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# Differential modulation of $I_h$ by 5-HT receptors in mouse CA1 hippocampal neurons

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## Abstract

CA1 pyramidal neurons of the hippocampus express various types of serotonin (5-HT) receptors, such as 5-$HT_{1A}$, 5-$HT_4$ and 5-$HT_7$ receptors, which couple to $G\alpha_i$ or $G\alpha_s$ proteins and operate on different intracellular signalling pathways. In the present paper we verify such differential serotonergic modulation for the hyperpolarization-activated current $I_h$. Activation of 5-$HT_{1A}$ receptors induced an augmentation of current-induced hyperpolarization responses, while the responses declined after 5-$HT_4$ receptors were activated. The resting potential of neurons hyperpolarized (–2.3 ± 0.7 mV) after 5-$HT_{1A}$ receptor activation, activation of 5-$HT_4$ receptors depolarized neurons (+3.3 ± 1.4 mV). Direct activation of adenylyl cyclase (AC) by forskolin also produced a depolarization. In voltage clamp, the $I_h$ current was identified by its characteristic voltage- and time-dependency and by blockade with CsCl or ZD7288. Activation of 5-$HT_{1A}$ receptors reduced $I_h$ and shifted the activation curve to a more negative voltage by –5 mV at half-maximal activation. Activation of 5-$HT_4$ and 5-$HT_7$ receptors increased $I_h$ and shifted the activation curve to the right by +5 mV. Specific activation of 5-$HT_4$ receptors by BIMU8 increased membrane conductance and showed an increase in $I_h$ in a subset of cells, but did not induce a significant alteration in the activation curve. In order to verify spatial differences, we applied BIMU8 selectively to the soma and to the dendrites. Only somatic application induced receptor activation. These data are confirmed by immunofluorescence stainings with an antibody against the 5-$HT_{4A}$ receptor, revealing receptor expression at the somata of the CA1 region. A similar expression pattern was found with a new antibody against 5-$HT_7$ receptors which reveals immunofluorescence staining on the cell bodies of pyramidal neurons.

## Introduction

Serotonin (5-HT) is a ubiquitous transmitter and neuromodulator which interacts with at least 14 different receptor isoforms in the brain (for review see Barnes & Sharp, 1999). So far, seven subgroups of 5-HT receptors have been classified: 5-$HT_1$ receptors are negatively coupled to adenylyl cyclases (ACs), 5-$HT_2$ receptors are coupled to phospholipase C, 5-$HT_3$ receptors form an ion channel and 5-$HT_4$ receptors are coupled positively to ACs via $G\alpha_s$ proteins (Dumuis *et al*., 1988; Bockaert *et al*., 1998; Ponimaskin *et al*., 2001; Heine *et al*., 2002). 5-$HT_6$ and 5-$HT_7$ receptors are also $G\alpha_s$-coupled receptors and increase the production of 3′,5′-cyclic adenosine monophosphate (cAMP). The function of 5-$HT_5$ receptors remains as yet unclear.

In the hippocampus, the mRNAs of 10 of these 14 receptor isoforms have been identified using *in situ* hybridization (for review see Andrade, 1998; Heidmann *et al*., 1998). A clear electrophysiological function of 5-$HT_{1A}$ and 5-$HT_4$ receptors has been demonstrated for CA1 pyramidal neurons, while GABAergic interneurons are modulated by 5-$HT_1$, 5-$HT_2$ and 5-$HT_3$ receptors (Andrade & Nicoll, 1987; Roychowdhury *et al*., 1994; Freund & Buzsaki, 1996; Schmitz *et al*., 1998; Barnes & Sharp, 1999). 5-$HT_{1A}$ receptors hyperpolarize neurons either through direct activation of potassium conductances by the βγ subunits of the $G\alpha_i$ complex (Ehrengruber *et al*., 1997; Luscher *et al*., 1997) or indirectly by the $G\alpha_i$–AC pathway (Karschin, 1999). The 5-$HT_4$ receptors induce a depolarization of CA1 neurons via an as yet unidentified cationic conductance (Andrade, 1998) and induce a reduction of afterhyperpolarizations following action potentials (Torres *et al*., 1996). The physiological role of 5-$HT_7$ receptors in hippocampal neurons remains to be investigated. In thalamic neurons, however, 5-$HT_7$ receptors modulate the hyperpolarization-activated current $I_h$ (Chapin & Andrade, 2001).

