brain slices

Brain slices were prepared as previously described [1]. In brief, young adult male albino rats (120–170 g; Camm, Charles River, or Harlan) were anesthetized with chloral hydrate (400 mg/kg) prior to decapitation. Brains were then removed and trimmed in ice-cold artificial cerebrospinal fluid (ACSF); coronal prefrontal cortical slices (500 μm) were cut with an oscillating-knife microtome and placed in an interface-type chamber through which ACSF flowed at ~1–1.5 ml/min. The ACSF and chamber were equilibrated with 95% O_2/5% CO_2 and maintained at 33°–34°C. ACSF was composed of (in mM): NaCl 128, KCl 3, NaH_2PO_4 1.25, D-glucose 10, $NaHCO_3$ 25, $CaCl_2$ 2, $MgSO_4$ 2 (pH ~ 7.35).

2.2. Recording and electrical stimulation

Intracellular recordings (used for the electrically evoked response experiments) were conducted using sharp electrodes containing 1 M K-acetate (30–60 MΩ); voltage-clamping was carried out in the discontinuous single-electrode voltage-clamp mode of the Axoclamp 2A (Axon Inst.) at switching frequencies of 4–6 kHz and a loop gain of 10 nA/mV (30% duty cycle). The headstage voltage was monitored continually to ensure that voltage transients decayed fully before voltage was sampled; false clamping was avoided by using optimal capacitance compensation and by selecting switching frequencies that allowed the input voltage to settle to a horizontal baseline.

Whole-cell recordings (used for analysis of spontaneous EPSCs) were performed with an Axoclamp 2A amplifier using low-resistance patch pipettes (2.5–3 MΩ) containing (in mM): K-gluconate 120, Hepes 10, BAPTA K_4 5, sucrose 20, $CaCl_2$ 2.38, $MgCl_2$ 1, K_2ATP 1, and GTP 0.1 (pH ~ 7.35). Cells were located by passing hyperpolarizing pulses (0.2 nA/200 ms) in current clamp mode using the blind method [4]. Following giga-seal formation (1–5 GΩ) induced by gentle suction, an additional pulse of suction was applied to attain whole cell mode. Series resistance, monitored continually throughout the experiment (except during periods of data collection), was usually between 4 to 8 MΩ. To minimize series resistance errors, cells were discarded if access resistance rose above 10 MΩ. As noted previously [1], when series resistance was maintained in this low range (i.e., close to full access), the input resistance of cells recorded by whole cell recording were not different from values obtained with intracellular recording provided that in the latter case adequate time is allowed for a stable seal to form after impalement. Postsynaptic currents were studied in the continuous single-electrode voltage-clamp mode (3 kHz low-pass filter cutoff frequency); to minimize holding currents, cells were clamped near their resting potential (typically −70 to −80 mV. At these holding potentials, $GABA_A$ and NMDA currents were minimized.

Layer V pyramidal cells were recorded in the medial prefrontal/anterior cingulate cortex in a zone approximately 1/2 to 2/3 the distance between the pial surface and the subcortical white matter. Pyramidal cells in this study had the following electrophysiological characteristics (intracellular and whole cell groups combined): resting potential, -72 ± 1 mV; action potential amplitude, 96 ± 2 mV; action potential duration (at half amplitude), 0.77 ± 0.1 ms; input resistance (−0.2 nA test pulse), 33 ± 3 MΩ ($n = 38$). Electrically evoked EPSPs were induced with a bipolar tungsten electrode placed on the subcortical white matter (forceps minor) on a line approximately perpendicular to the recorded layer V pyramidal cell (see Fig. 2, inset). The monophasic constant-current stimulus (0.2 ms) was set at an intensity (range: 0.06 to 0.2 mA) sufficient to induce a monosynaptic EPSC of ~0.5 to 1 nA. If antidromic spikes were observed from a given stimulation site, the position of the stimulating electrode was shifted along the forceps minor until a site was found that did not give rise to antidromic spikes.

2.3. Data collection and analysis

Data collection was by means of pClamp software via a Digidata 1200 interface (Axon Inst.). EPSC frequency and amplitude were determined with Axograph peak detect software; signals < 5 pA were excluded from the measurements. Statistical comparisons of within-cell changes in response were made using either two-tailed paired *t* tests or the nonparametric Kolmogorov–Smirnov test for distributions (Statistica, Statsoft). Mean values are given as $\pm$S.E.M.

2.4. Test substances

Substances were applied at known concentrations in normal ACSF via stopcocks placed close to the recording chamber ($\sim$20 s dead space). Peak responses to 5-HT occurred within 1–2 min of bath application; routinely, exposure to 5-HT was limited to 1–2 min to avoid desensitization which can result from more prolonged exposure to 5-HT. Serotonin creatinine sulfate (5-HT) was from Sigma; DOI and bicuculline methiodide were from RBI; MDL 100,907 was a gift from Marion Merrell Dow.

