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5-hydroxyindole causes convulsions and increases transmitter release in the CA1 region of the rat hippocampus

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1 5-hydroxyindole (5-OHi) is a proposed tryptophan metabolite able to cause convulsions when systemically injected into rodents. We studied its effects using microdialysis *in vivo* and electrophysiological approaches *in vitro*.

2 Local administration of 5-OHi into the CA1 region of the rat hippocampus, *via* a microdialysis probe, significantly increased glutamate concentrations in the dialysates.

3 In rat hippocampal slices, using extracellular recordings in the CA1 region, 5-OHi (30–300 μM) increased the amplitude of population spikes and fEPSPs.

4 In the same preparation, using intracellular recordings in CA1 pyramidal neurons, 5-OHi reduced the latency of firing induced by direct depolarization and increased both evoked excitatory and slow inhibitory postsynaptic potential amplitudes, without affecting the resting membrane potential, the after-hyperpolarization or the neuronal input resistance. It also altered $GABA_A$-mediated neurotransmission by increasing the frequency and the amplitude of pharmacologically isolated spontaneous inhibitory postsynaptic currents (sIPSC).

5 In separate experiments, performed by measuring AMPA or NMDA-induced depolarization in cortical wedges, 5-OHi did not modify glutamate receptor agonist responses.

6 Our results show that 5-OHi causes convulsions, modifies the properties and the function of the hippocampal circuitry, and facilitates the output of both excitatory and inhibitory transmitters.

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Abbreviations: AP, action potential; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; D-AP5, D(-)-2-amino-5-phosphonopentanoic acid; EPSP, excitatory postsynaptic potential; fEPSP, field EPSP; GABA, γ-aminobutyric acid; MLA, methyllycaconitine; NMDA, N-methyl-D-aspartate; PS, population spike; p.f., probability of firing; TTX, tetrodotoxin

Introduction

Oxindole (2-indolinone) and its analogue 5-hydroxyindole (5-OHi) are putative tryptophan metabolites requiring tryptophanase, an enzyme present in gut bacteria, for their synthesis (see: Figure 1 and Carpenedo *et al*., 1998). Oxindole has sedative and anticonvulsant actions and, when tested in hippocampal slices, it significantly decreases neuronal excitability (Mannaioni *et al*., 1998). Its presence in mammalian tissues has been clearly demonstrated (Carpenedo *et al*., 1997) and it has also been shown that, when liver function is impaired, blood and brain concentrations of this metabolite significantly increase (Carpenedo *et al*., 1998; Moroni *et al*., 1998).

In preliminary experiments in rodents, we noticed that 5-OHi administration caused convulsions and loss of consciousness and we suggested the possibility of its involvement in the patho-physiology of hepatic encephalopathy (Albrecht & Jones, 1999; Moroni *et al*., 1998). However, the presence of 5-OHi in mammalian tissues has not been clearly demonstrated, possibly because the molecule is rather unstable at physiological pH and because it is rapidly metabolized into sulphate or glucuronide derivatives (King *et al*., 1966). The compound has been identified in rat urine and faeces after systemic administration of labelled indole (King *et al*., 1966) and we noticed that 5-OHi is formed by incubating rat liver homogenates with indole, the direct precursor (personal unpublished observations). When applied to neuroblastoma cells or to cells expressing recombinant $5\text{-}HT_3$ receptors, 5-OHi facilitates the 5-HT evoked ion currents by modifying $5\text{-}HT_3$ receptor-ion channel properties suggesting that it may have a role in the regulation of the serotoninergic neurotransmission (Kooyman *et al*., 1993; 1994). Moreover, 5-OHi potentiates nicotine responses in α_7 ACh receptors expressed in *Xenopus* oocytes and in hippocampal interneurones (Gurley *et al*., 2000). In order to further investigate the mechanism(s) of 5-OHi actions we studied its effects on: (i) glutamate output in microdialysis experiments in freely moving animals; (ii) electrically evoked potentials and cell excitability in the CA1 region of rat hippocampus using both extra- and intra-cellular recordings; (iii) ionotropic glutamate receptor function in cortical slices (Harrison & Simmonds, 1985).

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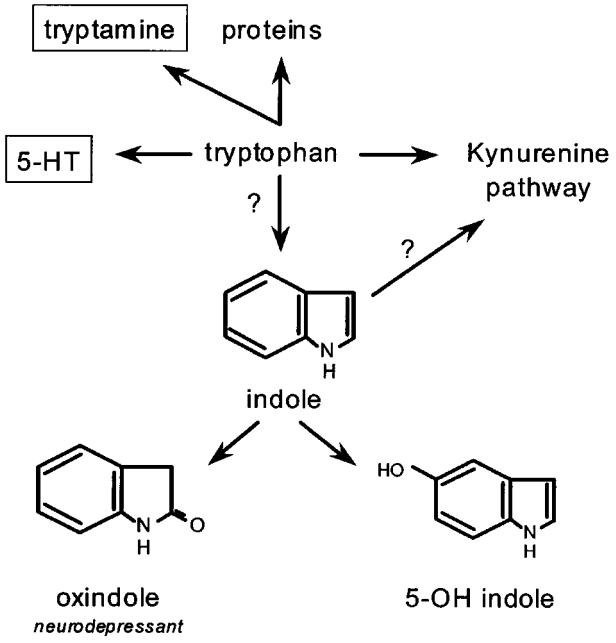

Figure 1 A schematic diagram showing the possible metabolic pathway for the synthesis of oxindole and 5-OHi. Formation of indole from tryptophan requires gut bacteria. The diagram also shows the possible metabolism of indole into kynurenine.