5-HT-induced modulation of CA1 neurons involves $I_h$ channels (for review see Pape, 1996; Santoro *et al*., 2000), which are sensitive to cAMP in a voltage-dependent manner (Larkmann & Kelly, 1997; Gasparini & DiFrancesco, 1999; Bickmeyer *et al*., 2000; Chapin & Andrade, 2001). $I_h$ channels are expressed in the soma as well as in the dendrites of CA1 neurons (Magee, 1998). The $I_h$ current is effective at the resting potential and modifies postsynaptic signal processing (Magee, 1998; Magee, 1999). In the present paper, we address two questions, the first about a differential spatial expression of 5-HT receptors and the second about subtype-specific modulation of the $I_h$ current.

## Materials and methods

### *Electrophysiology*

Experiments were performed on 9–14-day-old-mice, which were killed by decapitation under deep ether narcosis. The brain was quickly removed and transferred into cold (4 °C) artificial cere-

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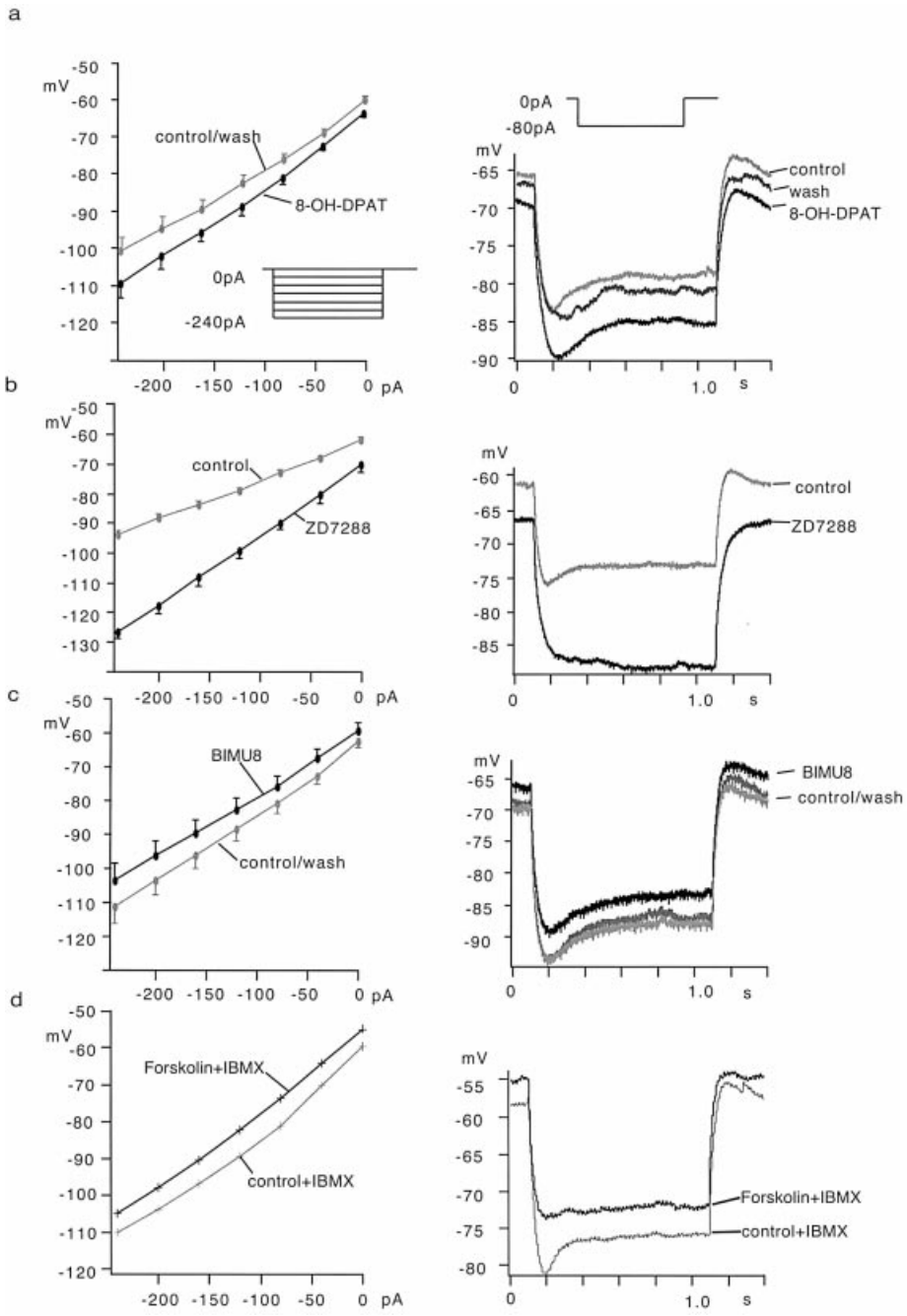