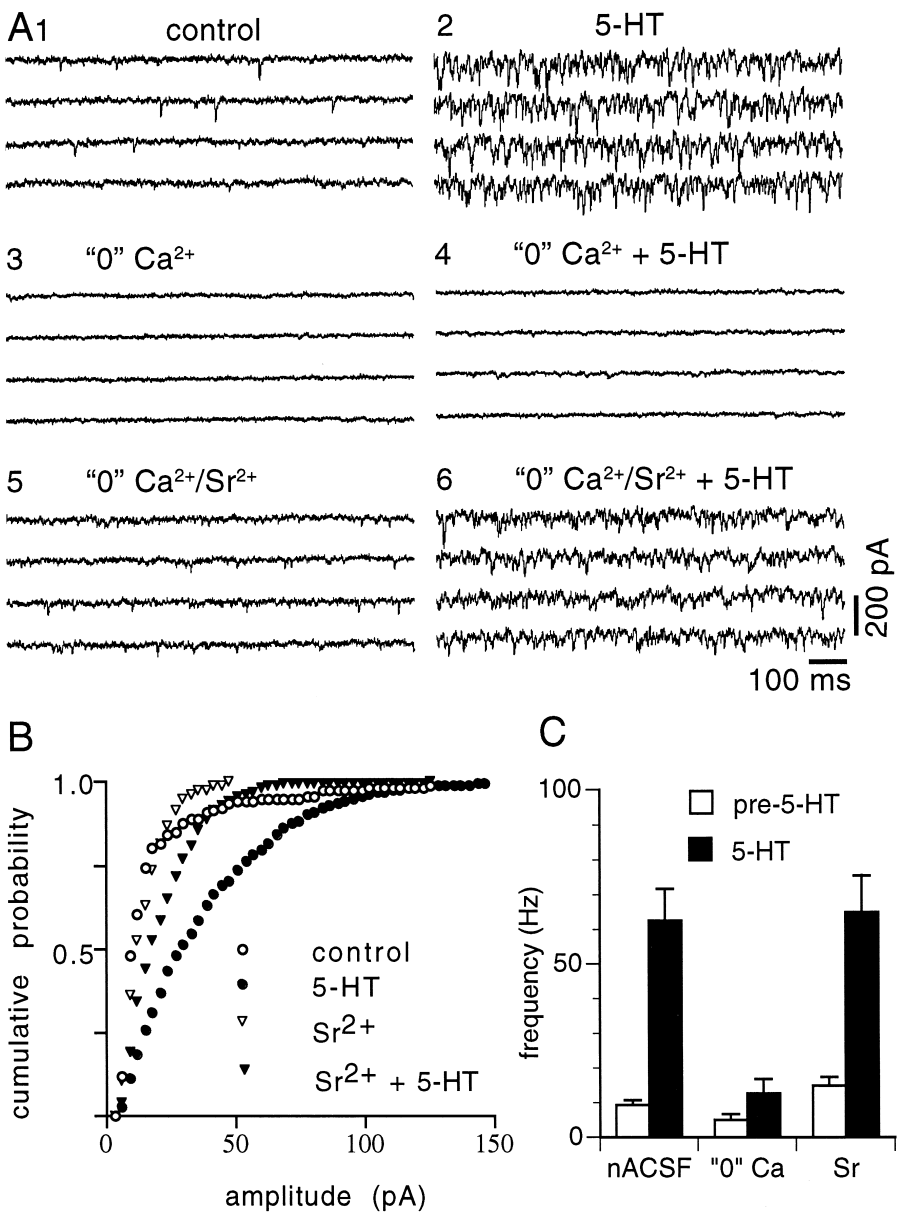

Fig. 1. Effect of Sr^{2+} on 5-HT-induced spontaneous EPSCs in layer V pyramidal cells of medial prefrontal cortex. (A1–6) Whole cell patch-clamp recording showing responses of an individual pyramidal cells to a 1-min application of 5-HT (100 μM) in normal ACSF (control/5-HT), after 9–10 min of ACSF with '0' added Ca^{2+} (2 mM Mg^{2+} substituted for Ca^{2+}), or after 9–10 min in Sr^{2+} (1 mM) substituted for Ca^{2+}. (B) Cumulative probability plot for the amplitude distribution of spontaneous EPSCs for the cell illustrated in (A); 5-HT induces a significant shift to higher amplitudes both in the nACSF and Sr^{2+} conditions ($p < 0.001$, Kolmogorov–Smirnov test). (C) Summary of results for five cells showing increased frequency of spontaneous EPSCs induced by 5-HT in normal ACSF or Sr^{2+}/'0' Ca^{2+} conditions ($p < 0.004$, paired *t* test); spontaneous EPSCs were not increased over the normal ACSF baseline by 5-HT in the '0' Ca^{2+} condition.

3. Results

3.1. Effect of Sr^{2+} on 5-HT-induced spontaneous EPSCs in layer V pyramidal cells of medial prefrontal cortex

As illustrated in Fig. 1A and summarized in Fig. 1C, application of 5-HT (100 μM) in normal ACSF to layer V pyramidal cells, induced a large increase in the frequency of spontaneous EPSCs ($n = 5$). As previously reported [1], the 5-HT-induced increase in spontaneous EPSCs was highly dependent on the presence of Ca^{2+} in the extracellular medium (Fig. 1A,C). A cumulative probability plot, using data taken from the cell in Fig. 1A, shows that 5-HT also induced an increase in the amplitude of spontaneous EPSCs (Fig. 1B); this increase was observed in all cells tested ($n = 5$; $p < 0.025$ to 0.001, Kolmogorov–Smirnov test). When Sr^{2+} (1 mM) was substituted for Ca^{2+}, the ability of 5-HT to induce spontaneous EPSCs was restored (Fig. 1A). In fact, the average frequency of EPSCs in the presence of Sr^{2+} was virtually identical with that in the normal medium containing Ca^{2+} ($n = 5$; Fig. 1C). In pilot experiments ($n = 4$), at a higher concentration of Sr^{2+} (2 mM) an even greater than normal increase in the frequency of 5-HT-induced spontaneous EPSCs was seen, but because of an unstable baseline, this concentration was not used routinely. The amplitude of 5-HT-induced spontaneous EPSCs was also increased in the Sr^{2+} solution relative to the Sr^{2+} baseline (Fig. 1B). These results indicate that Sr^{2+} can substitute for Ca^{2+} in supporting the ability of 5-HT to increase both the frequency and amplitude of spontaneous EPSCs. However, in 4/5 cells tested, amplitudes in the Sr^{2+} condition (both basal and 5-HT-induced) were reduced relative to those in normal Ca^{2+} ($p < 0.01$ to 0.001, Kolmogorov–Smirnov test) (Fig. 1B); this reduction in amplitude did not occur when Sr^{2+} was added together with normal Ca^{2+} (not shown). Thus, Sr^{2+} substituted fully for Ca^{2+} in supporting the 5-HT-induced increase in frequency of spontaneous EPSCs, but only partially with respect to amplitude. An antagonist of AMPA/kainate glutamate receptors, LY293558 (3 μM), totally blocked the 5-HT-induced spontaneous EPSCs in Sr^{2+} ($n = 2$; not shown), as previously found in normal Ca^{2+}-containing perfusate [1].