Methods

Materials

5-OHi, obtained from Aldrich Sigma, was dissolved in DMSO. The maximal concentration of DMSO used in electrophysiological studies was 0.003% (v v^{-1}) and had no measurable effects on evoked potentials. (−)bicuculline methiodide and D(−)-2-amino-5-phosphonopentanoic acid (D-AP5) were from Sigma-Aldrich; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), methyllycaconitine (MLA) and NMDA were from Tocris; tropisetron and tetrodotoxin (TTX) were from Calbiochem.

Behavioural actions and convulsive activity

5-OHi was dissolved in small volume of DMSO and injected i.p. (maximal injected volume was 0.1 ml) to male Wistar rats (100–200 g body weight, 3–4 weeks old, Charles River, Como, Italy). Rats were placed in an open field (1 square meter) and observed for at least 30 min after the injection. Clonic seizure, defined as the appearance of automatisms such as chewing, head bobbing and wet-dog shakes (Racine, 1972) tonic seizures, defined as tonic forelimb flexion/extension followed by whole-body clonus (Racine, 1972), or death were recorded. The Animal Care Committee of the Department of Pharmacology of the University of Florence and the Emory University Institution of Animal Care and Use Committee (IACUC) approved all procedures for both *in vivo* and *in vitro* experiments.

Microdialysis experiments and glutamate assay

Male Wistar rats (200–250 g body weight, Charles River, Como, Italy) were anaesthetized with chloral hydrate (300–mg kg^{-1}) and placed in a stereotaxic frame and transcerebral microdialysis tubing placed in their dorsal hippocampus as previously described (Carpenedo *et al*., 1994). Twelve to eighteen hours after surgery, the membranes were perfused at a flow rate of 2 μl min^{-1} with an iso-osmotic solution (in mM): NaCl 155, KCl 5.5 and $CaCl_2$ 2.3. Glutamate and GABA in the dialysate were measured using HPLC separation with pre-column derivatization and fluorimetric detection (Cozzi *et al*., 1997). Experiments were performed by collecting, under basal conditions and then in the presence of 5-OHi (30 or 300 μM), several 30 μl fractions of the dialysate.

Preparation of hippocampal slices

Experiments were carried out using *in vitro* hippocampal slices as previously described (Mannaioni *et al*., 1998). Slices (400 μM thick) were cut from the hippocampus, kept in oxygenated aCSF for at least 1 h at room temperature (20–23°C). A single slice was then completely submerged in a small chamber and superfused with oxygenated aCSF (30–32°C) at a constant flow rate of 2–3 ml min^{-1}. Drugs were administered through a three-way tap and a complete exchange of the chamber volume occurred in 1 min.

Extracellular recordings

Test pulses (80–110 μs duration; 0.017–0.05 Hz) were delivered through bipolar nichrome electrodes positioned in the CA1 stratum radiatum. Evoked extracellular potentials were recorded with glass microelectrodes (2–10 MΩ) filled with 3 M NaCl, either from the pyramidal cell layer of CA1 (population spike, PS) or from stratum radiatum (field excitatory postsynaptic potential, fEPSP) in the CA1 area. Responses were amplified (Neurolog NL 104, Digitimer Ltd), displayed on a computer monitor, digitized (sample rate 33 kHz), and analysed off-line using pClamp 6 software (Axon Instruments Ltd.).

Stimulus–response relationships were investigated at the beginning of each experiment by gradually increasing the stimulus strength. The test stimulus pulse was adjusted to produce a population spike whose amplitude was 40–50% (2–3 mV) of the maximum and subsequently kept constant throughout the experiment. After 30 min of equilibration, a control period (10–15 min) was recorded and used to generate baseline values before experimental tests.

In the recordings from the pyramidal cell layer, the population spike of the evoked responses was measured as the peak-to-peak amplitude of the first negative phase of the population spike (Mannaioni *et al*., 1998). In recordings from the stratum radiatum, the amplitude of both fEPSP and afferent volley were measured. Parameters were evaluated before, during, and after drug treatment.

Intracellular recordings

CA1 pyramidal neurones were recorded in current-clamp mode with 3 M KCl-filled sharp electrodes (35–50 MΩ). Electrical signals were amplified with an Axoclamp 2A (Axon Instruments, Foster City, U.S.A.) and displayed on an oscilloscope and chart recorder (2800 Gould, Valley View, U.S.A.). Traces were stored on a digital tape (DTR 1200, BioLogic, Claix, France; sampling frequency 48 kHz) and on

a computer using pClamp6 software (Axon Instruments) for off-line analysis. Several criteria were used to select cells for the experiments: stable resting membrane potential (r.m.p.) of at least −60 mV and no spontaneous firing of action potentials; no sudden drops in the neuronal input resistance (R_{in}), which are indicative of cell damage; constant amplitude of the action potential (>80 mV), obtained by direct activation of the cell. The slow afterhyperpolarization was measured at the peak (peak AHP), following a repetitive firing elicited by injection of depolarizing currents (+300/+500 pA, 400 ms) through the recording electrode. A series of current steps (400 ms, −200 pA) were constantly injected to monitor cell membrane resistance. Probability of firing (p.f.) upon direct depolarization of the neuron was calculated on five subsequent responses. To study the effects of 5-OHi on cell discharge, direct activation of the impaled neuron was obtained by the injection of short (40 ms, range 0.4–0.8 nA to elicit a probability of firing of 1 in control conditions) depolarizing current steps through the recording electrode. The intensity of the depolarizing current step was kept constant during the experiment.