FIG. 1. (a) Voltage response to hyperpolarizing current pulses before and after activation of 5-$HT_{1A}$ receptors. *I–V* curve in current clamp with and without 20 μM 8-OH-DPAT ($n$ = 11). (b) Voltage response to hyperpolarizing current pulses before and after application of 10 μM ZD7288 ($n$ = 3). (c) Voltage response to hyperpolarizing current pulses with and without activation of 5-$HT_4$ receptors. *I–V* curve in current clamp before and after application of 20 μM BIMU8. ($n$ = 8). (d) Forskolin (50 μM) and IBMX (50 μM) evoked a depolarization of membrane potential and changed the voltage response to a hyperpolarizing current pulse.

brospinal fluid (ACSF). The hemispheres were separated and their medial plane fixed on a tissue slicer (vibro slicer Leica VT1000S; Solms, Germany) to cut 300-μm transverse slices, which were transferred into a chamber and superfused with ACSF that was gassed with carbogen (95% $O_2$ and 5% $CO_2$) at room temperature. A minimum of 60 min was allowed for recovery from mechanical trauma. CA1 pyramidal cell were identified by their shape and by their characteristic discharge pattern in current-clamp experiments (Freund & Buzsaki, 1996). For electrophysiological measurements, slices were transferred into the experimental chamber mounted on a Zeiss Axioskop (Zeiss, Göttingen, Germany). ACSF contained (in mM) NaCl, 125; KCl, 2.5; $MgCl_2$, 1.0; $CaCl_2$, 2; $NaH_2PO_4$, 1.3; Na-Pyruvate, 2; $NaHCO_3$, 24; and D-glucose, 30. We used borosilicate pipettes to produce patch electrodes with a tip diameter of 1.5–2 μm and resistances of 4–6 MΩ. Our standard pipette solution contained (in mM) K-gluconate, 140; $MgCl_2$, 2; $CaCl_2$, 1; Na-adenosine

5′-triphosphate (ATP), 2; ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 10; (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonicacid]) (HEPES), 10; and guanosine 5′-triphosphate (GTP), 0.4. EGTA (10 mM) was used to minimize the influence of free calcium on $I_h$ (Lüthi & McCormick, 1998). For selective blockade of $Na^+$, $Ca^{2+}$ and $K^+$ conductances, but not $I_h$, in some voltage-clamp experiments an 'isolation buffer' was used containing 1 μM TTX and (in mM) $BaCl_2$, 2; $CdCl_2$, 0.1; tetraethylammonium-Cl, 10; 4-aminopyridine, 2; $MgCl_2$, 1; KCl, 3; NaCl, 110; $NaHCO_3$, 20; and D-glucose, 30.

Local drug application was performed by pressure ejection (PDES2l NPI, Tamm, Germany) through a patch pipette with a tip diameter of 3–4 μm. There was no detectable leak of drugs out of the pipette. This was verified with different dyes such as methylene blue. We never observed any drug effect before onset of pressure application. Voltage- and current-clamp measurements were performed with a SEC05 discontinuous single-electrode voltage-clamp amplifier (NPI; Tamm, Germany) or an EPC9 patch clamp amplifier (HEKA, Lambrecht, Germany). A liquid junction potential of 4 mV was measured according to Neher (1992), but data were not corrected. Statistical evaluation was performed with Graphpad Prism software using Student's *t*-test. Data are presented as means ± SEM. The measurements were unlikely to have been seriously affected by washout of cellular components during whole-cell recordings because drug effects were at least partially reversible.

### *Immunofluorescence*

We developed a new antibody from rabbits immunized with a synthetic peptide with the sequence CKHERKNISSFKREQK (amino acid positions: 348–363). This corresponds to a sequence of the third intracellular loop of the mouse 5-$HT_7$ receptor (see Vanhoenacker *et al*., 2000). For detection of the 5-$HT_{4A}$ receptor, we used the antibody AS9459 as described by Ponimaskin *et al*. (2001) and Heine *et al*. (2002), which is directed against a characteristic sequence close to the C-terminus of the receptor. After fixation in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), brains were cryoprotected in 30% sucrose, frozen at –24 °C and cut into 30-μm slices by a cryoslicer (Reichert-Jung, Wetzlar, Germany). Cells were permeabilized with 0.5% Triton X-100 and nonspecific binding was blocked with 2% bovine serum albumin (BSA) and 10% goat serum in PBS. Slices were incubated overnight in PBS containing the primary antibody at 1 : 400 dilution. After washing and blocking with 2% BSA, 2% goat serum in PBS, Alexa 488 was used as second fluorescent antibody (Molecular Probes, Leiden, Netherlands) in a concentration of 1 : 200 in 2% goat serum in PBS. Incubation in the dark was done for 1 h at room temperature. Immunofluorescence analysis was performed using a laser scanning confocal microscope (LSM 510; Zeiss, Göttingen, Germany). As a negative control, we used the preimmune serum of the rabbit processed as described above. Staining of cell nuclei was achieved with a nuclear acid staining with propidium iodide in a final concentration of 25 μg/mL.