3.2. Electrically evoked EPSCs in normal Ca^{2+} or after Sr^{2+} substitution

To evaluate the effect of Sr^{2+} substitution upon electrically evoked EPSCs, constant-current stimulation was applied at 10-s intervals (0.1 Hz) to the subcortical white matter directly beneath the area of cortex from which a layer V pyramidal cell was being recorded (Fig. 2, inset); stimulation sites were chosen so as to avoid antidromic responses. Typically, such close-proximity stimulation elicited a short-latency (~ 2 ms), synchronous EPSC (Figs. 2–6). As expected, the removal of Ca^{2+} (2 mM) from the medium resulted in the virtual disappearance of the evoked EPSCs ($n = 5$; Fig. 2B). In accord with Goda and Stevens [9], the substitution of Sr^{2+} (1 mM) for Ca^{2+} resulted in the appearance of late, asynchronous evoked EPSCs fol-

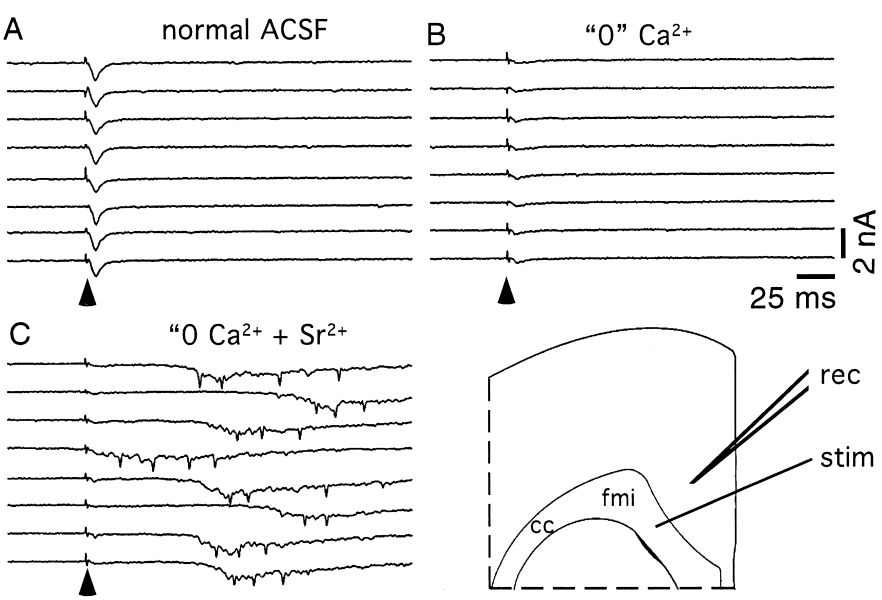

Fig. 2. Evoked EPSCs (evoked EPSCs) in a layer V pyramidal cells of medial prefrontal cortex: effects of Sr^{2+} substitution for Ca^{2+}. Electrical stimuli (0.2 ms; arrowheads) were applied at 10-s intervals to the underlying white matter of the forceps minor (fmi), medial to the corpus callosum (cc; see inset). (A) shows 10 consecutive responses in normal ACSF where only a short latency (~ 2 ms) synchronous EPSCs was evoked. (B) All EPSCs, both synchronous and asynchronous, are suppressed markedly in ACSF with '0' added Ca^{2+} applied for 6–7 min. (C) The inclusion of Sr^{2+} in '0' Ca^{2+} ACSF results in sweeps of asynchronous evoked EPSCs following each stimulus in the absence of synchronous EPSCs. Downward deflections indicate inward currents.