Modifications in synaptic efficacy by 5-OHi were evaluated by measuring, in the presence of the $GABA_A$ receptor antagonist bicuculline (10 μM), the changes in amplitude of both the evoked excitatory postsynaptic potential (EPSP) and the slow inhibitory postsynaptic potential (slow IPSP) upon stimulation of afferent pathway. The effects of 5-OHi on evoked EPSPs and on the slow $GABA_B$-mediated IPSPs were evaluated. The slow IPSP amplitude was determined at its maximal amplitude, e.g. 200 ms after stimulus onset as previously reported (Solis & Nicoll, 1992). Due to the different timecourse of the fast EPSP and the slow IPSP, no significant overlap between these two components was introduced in our measurements (Buonomano & Merzenich, 1998).

Whole-cell patch recordings

Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded using conventional visually guided whole-cell patch recordings in voltage clamp configuration using an Axopatch 200 A amplifier (Axon Instruments), a Zeiss Axioskope equipped with DIC and a pipette of 5–7 MΩ of resistance. Electrodes were filled with (in mM): KCl 140, HEPES 10, Mg-ATP 4, Na_3-GTP 0.3, and QX3143 5. The ionotropic glutamate receptor antagonists APV (50 μM) and CNQX (25 μM) were included in the ACSF. Spontaneous IPSCs were automatically detected from a holding potential of −60 mV by using the Mini Analysis Program (Synaptosoft Inc., http://www.synaptosoft.com). Due to the high intracellular chloride concentration, IPSCs were inward currents at −60 mV.

Both the frequency and the peak amplitude of detected events were analysed. The $GABA_A$ receptor blocker bicuculline (10 μM) was routinely added at the end of experiments, to verify that the spontaneous IPSCs were completely abolished, confirming that they were $GABA_A$ receptor-mediated.

Preparation of cortical wedges

The cortical wedge preparation described by (Harrison & Simmonds, 1985) was used as previously described (Mannaioni *et al*., 1996). AMPA and NMDA-induced variations of the D.C. potentials between the two compartments were monitored *via* Ag/AgCl electrodes and displayed on a chart recorder. The preparations were initially tested by repeated application of 10 μM NMDA, a concentration which gave a sub-maximal response but which did not significantly reduce the response of subsequent applications of the agonist (Mannaioni *et al*., 1994).

Data analysis

All numerical data are expressed as mean ± s.e.mean. EC_{50} s and sigmoidal curve fitting were obtained by using Microcal Origin 6 software. GraphPad Prism software was used for calculating Hill slope. In extracellular recordings, average amplitude of five evoked responses in control condition and of three evoked responses before and after the peak during the drug application was used. Data were analysed statistically by paired or unpaired Student's *t*-test, by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test or by Kolmogorov-Smirnov test. A value of $P<0.05$ was considered statistically significant. 5-OHi-induced seizures data were analysed using the χ^2-test.

Results

5-OHi and convulsions

In preliminary experiments, we observed that intra-peritoneal administration of 5-OHi (50–100 mg kg^{-1}) to rats caused convulsions. In rats, at the dose of 50 mg kg^{-1} i.p., 5-OHi caused immobility in the open field; larger doses, however, caused clonic and tonic convulsions after a latency period of approximately 10 min (see Table 1). The total duration of the convulsive activity was 15–20 min and the animals that survived had no obvious behavioural changes.

Effects of 5-OHi administration on glutamate output in hippocampal dialysates

When 5-OHi (300 μM) was added to the dialysis solution, a biphasic increase of glutamate output was detected. A lower 5-OHi concentration (30 μM) did not change the extracellular concentrations of the amino acid (Figure 2). Interestingly, no variations in GABA extracellular concentrations were detected at the same 5-OHi concentrations used (105 ± 10% of control in 5-OHi, $n=3$; $P>0.05$).

5-OHi and extracellular recordings in CA1 hippocampal region

In hippocampal slices *in vitro*, 5-OHi (30–300 μM; 10 min application) increased the amplitude of evoked synaptic potentials recorded in the CA1 region (Figure 3A,C) with an EC_{50} of 33 ± 4 μM (Hill slope 1.7) (Figure 3B). A typical experiment showing the time-course and the effects of three 5-OHi concentrations is shown in Figure 3A,C. The increase of synaptic responses amplitude was reversible within 15 min of drug washout (Figure 3C). 5-OHi applications increased both the population spike component of evoked synaptic responses (i.e. the compound action potential generated by synchronous

Table 1 Effects of 5-OHi administrations

	Clonic convulsions	*Tonic convulsions*	*Death*
Saline	0/6	0/6	0/6
5-OHi 50 mg kg^{-1}	0/6	0/6	0/6
5-OHi 80 mg kg^{-1}	3/5*	0/5	0/5
5-OHi 100 mg kg^{-1}	4/5*	3/5*	3/5*

5-OHi was injected i.p. and the animal behaviour monitored for 30 min. Values represent number of animals showing clonic or tonic convulsions or death over the total number of animals tested. *$P<0.05$ *vs* saline χ^2-test.