ELISA testing did not reveal cross-reactivity between the peptides used for immunization against 5-$HT_{4a}$ and 5-$HT_7$ receptors.

For Western blots we used nitrocellulose transfer membranes (PVDF; Amersham, Braunschweig, Germany) and probed for 5-$HT_7$ peptides with a polyclonal rabbit antiserum (1 : 1000 dilution) and antirabbit IgG antibody coupled to peroxidase (1 : 2000 dilution; Sigma). Detection was performed with an enhanced chemoluminescence kit (ECL Plus; Amersham). Film exposure times ranged between 1 and 30 s.

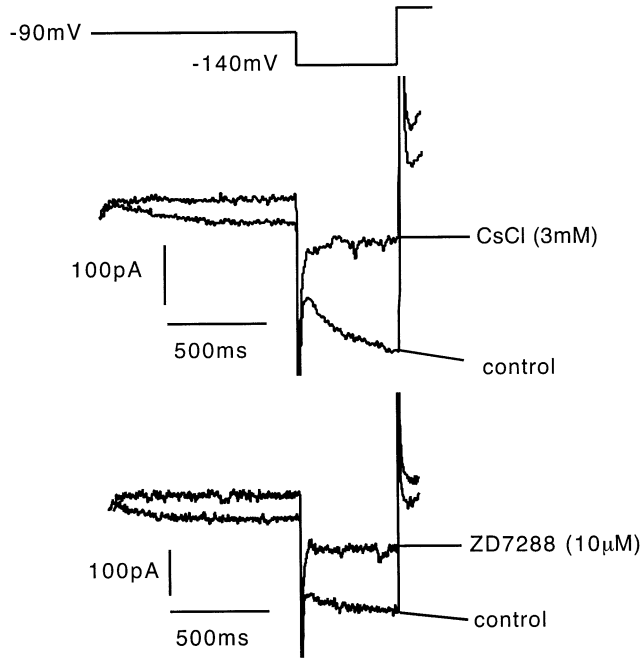


FIG. 2. Blockade of $I_h$ with 3 mM CsCl and 10 μM ZD7288.

### *Chemicals*

BIMU8 [4-ethylphenylamino-1,2-dimethyl-6-((endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dehydro-2-oxo-3-(prop-2-yl)-ICH-benzimid-azole-1-carboxamide)] was kindly provided by Boehringer Ingelheim (Ingelheim, Rhineland-Pallatinate, Germany), GR113808 ({1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl}methyl-1-methyl-1-H-indol-3-carboxylate, maleate salt) was a gift from Glaxo-Wellcome (Herts, UK) and other chemicals were purchased from Biotrend (Cologne, North Rhine–Westphalia, Sigma (Taufkirchen, Bavaria, Germany), Merck (Darmstadt, Hesse, Germany) or MoBiTec (Göttingen, Lower Saxony, Germany). The company Bioscience (Göttingen, Lower Saxony, Germany) helped us to produce the antibodies.

## Results

### *Current-clamp analysis of receptor-specific effects*

To activate 5-$HT_{1A}$ and $5HT_4$ receptors we used BIMU8 as a selective 5-$HT_4$ receptor agonist (Eglen *et al*., 1995) and 8-OH-DPAT [8-hydroxy-2-(di-n-propylamino)tetralin] as a 5-$HT_{1A}$ receptor activator.