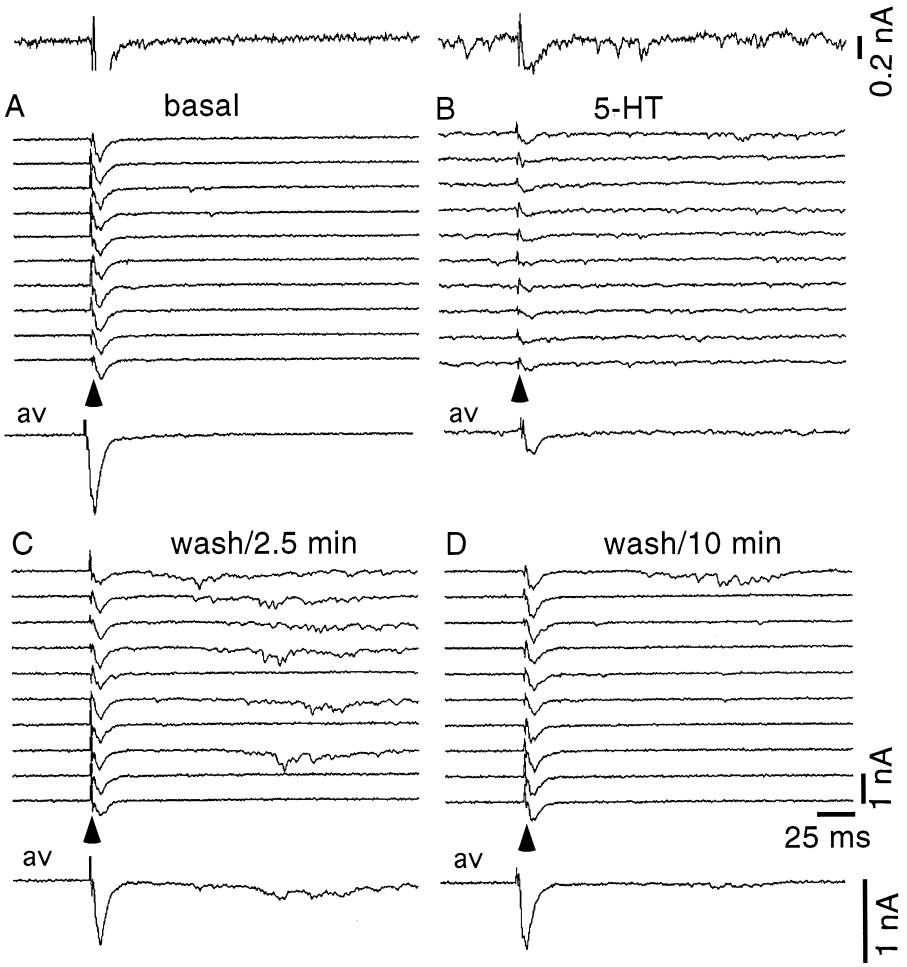

Fig. 3. Effects of 5-HT and 5-HT washout on electrically evoked EPSCs (evoked EPSCs) in a layer V pyramidal cell of medial prefrontal cortex. (A) shows responses to 10 consecutive stimuli (arrowheads) where only short latency synchronous EPSCs are evoked. (B) After a 1-min application of 5-HT (100 μM) there is a reduction in the amplitude of the synchronous evoked EPSC, as previously described [1,28], but a large increase in spontaneous EPSCs both before and after the stimulus (see top inset). (C) After a short washout of 5-HT (2.5 min), sustained late or nonsynchronous EPSCs appear after 6/10 stimuli; note that at this point the synchronous evEPSC is recovering and the increase in spontaneous EPSCs is subsiding. (D) There is a return toward basal responses after a longer washout period (10 min). The lower insets show the averaged responses (av) for each set of 10 traces.

lowing each stimulus with a virtual absence of synchronous EPSCs ($n = 5$; Fig. 2C). In the cell illustrated in Fig. 2C, there was considerable variability in the onset of this late component; in other cells (3/5) the onset of the asynchronous evoked EPSCs was more uniform but always delayed in relation to the control synchronous EPSC.

3.3. Effect of 5-HT on the occurrence of the late component of electrically evoked EPSCs

In previous studies on prefrontal cortical neurons, 5-HT was noted to reduce the amplitude of electrically evoked EPSPs/EPSCs through an action upon *non*-5-HT_{2A} (possibly 5-HT_1) receptors [1,22,28]; the latter studies only examined synchronous evoked responses. In the present study, it was similarly noted that while 5-HT was being applied, there was a reduction in the amplitude synchronous evoked EPSCs (Fig. 3B). Also, as expected, during the application of 5-HT there was an increase in the frequency of spontaneous EPSCs. Thus, in the example shown in Fig. 3, at a near maximal concentration of 5-HT (100 μM), there were a large number of spontaneous EPSCs both before and after the electrical stimulus (see inset); late evoked EPSCs were not evident at this point in time. However, during 5-HT washout, as the amplitudes of synchronous evoked EPSCs were recovering and the frequency of spontaneous EPSCs was returning toward normal, sweeps with sustained, late evoked EPSCs began to appear in an all-or-none fashion following some of the stimuli. As seen in Fig. 3C, late evoked EPSCs occurred in 6/10 sweeps during the washout period; these resembled asynchronous evoked EPSCs seen after Sr^{2+} substitution (Fig. 2) but not polysynaptic evoked EPSCs seen in the presence of bicuculline (see below). This 5-HT washout effect was seen in 7/12 cells tested. The fact that the late component of evoked EPSCs was enhanced only during

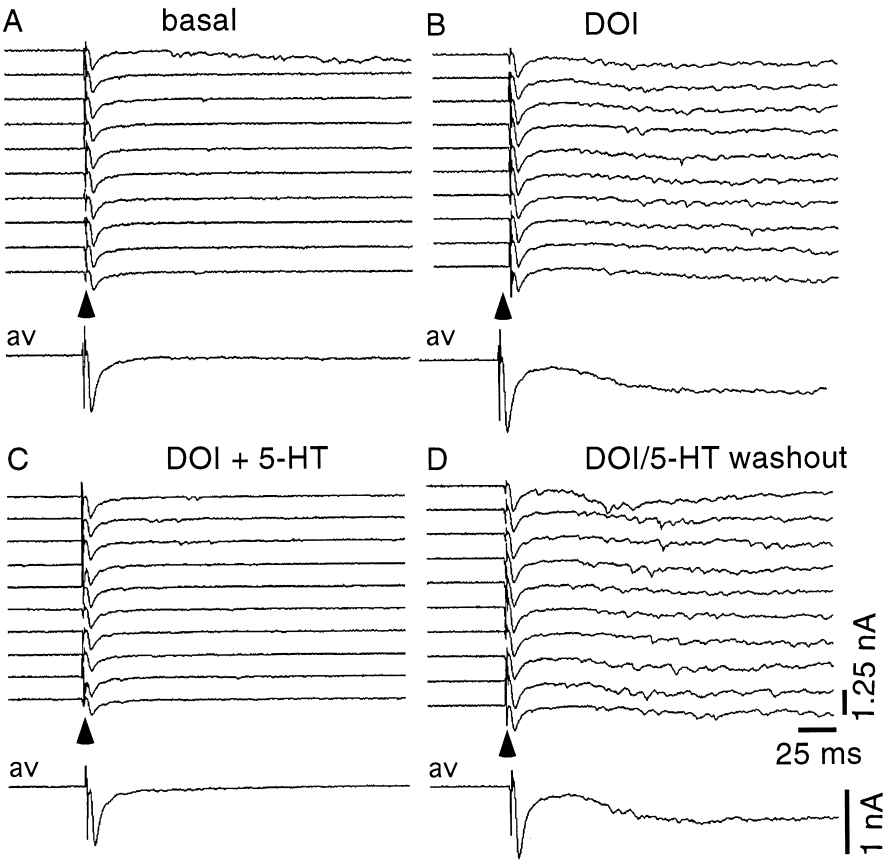

Fig. 4. DOI increases the occurrence of late components of evoked EPSCs in a layer V pyramidal cell. (A) Under basal conditions, there is an all-or-none sweep of a late component of the evEPSC only after the first in a series of 10 stimuli. (B) Following DOI (3 μM) applied for 10–12 min there is a progressive increase in the proportion of sweeps with a persistent late component of EPSCs; at the time point shown (~ 10 min following the end of DOI application), all sweeps show a late component following the synchronous response. (C–D) During the application of 5-HT (100 μM) for 1 min there is a suppression of this late component; the latter return after ~ 10 min of washout.

washout suggested that 5-HT, through an action upon non-5-HT_{2A} receptors, might interfere with the coupling of impulses to the production of the late evoked EPSCs; during washout, the non-5-HT_{2A} effects may subside, allowing the 5-HT_{2A}-mediated late component to emerge. Consistent with the interpretation, previous studies on cortical pyramidal cells have shown that postsynaptic depolarizing effects of 5-HT mediated by 5-HT_{2A} receptors outlast 5-HT_{1A}-mediated hyperpolarizations (see Fig. 4, Araneda and Andrade [2]). Studies in piriform cortex have also shown that direct 5-HT_{2A}-receptor mediated excitatory effects on interneurons are prolonged, outlasting the 5-HT application period by several minutes [15,25].