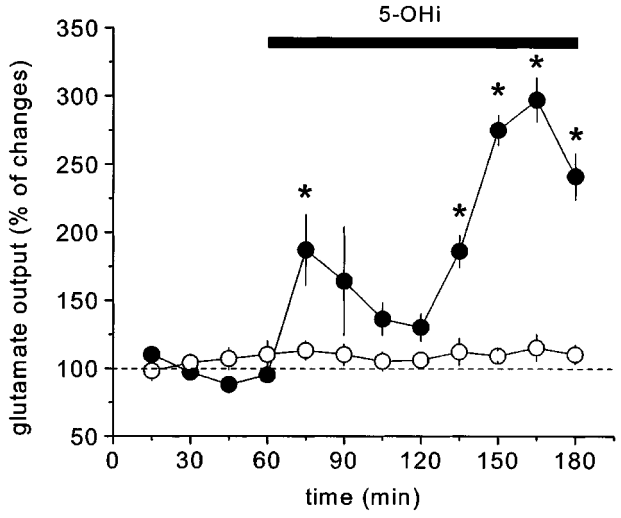

Figure 2 5-OHi increases glutamate output from rat hippocampus. Time courses of the effect of constant injection throughout the implanted micro-dialysis tubing of 5-OHi (30 μM open circle and 300 μM filled circle) on glutamate concentrations in the dialysis fluid. The amino acid was measured in fractions collected every 15 min beginning 15–18 h after the implantation of the dialysis probe. The mean value obtained for each animal just before the treatment with 5-OHi was defined spontaneous output (100%) and was 6.5 ± 1.2 μM (mean $\pm$ s.e.mean of values obtained in 20 rats). Glutamate recovery in the probes was 50 ± 4%. Data are the average values obtained in four rats. Filled bar indicates the time of drug applications. *$P<0.05$ *vs* saline (ANOVA).

discharge of CA1 pyramidal cells) and the fEPSP. When 5-OHi was used at 30 μM, the population spike amplitude increased by 34 ± 4% ($n=5$, $P<0.05$), at 100 μM by 66 ± 8% ($n=10$, $P<0.05$) and at 300 μM, increased by 78 ± 9% ($n=5$, $P<0.05$).

5-OHi also increased the evoked fEPSP amplitude recorded in the dendritic region of CA1 pyramidal cells ($EC_{50}=38\pm8$ μM, Hill slope 2, Figure 4B). Figure 4C shows a typical experiment in which three 5-OHi concentrations were tested. The increase of fEPSP amplitude was associated with an increase of afferent volley amplitude (from 135 ± 15 to 184 ± 13 μV; $P<0.05$).

5-OHi and intracellular recording in CA1 hippocampal region

To elucidate 5-OHi effects on excitability of CA1 pyramidal cells, we measured the number of spikes generated by a current pulse and the latency of the first spike generation. 5-OHi (100 μM) reversibly increased the number of action potentials elicited by a direct excitation of the cell produced by the injection of a constant current step through the recording electrode (Figure 5A upper, middle and lower panel) (p.f. 1 in control *vs* 1.9 ± 0.05 in 5-OHi, $n=5$, $P<0.05$). This result was associated with a significant and reversible reduction of the latency of the first action potential (Figure 5B and Table 2) (30.4 ± 0.46 ms in control *vs* 22.1 ± 0.7 ms in 5-OHi and 29.1 ± 0.5 ms in wash out; $n=5$; $P<0.05$) whereas action potentials amplitude, action potential duration, cell input resistance, peak AHP and membrane potential were not significantly changed (Table 2). It is interesting to note that these effects may underlie the increased fibre volley observed in the field components. However, the increase of the fEPSP indicates that 5-OHi has additional effects on synaptic transmission and we investigated this issue with intracellular recordings as shown in Figure 6.

Stimulation of the CA1 stratum radiatum in the presence of the $GABA_A$ channel blocker bicuculline (10 μM) induced an evoked EPSP followed by a slow $GABA_B$-mediated IPSP (Figure 6A, left panel). Application of 5-OHi (100 μM) significantly increased the amplitude of evoked EPSP (149 ± 7% of control in 5-OHi, $n=4$; $P<0.05$) (Figure 6A, middle panel and right panel, Figure 6B) as well as the amplitude of slow-IPSP (214 ± 8% of control in 5-OHi, $n=4$; $P<0.05$) (Figure 6A, middle and right panel, Figure 6B). Thus, it appears that 5-OHi applications potentiated synaptic transmission.

To rule out the possibility that the increase of IPSP amplitude is mediated by an increased excitatory drive to GABAergic interneurones, we checked the effects of 5-OHi on pharmacologically isolated sIPSCs. Figure 7A shows an example of spontaneous IPSCs recorded under control conditions and during bath application of 5-OHi. A 3-min bath application of 5-OHi (100 μM), in the presence of APV (50 μM) and CNQX (25 μM), caused a robust and reversible increase in spontaneous IPSC frequency (5.6 ± 1 Hz in control *vs* 8.4 ± 1.4 Hz in 5-OHi, $n=8$; $P<0.05$) (Figure 7A,B, left panel and D, left panel) and produced a leftward shift of the cumulative probability distributions of sIPSC inter-event intervals (Figure 7C, left panel), indicating a 5-OHi-induced increase in the frequency of sIPSCs. Application of 100 μM 5-OHi also produced a significant effect on sIPSC amplitudes (53 ± 6 pA in control *vs* 75 ± 8 pA in 5-OHi, $n=8$; $P<0.05$) (Figure 7B, right panel and E, left panel) and a rightward shift of the cumulative fraction distribution of sIPSCs amplitudes (Figure 7C, right panel). The average frequency and amplitude was calculated for each cell, and the means of these values are shown in Figure 7D and E.