The resting membrane potential of CA1 neurons was –60.7 ± 1.4 mV. During application of 20 μM 8-OH-DPAT, neurons slightly hyperpolarized by –2.3 ± 0.7 mV ($P < 0.01$, $n = 25$). After 20 μM BIMU8 was applied, they depolarized by +3.3 ± 1.1 mV ($P < 0.01$, $n = 23$; Fig. 1). During 5-$HT_{1A}$ receptor activation with 8-OH-DPAT, hyperpolarizing responses to negative current pulses were augmented. The steady state *I–V* curves (measured 1 s after beginning of a stimulus) showed a significant shift towards more negative potentials (Fig. 1a). This increase in the voltage responses originated from a reduction in conductances, which involves $I_h$. The voltage change induced by $I_h$ produced a characteristic decay after an initial peak ('sag') as shown in Fig. 1a and b. The voltage decay

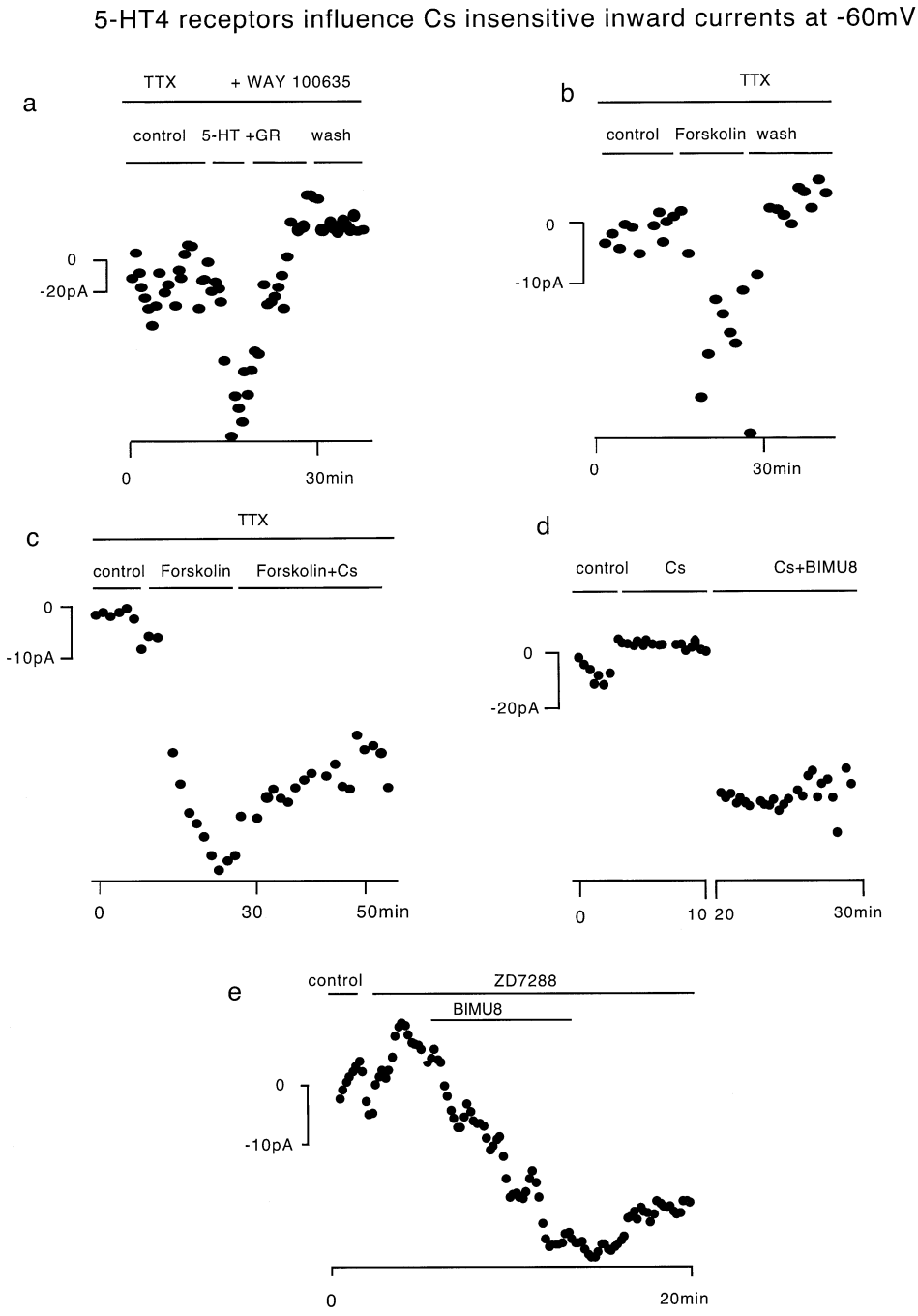


FIG. 3. (a) The 5-HT effect on holding current close to resting membrane potential (≈ –60 mV). The 5-HT (50 μM)-induced inward current was blocked by the 5-$HT_4$ receptor antagonist GR113808 (10 μM). (b) The 5-HT effect was mimicked by 50 μM forskolin. (c) Forskolin (50 μM)-induced inward current was not fully blocked by 3 mM CsCl. (d) 5-$HT_4$ receptor-induced inward current in the presence of 3 mM CsCl. (e) 25 μM ZD7288 blocked $I_h$ and therefore induced a net outward current. 50 μM BIMU8 in the presence of 25 μM ZD7288 induced an inward current.