3.4. The effect of DOI on late components of evoked EPSCs

To avoid activating non-5-HT_2 receptors, the effect of the selective 5-$HT_{2A/2C}$ agonist DOI [30] was tested in a subgroup of five cells that displayed a late component of evoked EPSCs during 5-HT washout (see above). Based on earlier studies [1,16], DOI was applied in the perfusate at a concentration of 3 μM for a period of 10–12 min, which produces a near maximal effect persisting for > 2 h. Prior to DOI (Fig. 4A), a sustained late component of the evoked EPSC was seen only in the first of 10 sweeps. However, following the application of DOI, the late component was evoked by every stimulus in all cells tested ($n = 5$; Fig. 4B). When 5-HT (100 μM) was applied after DOI, the late component evoked EPSCs was suppressed (Fig. 4C); the late component reappeared during washout of 5-HT (Fig. 4D). Note that after DOI, 5-HT (Fig. 4C) did not produce the usual increase in spontaneous EPSCs (compare with Fig. 3B). We interpret this failure as being due to the occupation of 5-HT_{2A} receptors by DOI; DOI itself, as a partial agonist [16], induces only about 10% of the maximal induction of spontaneous EPSCs by 5-HT (not shown). In contrast, 5-HT would be free to suppress the late component of the evoked EPSCs through non-5-HT_2 receptors since they would not be occupied by DOI [30].

3.5. Interstimulus interval and the occurrence of the late component of evoked EPSCs induced by DOI

Typically, under baseline conditions, late evoked EPSCs tended to occur mainly after a pause between trains of

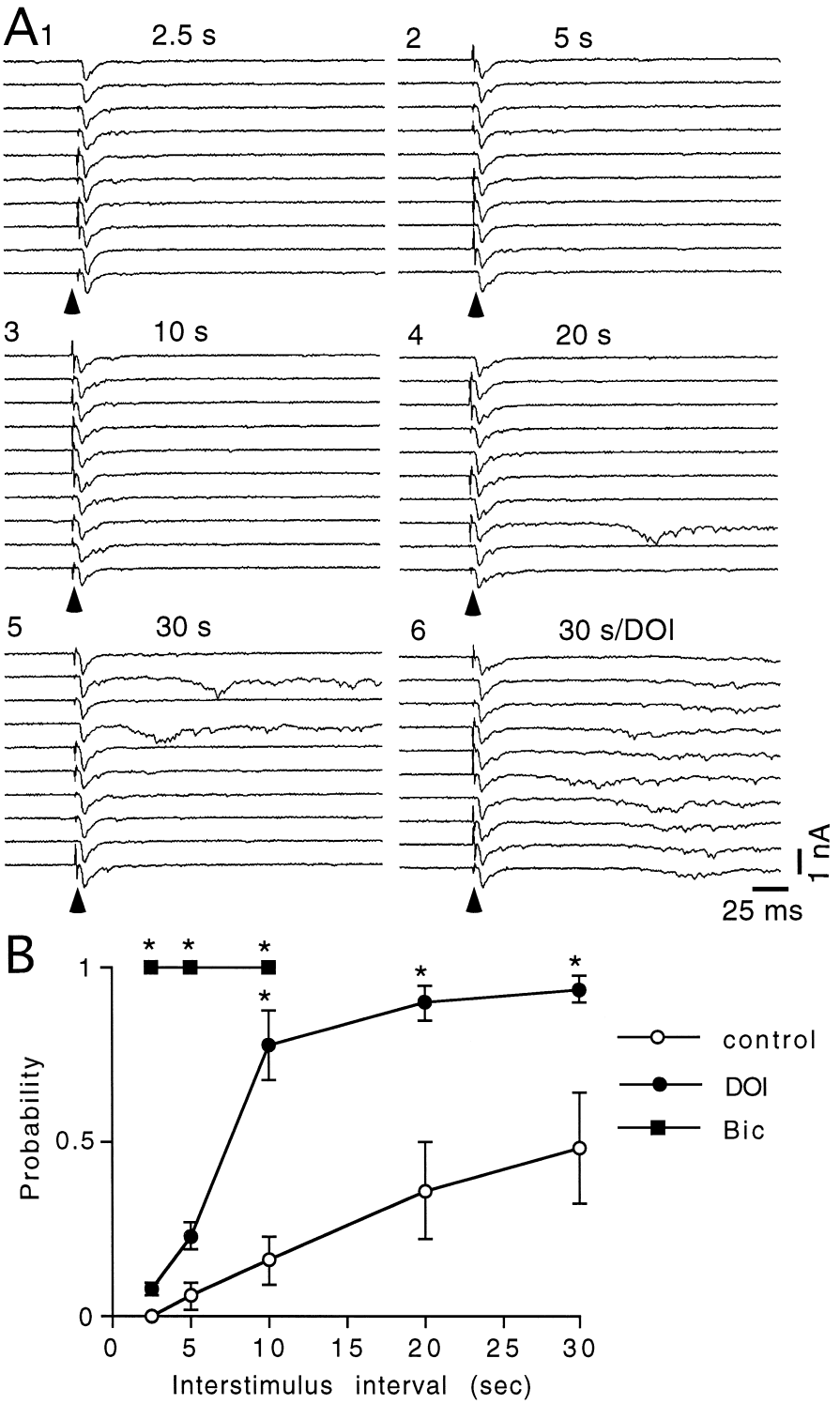

Fig. 5. The influence of interstimulus interval on DOI-induced late evoked EPSCs. (A_{1-5}) shows a cell in which the occurrence of all-or-none late components occurred only at the longer intervals (20 or 30 s). (A_6) Following application of DOI (3 μM), all sweeps show a late component at the 30-s interval. (B) Summary of data for five cells; only cells/stimulation sites that displayed late evoked EPSCs during 5-HT washout were included. Also included are data from five additional cells in which evoked responses were obtain following treatment with bicuculine (Bic) (see Fig. 6); the Bic controls, which were close to zero, are not shown. $^{*}p < 0.01$ to $p < 0.001$ with respect to controls, paired t test.