Due to the previously described potentiating effects of 5-OHi on 5-HT_3 and α_7 nAChR (Gurley *et al*., 2000; Kooyman *et al*., 1993; 1994), we tested the ability of tropisetron (30 nM) or methyllycaconitine (MLA 3 μM), a selective 5-HT3 receptor and nAChR α_7 antagonist respectively, to antagonize 5-OHi mediated effects on sIPSCs. Pre-incubation (8–10 min) with either tropisetron or MLA did not block the 5-OHi-induced increase either in sIPSCs frequency (4.7 ± 0.9 Hz in control *vs* 7 ± 0.6 Hz in 5-OHi and tropisetron, $n=6$, $P<0.05$ and 3.9 ± 1 Hz in control *vs* 5.7 ± 1 Hz in 5-OHi and MLA, $n=3$, $P<0.05$) (Figure 7D, middle and right panel) or amplitude (30 ± 3 pA in control *vs* 40 ± 4 pA

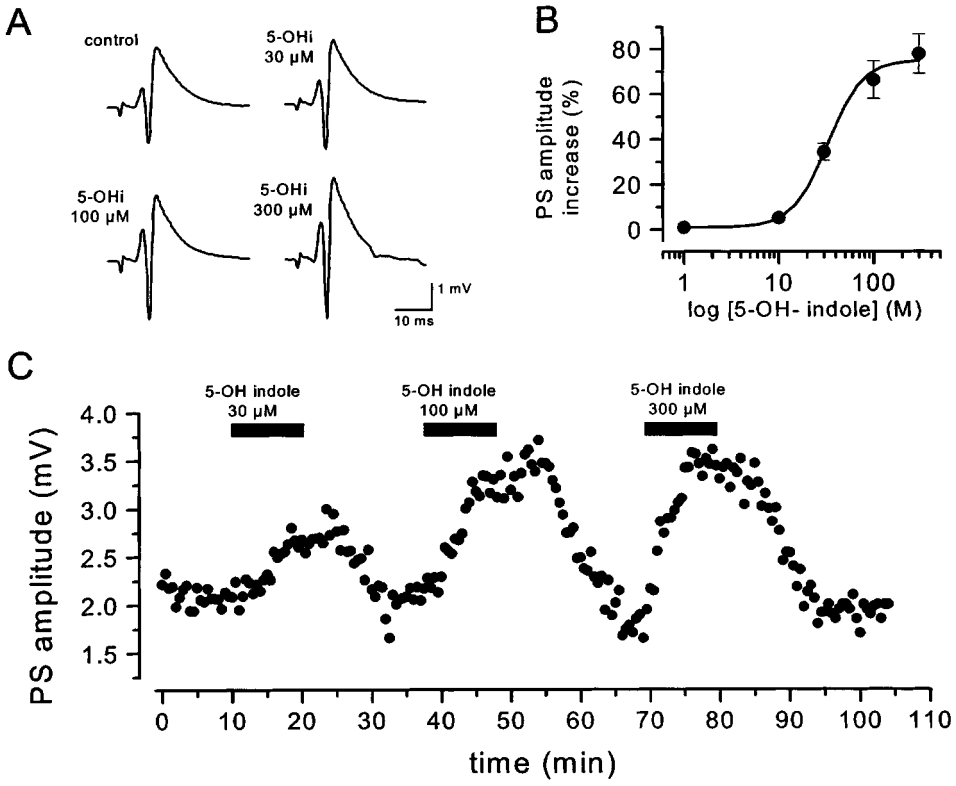

Figure 3 5-OHi increases the amplitude of population spike in CA1 hippocampal region. (A) Application of 5-OHi increases the amplitude of somatic potentials in a concentration-dependent manner. Traces are single responses evoked by constant strength (16 V) stimuli (biphasic fast transients) in control and at the end of drug application. (B) Concentration-response curve of 5-OHi-induced increase in amplitude of population spike. Values are means ± s.e.mean of five experiments. (C) Time-course from a single experiment showing the increase of amplitude of population spike induced by 5-OHi. Points represent the amplitude of the population spike expressed in mV. Filled bars indicate the time of drug applications. Data shown are obtained from the same experiment, which was repeated five times with similar results (see B).

in 5-OHi and tropisetron, $n=6$, $P<0.05$ and 40 ± 1 pA in control *vs* 49 ± 0.3 pA in 5-OHi and MLA, $n=3$, $P<0.05$) (Figure 7E, middle and right panel).

Cortical wedge preparations

Finally, in order to evaluate the possibility that the 5-OHi-induced effects on cell excitability and on synaptic transmission were mediated by an interaction with glutamate receptor, we tested the effects of 5-OHi on AMPA/kainate or NMDA receptor responses using 'cortical wedges', a technique which allows the study of excitatory amino acid agonists and antagonists (Mannaioni *et al*., 1996). In the presence of TTX (1 μM), 5-OHi application (300 μM) did not affect either AMPA (4 and 2 μM) or NMDA responses (10 and 5 μM) (Figure 8).

Discussion

While oxindole administration to rodents causes sedation and coma (Mannaioni *et al*., 1998), our results show that the administration of similar doses of 5-OHi, an oxindole analogue, caused clonic and tonic convulsions. When 5-OHi was locally applied, through reverse microdialysis in the dorsal hippocampus *in vivo*, it increased glutamate output suggesting that it was able to increase excitatory transmitter release. On the other hand, no increase in GABA output was detected in the microdialysis experiments. The 5-OHi induced increase of neuronal activity could be responsible for an augmented neuronal re-uptake of GABA (Moroni *et al*., 1982) modulating therefore GABA concentration in the synaptic cleft and offsetting any potential increase in GABA release due to the increased firing of interneurones. In electrophysiological experiments, in rat hippocampal slices *in vitro*, 5-OHi enhanced both excitatory and inhibitory neurotransmission in CA1 hippocampal pyramidal cells. In extracellular recording experiments, it caused an increase of the PS amplitude, a parameter reflecting the synchronous discharge of neuron action potentials located close to the recording electrode. Since this amplitude is proportional to the number of discharging cells (Lynch & Schubert, 1980), our results suggested that 5-OHi increased the excitation of the pyramidal cells after Shaffer collateral stimulation. This effect could be due to an increase of the afferent excitatory input, an increase of neuronal excitability or a combination of both mechanisms. Since 5-OHi also increased fEPSP and fibre volley amplitude, an index of afferent recruitment, we assumed that 5-OHi effects were mediated by an increase of the afferent excitatory input. In support of this possibility stand the above mentioned 5-OHi-induced increase of glutamate concentration in the extracellular hippocampal spaces and the lack of direct interaction between 5-OHi and AMPA or NMDA receptors (see the experiments in cortical