induced by $I_h$ shifted significantly to negative voltages at all points of the *I–V* curve ($P < 0.05$, $n = 11$). Such findings suggest that activation of 5-$HT_{1A}$ receptors induced a negative shift in $I_h$ activation and/or a reduction in a 'leakage' current.

To demonstrate the influence of the $I_h$ current we blocked it with 10 μM methylaminopyrimidiumchloride (ZD7288). A similar change in voltage responses in comparison to activation of 5-$HT_{1A}$ receptors was actually seen when the $I_h$ current was partially blocked by 0.5 mM Cs (data not shown).

Bath application of the 5-$HT_4$ agonist BIMU8 (20 μM) produced an opposite effect, i.e. there was a shift of the voltage response towards more positive values (Fig. 1c). The changes were significant ($P \leqslant 0.05$, $n = 8$) at every potential value along the *I–V* curve. A similar change in voltage responses was seen when cAMP levels were

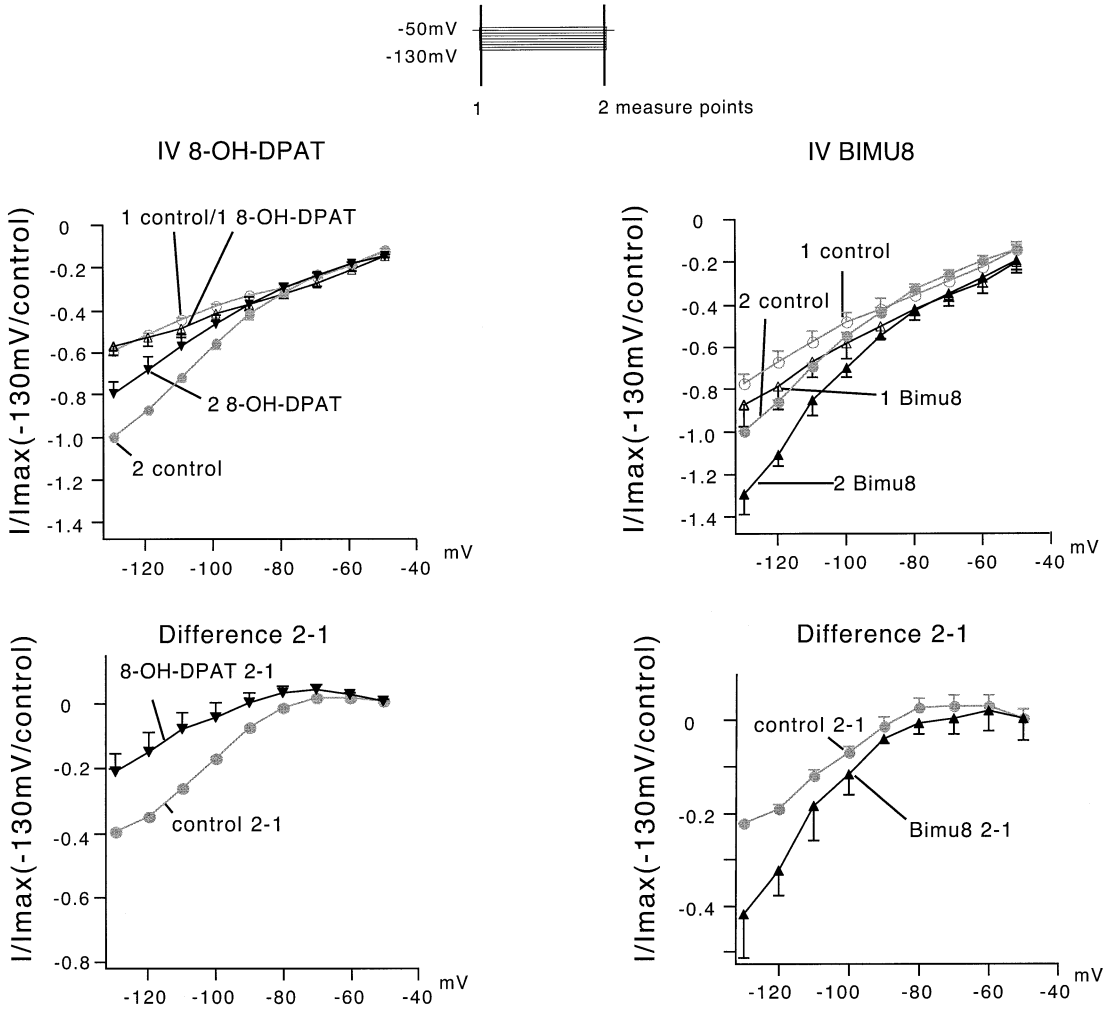


FIG. 4. Normalized *I–V* curves after activation of 5-$HT_{1A}$ receptors and 5-$HT_4$ receptors. (Left) Activation of 5-$HT_{1A}$ (20 μM 8-OH-DPAT) receptors mainly decreased the fully activated $I_h$ current (2 – 1) and not the instantaneous current (1) ($n$ = 3). (Right) Activation of 5-$HT_4$ receptors (20 μM BIMU8) increases the instantaneous current (1) as well as the fully activated $I_h$ current (2 – 1) ($n$ = 3).

increased by forskolin (50 μM) application in the bath and/or isobutylmethylxanthine (IBMX) (50 μM) applied via the pipette (Fig. 1). During this receptor-independent elevation of cAMP by forskolin and IBMX, other channel types are modulated in addition to $I_h$ (see below).

### *Voltage-clamp analysis of receptor-specific effects*

We performed voltage-clamp experiments to measure the $I_h$ current directly. The current was pharmacologically identified as $I_h$ due to its blockade by 10–25 μM ZD7288 and 3 mM CsCl and because it persisted in $I_h$ isolation buffer (Fig. 2).