stimuli (e.g., see Fig. 3D and Fig. 4A). To investigate this phenomenon, data were collected during ongoing stimulation at a given interstimulus interval. Fig. 5A shows results from one cell prior to the administration of DOI in which sustained, late evoked EPSCs occurred spontaneously only after long intervals (20 or 30 s). Following DOI, sweeps

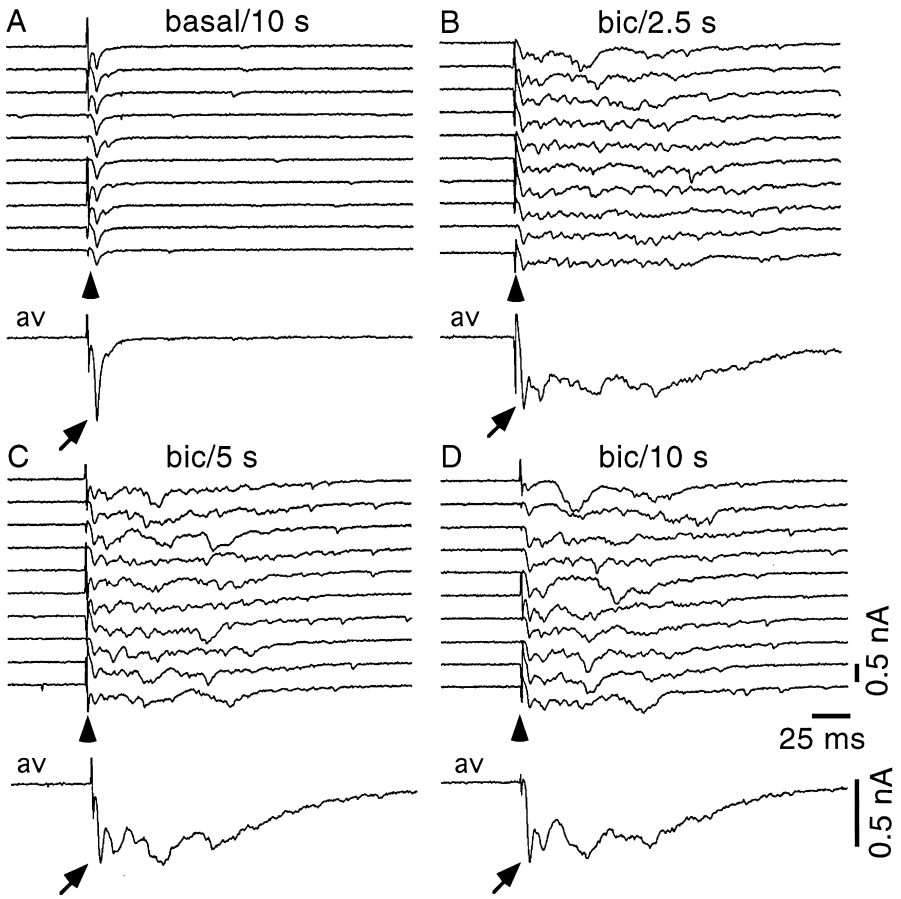

Fig. 6. The influence of interstimulus interval on polysynaptic evoked EPSCs in the presence of bicuculline. (A) Prior to the application of bicuculline, only a short latency, synchronous EPSC were observed in this cell. (B–D) Following a 10-min application of bicuculline (1 μM) to disinhibit polysynaptic transmission, late components of the evEPSC emerged uniformly at all interstimulus intervals tested (i.e., 2.5, 5, and 10 s). Note that the onset of this late component was relatively rapid, beginning during the falling phase of the synchronous response. Also note that in the averaged evoked EPSCs (insets: av), the peak amplitude of the early, synchronous response (arrows) virtually unaltered by bicuculline. See Fig. 5B for a summary of results from five cells tested after bicuculline.

with late evoked EPSCs increased from 2/10 to 10/10 at the 30-s interval (Fig. 5A). Fig. 5B summarizes data from five such experiments: DOI dramatically increased late evoked EPSCs at intervals of 10 s and above; at shorter intervals (2.5 and 5 s) the increase in the late component was minimal. These results suggest that there is a relative refractory period for elicitation of the late component of evoked EPSCs that cannot be overcome by DOI. The mechanism for this refractory period remains to be determined.

3.6. Effect of interstimulus interval on polysynaptic evoked EPSCs induced by bicuculline

For purposes of comparison with DOI, the $GABA_A$ antagonist bicuculline, which suppresses feed-forward and feedback GABAergic inhibition in the cortex, was used to promote polysynaptic evoked EPSCs. In the presence of a relatively low concentration of this drug (1 μM) it was possible to produce a robust late component of the evoked response without inducing spontaneous epileptiform activity (Fig. 6; $n = 5$). In contrast to DOI, the occurrence of late evoked EPSCs after bicuculline was undiminished at the shorter intervals (i.e., 2.5 and 5 s; Fig. 5B). This result is not surprising since polysynaptic evoked EPSCs are presumably driven by successive waves of synchronous transmission which should not have such long refractory periods. Also in contrast to DOI, the onset of bicuculline-induced late EPSCs was quite rapid, beginning during the falling phase of the synchronous EPSC (see 'av' insets, Fig. 6). It should be noted that the amplitudes of the synchronous evoked EPSCs were not altered by bicuculline (controls, 731 ± 31 pA; bicuculline, 718 ± 23 pA; $n = 5$), indicating that this initial component represents predominantly a monosynaptic EPSP.