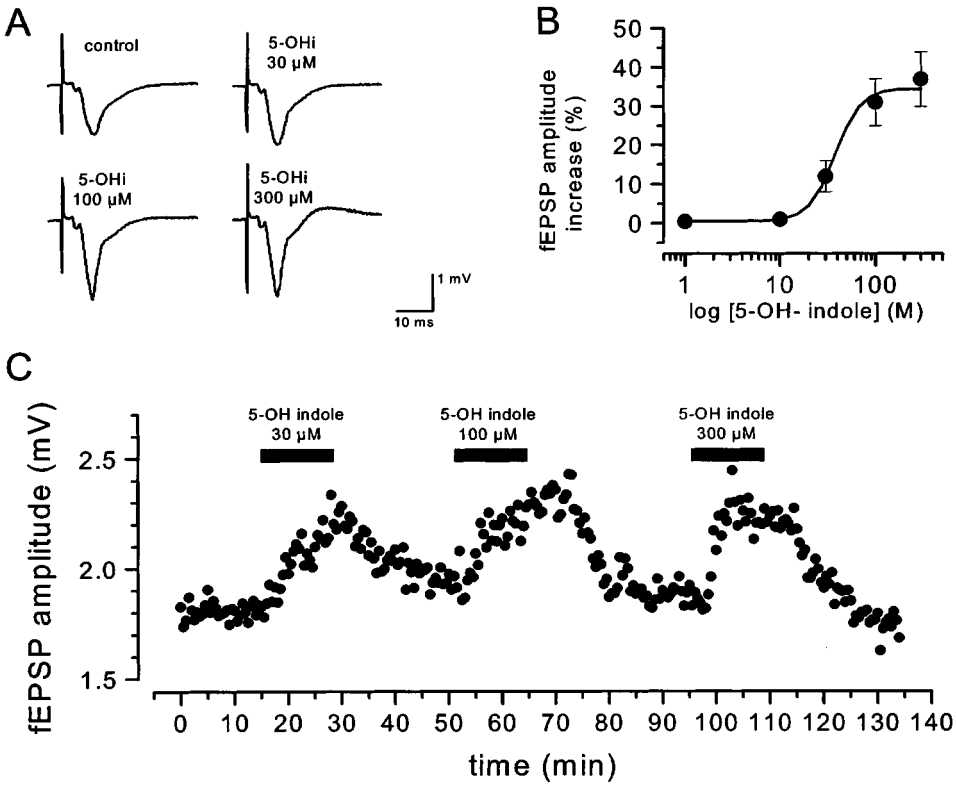

Figure 4 5-OHi increases the amplitude of field EPSP in CA1 hippocampal region. (A) Application of 5-OHi increases the amplitude of field EPSP (fEPSP) in a concentration-dependent manner. Traces are single responses evoked by constant strength stimuli (4 V) in control and at the end of drug application. (B) Concentration-response curve of 5-OHi-induced increase in amplitude of fEPSP. Values are means ± s.e.mean of five experiments. (C) Time-course from a single experiment showing the increase of amplitude of fEPSP induced by 5-OHi. Points represent the amplitude of the fEPSP expressed in mV. Data shown are obtained from the same experiment, which was repeated five times with similar results (see B).

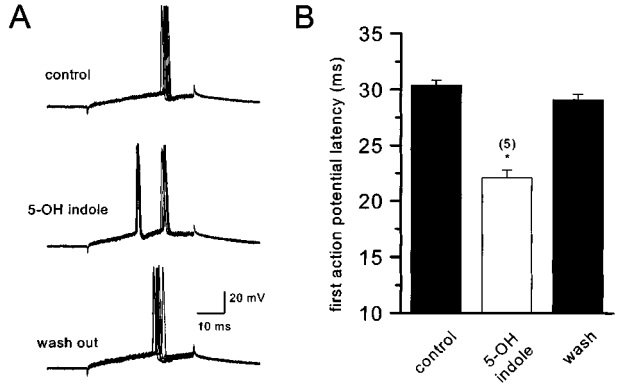

Figure 5 5-OHi increases the excitability of CA1 pyramidal neurones, induced by direct depolarization, by reducing the latency of firing an action potential. (A) Each panel shows five superimposed responses evoked by a depolarizing current injection (+400 pA, 40 ms) through the recording electrode; under control conditions (top), in the presence of 5-OHi 100 μM (middle) and after 15 min wash out (bottom). The amplitude and duration of the action potentials under control conditions and under 5-OHi treatment were not changed (not shown). (B) The bar graph represents the mean of the latency to first action potential fired, following a depolarizing step, in control conditions and after 5-OHi applications. Number of cells tested is indicated in parentheses. Values are mean (± s.e.mean). *$P<0.05$ *vs* control; *t*-test.

wedges reported in Figure 8) that are present in CA1 pyramidal neurones (Collingridge *et al*., 1983).

With the aim of further exploring the effects of 5-OHi on pyramidal cell excitability and synaptic transmission, we performed experiments using intracellular recordings and direct or synaptic stimulation of the pyramidal cells. With direct stimulation, 5-OHi increased the probability of firing action potentials and reversibly decreased the latency of the first spike appearance, but did not change cell input resistance, peak AHP and membrane potential. These data, obtained in the presence of bicuculline, could indicate that the 5-OHi-induced increase of endogenous extracellular glutamate could be responsible for the facilitation in action potential discharge as previously described (Sah *et al*., 1989). Nonetheless, a modulation of K^+ channels by 5-OHi cannot be ruled out.