Activation of 5-$HT_4$ receptors by BIMU8 (20 μM) induced an inward current of –21 ± 5.5 pA ($n$ = 11) when measured close to the membrane resting potential. A similar inward current became visible after application of BIMU8 (20 μM, –16 ± 5.8 pA) even at a voltage of –50 mV in $I_h$ isolation buffer ($n$ = 10). This effect is consistent with the depolarization of neurons as measured in current clamp. A comparable effect was seen after elevation of cAMP with forskolin, which induced an inward current of –40 ± 14.5 pA ($n$ = 5).

When 50 μM 5-HT was applied in the presence of 1 μM (N-[-(4-[2-Methoxyphenyl]-1-piperazinyl)ethyl]-N-2-pyridinylcyclohexanecarboxamide) (WAY 100635) to activate 5-HT receptors other than 5-$HT_{1A}$ receptors, an inward current was induced that was blocked by the 5-$HT_4$ receptor antagonist GR113808 (10 μM, $n$ = 3, Fig. 3). The shift in baseline to positive values after application of GR113808 (10 μM) might be explained by a high constitutive activity of the 5-$HT_4$ receptor which is blocked by GR113808 (Ponimaskin *et al.*, 2002). 5-$HT_4$ receptors show a strong desensitization after 5-HT has been applied in a heterologous expression system (Heine *et al.*, 2002). Therefore a second 5-HT application did not reveal a response (data not shown). However, it is important to note that the inward currents that were induced by either 50 μM BIMU8 or 50 μM forskolin were not fully blocked even by high concentrations of Cs (3 mM; Fig. 3). This indicates that another conductance was activated besides $I_h$. Additional experiments with 25 μM ZD7288 in the bath solution and application of 50 μM BIMU8 revealed an increase in an inward current in two out of four experiments (Fig. 3), which was partially reversible.

### I–V *relationship of BIMU8- and 8-OH-DPAT-induced membrane currents*

The BIMU8-induced increase of the net inward current did not seem to originate just from an activation of $I_h$, but also from coactivation of