3.7. The effect of MDL 100,907 on the enhancement of late evoked EPSCs by DOI

Previous studies have shown that MDL 100,907, a highly selective 5-HT_{2A} receptor antagonist [13,20], totally blocks 5-HT-induced spontaneous EPSCs [1]. In the pre-

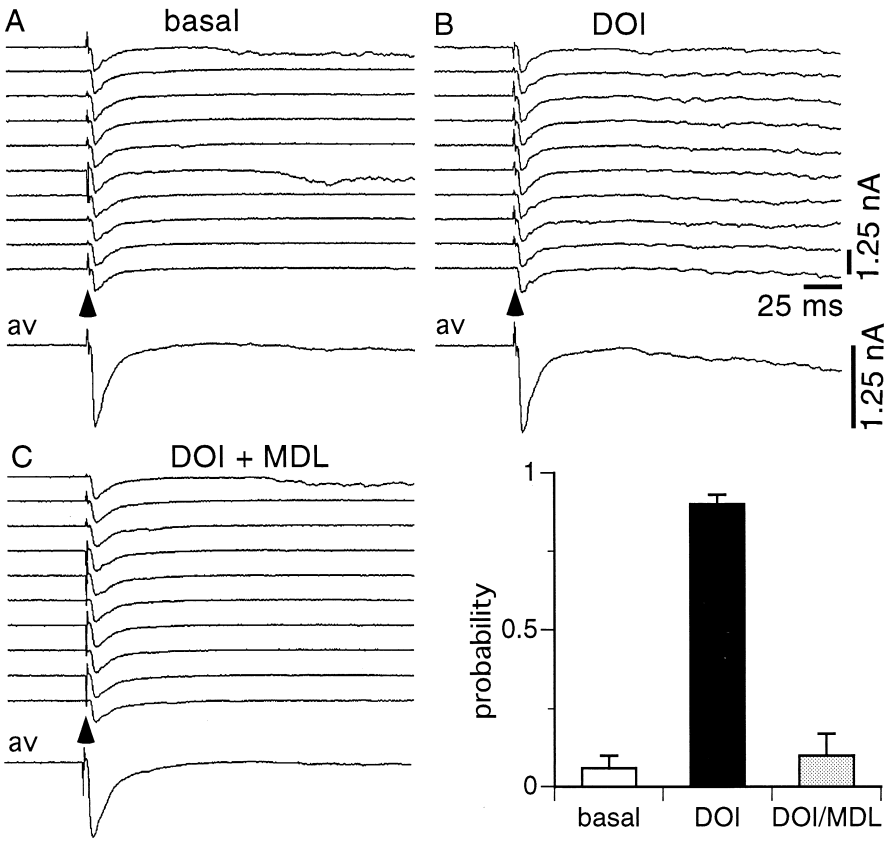

Fig. 7. Reversal by the 5-HT_{2A} antagonist MDL 100907 of the increase in evoked EPSCs by DOI. (A–C) Illustrate for a single layer V pyramidal cell the increase in evoked EPSCs induced by DOI (3 μM) and the reversal of this effect ($p < 0.001$ compared to basal or DOI/MDL) after a 35-min application of MDL 100907 (100 nM). (B) Summary of data for five cells.

sent study, after DOI (3 μM) was applied to achieve a high proportion of evoked sweeps with late evoked EPSCs, MDL 100,907 (100 nM) was applied for 30–40 min (Fig. 7). There was a gradual but almost complete (98%) reversal of the DOI effect over that period; as in previous studies [16], the effect of DOI persisted >2 h in the absence of antagonist ($n = 5$). The persistence of the effect of DOI has been interpreted as reflecting the slow off rate of the drug from 5-HT_{2A} receptors [16].

4. Discussion

The key finding of this study was that the 5-HT-induced increase in the *frequency* of spontaneous EPSCs in layer V pyramidal cells of prefrontal cortex is fully supported by Sr^{2+} in the absence of added Ca^{2+}. This result suggests that the effect of 5-HT is mediated through asynchronous rather than synchronous mechanisms of transmitter release since Sr^{2+} substitution has been shown to support only the asynchronous mode of transmission [9,14]. The 5-HT-induced increase in the *amplitude* of spontaneous EPSCs was only partially supported by Sr^{2+} (in the absence of Ca^{2+}), consistent with the proposed role of Ca^{2+} in the postsynaptic amplification of synaptic signals to apical dendrites of layer V pyramidal cells (for which Sr^{2+} is not known to substitute) [24]. The comparable ability of Ca^{2+} and Sr^{2+} to support the 5-HT-induced increase in frequency of spontaneous EPSC points to the involvement of a presynaptic site of action. However, a recent electron immunocytochemical study has shown that there is only a scattering of 5-HT_{2A}-labeled nerve terminals in prefrontal cortex, while the bulk of these receptors are located postsynaptically within pyramidal cell apical dendrites [12]. Thus, while an activation of postsynaptic 5-HT_{2A} receptors in layer V pyramidal cells might be expected to contribute to an increase in the amplitude of spontaneous by Ca^{2+} (but not Sr^{2+}), there is no simple way to explain how a postsynaptic action could result in such a marked increase the frequency of EPSCs. Given the evidence for a predominantly postsynaptic location of 5-HT_{2A} receptors, there is a need to explain how the interactions with presynaptic mechanisms may be occurring. One possibility would be that a retrograde messenger is generated through 5-HT's action upon postsynaptic 5-HT_{2A} receptors, which then could have a presynaptic effect upon excitatory nerve terminals. Alternatively, it is possible that the effect of 5-HT is mediated through a direct presynaptic action upon the relatively small subset of 5-HT_{2A}-positive terminals that have been demonstrated to exist by electron immuno-

cytochemistry [12]. These alternatives remain to be examined experimentally.