When studied in the evoked synaptic transmission, 5-OHi not only increases the evoked EPSP amplitude but it also increases the $GABA_B$-mediated slow IPSP amplitude suggesting a role of 5-OHi in modulating, in a non-specific manner, both excitatory and inhibitory neurotransmission. The finding that 5-OHi increases the frequency and the amplitude of spontaneous IPSCs, an effect mediated by activation of $GABA_A$ receptors (Ropert *et al*., 1990) as well as the $GABA_B$-mediated slow IPSP component, is consistent with the possibility that 5-OHi causes GABA release from hippocampal interneurones (Maccaferri *et al*., 2000).

Previous studies have shown that 5-OHi may facilitate the activation of both 5-HT3 (Kooyman *et al*., 1993; 1994) and nACh α_7 receptors (Gurley *et al*., 2000). Since activation of 5-

Table 2 Effects of 5-OHi on main properties and excitability of CA1 hippocampal pyramidal neurones

	Control	*5-OHi 100 μM*
V_m (mV)	−67.2±1.2	−68.9±1.7
R_{in} (MΩ)	34.3±5	35.8±6
AP latency (ms)	30.4±0.46	22.1±0.7*
AP amplitude (mV)	101±5	103±4
AP half width (ms)	0.85±0.04	0.83±0.06
peak AHP (mV)	−3.8±1	−3.9±1.2

Data are means±s.e.mean. 5-OHi was applied for 10 min at the indicated concentrations. V_m: resting membrane potential R_m: neuronal input resistance. Action potential (AP) amplitude was measured from V_m. AP half width was the time between 50% of the peak on the rising phase and 50% of the peak on the falling phase. AHP: peak amplitude of the AHP, which followed repetitive discharge cells elicited by positive step (range +300/+500 pA). *Significantly different from the control ($P<0.05$; *t*-test; $n=5$).

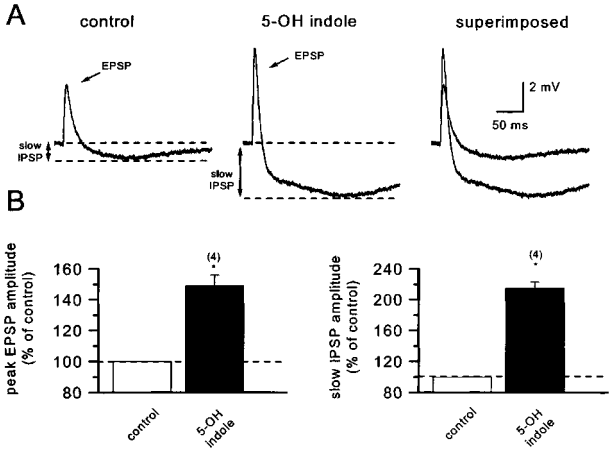

Figure 6 5-OHi increases evoked EPSP and evoked $GABA_B$-mediated slow IPSPs elicited by electrical stimulation of the stratum radiatum in CA1 hippocampal neurones. (A) Traces are the mean of five responses evoked by stimulation of the stratum radiatum (3.5 V, transient not shown in the presence of bicuculline 10 μm) in control (left), after a 10-min application of 5-hydroxyndole 100 μM (right) and superimposed. (B) Bar graphs showing the increase induced by 5-OHi application on the evoked EPSP amplitude (left panel) and on the $GABA_B$-mediated slow IPSP, calculated after 200 ms from the stimulus (right panel). Open bars are control conditions and filled bars are 5-OHi application. Number of cells tested is indicated in parentheses. Values are mean (±s.e.mean). *$P<0.05$ *vs* control; *t*-test.