We also observed that in some cells a *late* component of the electrically evoked EPSC was increased during 5-HT washout and also after treatment with the 5-$HT_{2A/2C}$ partial agonist DOI. In contrast, during the *ongoing* application of 5-HT there is a reduction, via non-5-HT_{2A}, receptors, of the *early*, synchronous component of electrically evoked EPSPs in medial prefrontal cortex [1,22,28]. The DOI enhancement of the late component of evoked EPSCs was blocked by the selective 5-HT_{2A} antagonist MDL 100,907 and, in preliminary studies, by presynaptic inhibitory group II/III metabotropic glutamate agonists [18]. The conventional interpretation of the 5-HT_{2A}-receptor mediated enhancement of the late EPSC would be that it represents an increase in polysynaptic or epileptiform activity. However, DOI and other hallucinogens are not pro-convulsant and, in fact, DOI has been shown to activate GABAergic interneurons in various cortical regions [16,25,28]. Moreover, we have presented evidence that the late component of the evoked response after DOI is distinct in several respects from conventional polysynaptic evoked EPSCs that occur in the presence of the $GABA_A$ antagonist bicuculline. For example, the late component after bicuculline, in contrast to DOI, did not exhibit a refractory period at the 2.5–5-s intervals and had a short latency, beginning on the falling phase of the synchronous EPSC. On the other hand, the long-latency, late EPSCs elicited by 5-HT_{2A} receptor stimulation resembled asynchronous EPSPs as seen in the presence of Sr^{2+}.

There is now compelling evidence that mechanisms of asynchronous transmitter release are distinct from that of synchronous transmitter release. Early studies at the frog neuromuscular junction showed that transmitter release is biphasic, with an initial fast component and a slow biphasic decay [3]. More recent studies have shown that these two components, a fast synchronous and slow asynchronous phase with differential Ca^{2+}/Sr^{2+} sensitivity, are present in central neurons cultured from the hippocampus [9]. The molecular basis for these differences is now becoming understood. A mutation in synaptotagmin I results in a loss of fast, synchronous transmitter release, whereas slow, asynchronous release is unaffected [7]. These studies suggest that synaptotagmin I is the main Ca^{2+} sensor involved in synchronous transmitter release in the forebrain. The coupling of synaptotagmin I to syntaxin, a component of the synaptic core complex which is important for vesicle fusion and neurotransmitter exocytosis [27], is triggered by high concentrations of Ca^{2+} (>200 μM) that would exist during the early phase of fast, synchronous release shortly following the arrival of a nerve impulse [14]. Sr^{2+} is able to trigger syntaxin binding by synaptotagmin III but not by synaptotagmin I, suggesting the former may be responsible for mediating slow, asynchronous transmitter release [14]. Synaptotagmin III is unique among the known synaptotagmins in allowing Sr^{2+} to substitute for Ca^{2+} and, in contrast to synaptotagmin I, has a high affinity for Ca^{2+}. Thus, synaptotagmin III is particularly well suited for mediating transmitter release during the late, asynchronous phase when relatively low levels of residual Ca^{2+} would be present [14].

Accordingly, we propose that 5-HT_{2A} receptors, either directly or indirectly, promote asynchronous EPSCs in layer V pyramidal cells by increasing residual Ca^{2+} in excitatory nerve terminals to levels sufficient for activation of synaptotagmin III (or of an unknown synaptotagmin with similar properties). Through what mechanisms could the activation of 5-HT_{2A} receptors cause an increase residual Ca^{2+}? It is known that 5-HT_{2A} receptors are coupled via G proteins to the phospholipase C/phophoinositide second messenger pathway, one limb of which leads to the formation of inositol trisphosphate, a releaser of Ca^{2+} from intracellular stores [6]. However, while the latter action may contribute to an elevation of intraterminal Ca^{2+} levels, the dependence on extracellular Ca^{2+} (or Sr^{2+}) for the enhancement of asynchronous EPSCs by 5-HT indicates that entry of extracellular Ca^{2+} is essential. In the case of evoked asynchronous EPSCs, Ca^{2+} entry through voltage-gated Ca^{2+} channels could then synergize with any increases in release from intracellular Ca^{2+} stores that may have occurred. However, in the case of the *spontaneous* EPSCs induced by 5-HT, it is more difficult to account for the entry of extracellular Ca^{2+} if, in the absence of an increase in impulse flow, voltage-gated Ca^{2+} channels are not activated (see Section 1). Previously, we have reported that 5-HT enhances a subtheshold, TTX-sensitive persistent Na^+ current in layer V pyramidal cells [1]. We have proposed elsewhere that if 5-HT acts similarly to induce an increase in a persistent, subthreshold Na^+ in excitatory nerve terminals, the resulting increase in intracellular Na^+ could activate reverse Na^+/Ca^{2+} exchange, resulting in increased Ca^{2+} influx [17]; this possibility remains to be evaluated experimentally.

Finally, the physiological significance of a 5-HT_{2A} -induced increase in asynchronous EPSCs in layer V pyramidal cells needs to be considered. The administration of DOI in vivo results in an increased expression of brain derived neurotrophic factor (BDNF) in cortical pyramidal cells [31]; it is possible that enhanced asynchronous EPSCs could contribute to this positive neurotrophic effect. On the other hand, it is likely that excessive asynchronous EPSCs are detrimental to cortical function since the hallucinogenic properties of DOI and related drugs are highly correlated with their affinity for 5-HT_2 receptors [8]. It is possible that an increase in asynchronous glutamatergic transmission is responsible for the hallucinogen-induced hyperfrontal metabolic pattern that has been found recently in human brain imaging studies; a similar hyperfrontal pattern has also been found in acute but not chronic schizophrenic patients [32]. A critical difference between hallucinogens and 5-HT appears to be that actions of 5-HT at non-5-HT_2 receptors suppress the coupling of impulses

to the induction of the late, nonsynchronous component of evoked EPSCs (see Figs. 3 and 4), possibly explaining why treatments that elevate extracellular 5-HT (e.g., inhibitors of monoamine oxidase and 5-HT uptake) are not by themselves hallucinogenic and may in fact attenuate the effects of hallucinogens [5,23].

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