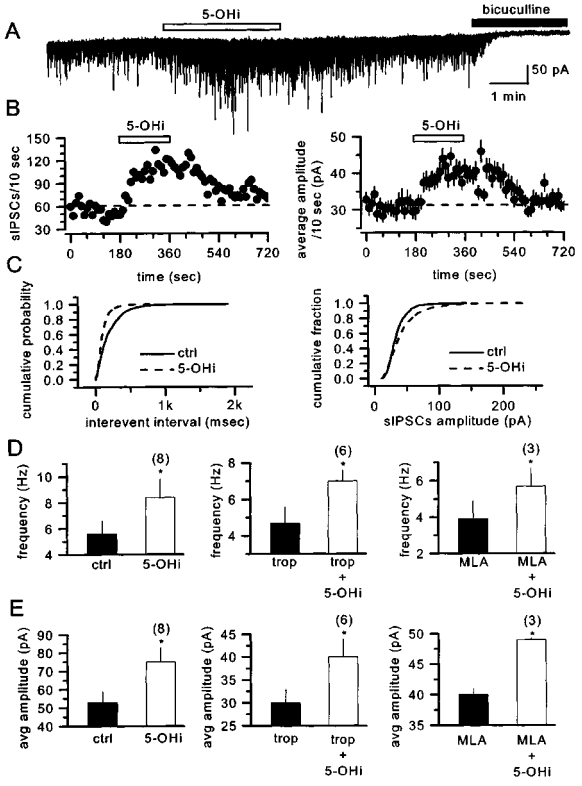

Figure 7 5-OHi increases the frequency and the amplitude of pharmacologically isolated spontaneous inhibitory postsynaptic currents (sIPSC) in CA1 pyramidal neurones. (A) Example of a typical experiment showing the increase in frequency of sIPSCs following 3 min application of 5-OHi (100 μM; open bar). The figure also shows the inhibitory action of bicuculline (10 μM; filled bar) on sIPSCs confirming $GABA_A$ dependency of the events. Trace is representative of results obtained in eight different cells tested. (B) Time course obtained from trace in (A) showing sIPSCs frequency (left panel) and amplitude (mean±s.e.mean; right panel) plotted *vs* the time of the experiment (10 s bins). Perfusion of the recording chamber with 100 μM 5-OHi induced an increase in both frequency and amplitude of sIPSCs. (C) Cumulative probability plots demonstrating the effect of 5-OHi on sIPSC inter-event interval and amplitude. Application of 5-OHi caused a significant shift in the inter-event interval distributions toward shorter interval (left panel) and a significant shift in the sIPSCs amplitude toward bigger amplitude (right panel), indicating a significant increase in the frequency and amplitude of sIPSCs respectively (Kolmogorov-Smirnov (K−S) statistic $P<0.01$). (D) and (E) Bar graphs demonstrating the increase in the frequency and amplitude of sIPSCs, induced by 5-OHi alone and in the presence of tropisetron (3 nM) or methyllycaconitine (MLA, 3 μM). Number of cells tested is in parenthesis; values are mean±s.e.mean. *$P<0.05$ *vs* control; *t*-test.

HT3 receptor may cause inhibitory effects in the CA1 region of the rat hippocampus (Passani *et al*., 1994; Ropert & Guy, 1991) and since nACh receptors may potentiate GABAergic transmission in multiple brain areas, such as thalamus, cortex, hippocampus and interpeduncular nucleus (Radcliffe *et al*., 1999; Fisher *et al*., 1998; Alkondon *et al*., 1997; Lena *et al*., 1993) we investigated whether tropisetron, a 5-HT3-antagonist, or MLA, a selective nACh α_7 antagonist, antagonized 5-OHi effects. The two antagonists were tested at fully active concentrations (Dobelis *et al*., 1999; Pei *et al*., 1993) but failed to modify 5-OHi-induced increase in frequency and amplitude of sIPSCs. It seems therefore that the described actions of 5-OHi on 5HT3 and nACh α_7 receptors cannot explain the results we obtained in hippocampal slices. However, possible interactions of 5-OHi on nACh receptors different from the α_7 subtypes have not been studied. Interestingly, it has recently been reported that nicotine increases sIPSCs in the ventral tegmental area through a α_7 insensitive mechanism (Mansvelder *et al*., 2002) and that several types of presynaptic nACh receptors enhance transmitter release from presynaptic terminals (Wonnacott, 1997). Therefore, we cannot rule out the possibility that the increased transmitter output observed in the presence of 5-OHi could be mediated by nACh receptors different from the α_7 subtype.

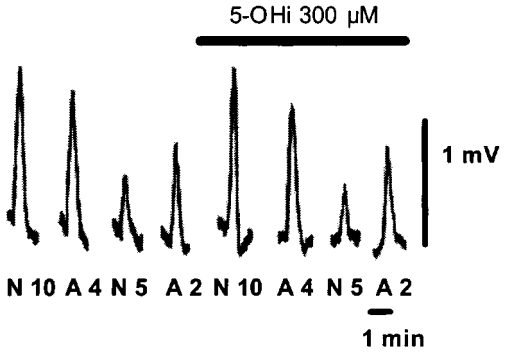

Figure 8 5-OHi does not affect NMDA or AMPA responses in wedges of mouse cerebral cortex. Records from a typical experiment show the response elicited by application (2 min, spaced by 15 min wash) of NMDA (indicated as N, 10 and 5 μM) or AMPA (indicated as A, 4 and 2 μM) in a Mg^{2+}-free aCSF (see Methods) at the indicated concentrations. The d.c. shifts produced by either agonist were not changed in the presence of 5-OHi (300 μM). Similar results were obtained in a total of three experiments.

Alternative hypothesis to explain 5-OHi actions are also possible. For example, the 5-OHi enolic group may undergo oxidative reactions with concomitant productions of oxygen derived free radicals. Since a number of free radical species increase transmitter release from hippocampal slice preparations with a mechanism that is not dependent upon direct receptor activation (Pellegrini-Giampietro *et al*., 1988; 1990), we cannot exclude this possibility to explain 5-OHi effects. Experiments are currently in progress to investigate this hypothesis.

We mentioned that 5-OHi is a tryptophan metabolite that may accumulate in blood and brain when indole levels are increased (King *et al*., 1966). In rats with liver failure and hepatic encephalopathy, indole is absorbed from the gut and then metabolized into several metabolites, including oxindole and 5-OHi (Carpenedo *et al*., 1998; Moroni *et al*., 1997) that may be measured in blood and brain. Hepatic encephalopathy is a complex disorder associated with changes in the balance between excitatory and inhibitory neurotransmission (Butterworth, 2001; Albrecht & Jones, 1999). Changes in GABA and glutamate metabolism and receptor functions have been described in several experimental models of this disorder (Gammal *et al*., 1990; Zeneroli & Baraldi, 1990; Moroni *et al*., 1983). Since 5-OHi increases glutamate release and the function of GABA interneurons, its participation in the processes leading to the clinical manifestation of the syndrome appears reasonable (Butterworth, 2001). In previous studies we noticed that oxindole levels increased in blood and brain of both rats and humans affected by hepatic encephalopathy (Mannaioni *et al*., 1999; Berqvist *et al*., 1999; Carpenedo *et al*., 1998) and the possibility that 5-OHi increases in parallel with oxindole should certainly be considered.

In conclusion, 5-OHi is a relatively potent indole metabolite able to cause convulsions and death when administered at relatively large doses to rodents. Its mechanism of action seems mainly based on potentiation of transmitter release, an effect that has been demonstrated here in hippocampal slices and with *in vivo* microdialysis.

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