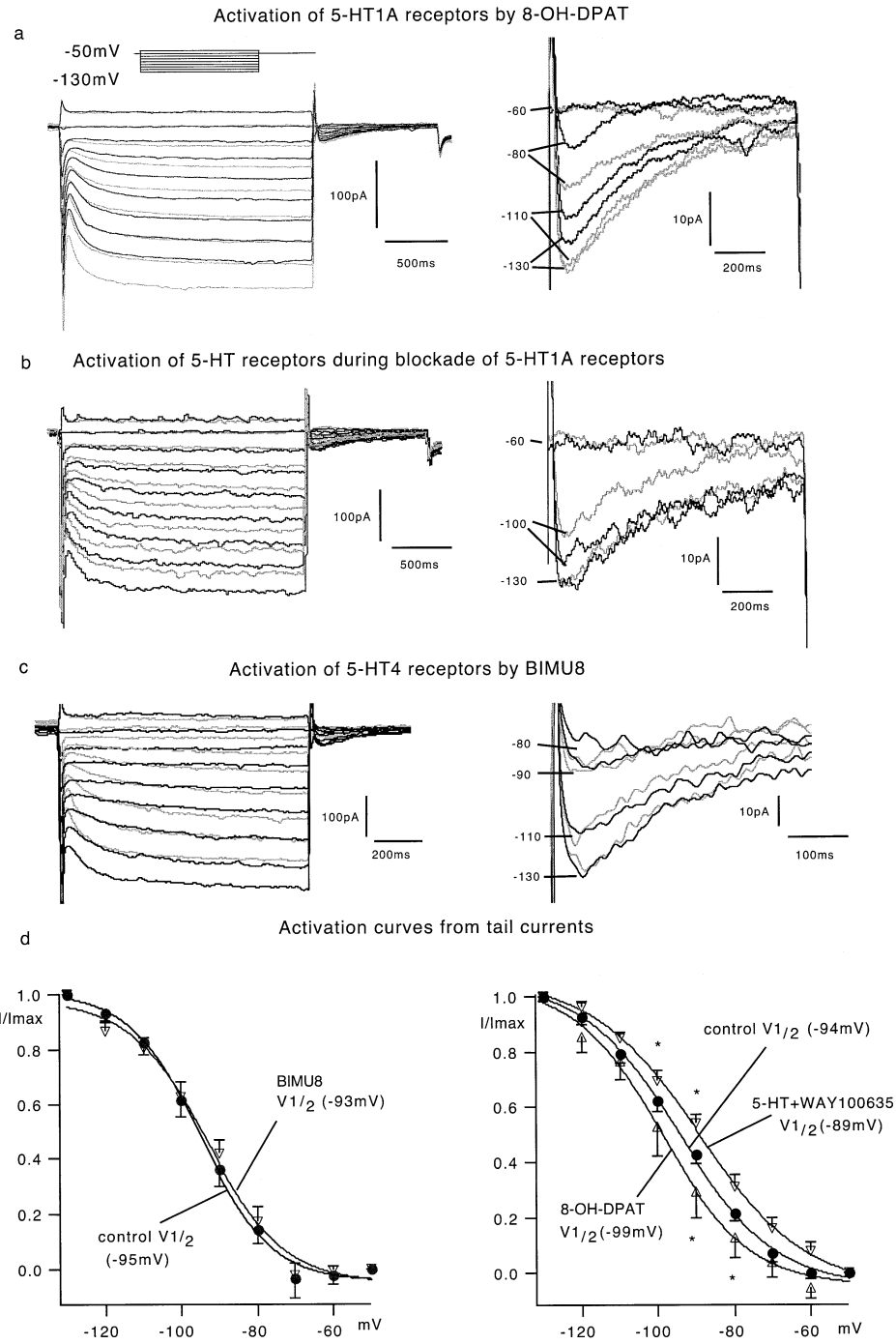


FIG. 5. (a) Family of currents before (grey traces) and after (black traces) application of 5-$HT_{1A}$ receptor agonist 20 μM 8-OH-DPAT. (b) During blockade of 5-$HT_{1A}$ receptors, 50 μM 5-HT increased $I_h$ (black traces). (c) Family of currents before (grey) and after (black) application of 5-$HT_4$ receptor agonist BIMU8 (20 μM). (d) Normalized tail current amplitudes plotted against the membrane voltage. The $V^1/_2$ of a Boltzmann fit to controls and after application of 20 μM BIMU8 ($n$ = 7) was nearly unchanged; 20 μM 8-OH-DPAT shifted the $V^1/_2$ of the activation curve –5 mV to the left ($n$ = 6), whereas 50 μM 5-HT (without activation of 5-$HT_{1A}$ receptors) shifted the $V^1/_2$ 5 mV to the right ($n$ = 8). Control curve represents data for both sets of experiments. Student's $t$-test was performed for each set of control data separately (*$P < 0.05$ at indicated voltages).

an as yet unidentified inward current (Fig. 4) which is insensitive to 3 mM CsCl as well as to 25 μM ZD7288 and to $I_h$ isolation buffer.

There was a difference between the currents measured at the very beginning of the voltage step, which ranged from –50 to –130 mV before and after application of BIMU8. This difference indicated a leakage current. To separate this leakage current from $I_h$, we determined the differences in current amplitudes measured at the very beginning of the voltage step and of the fully activated current at the

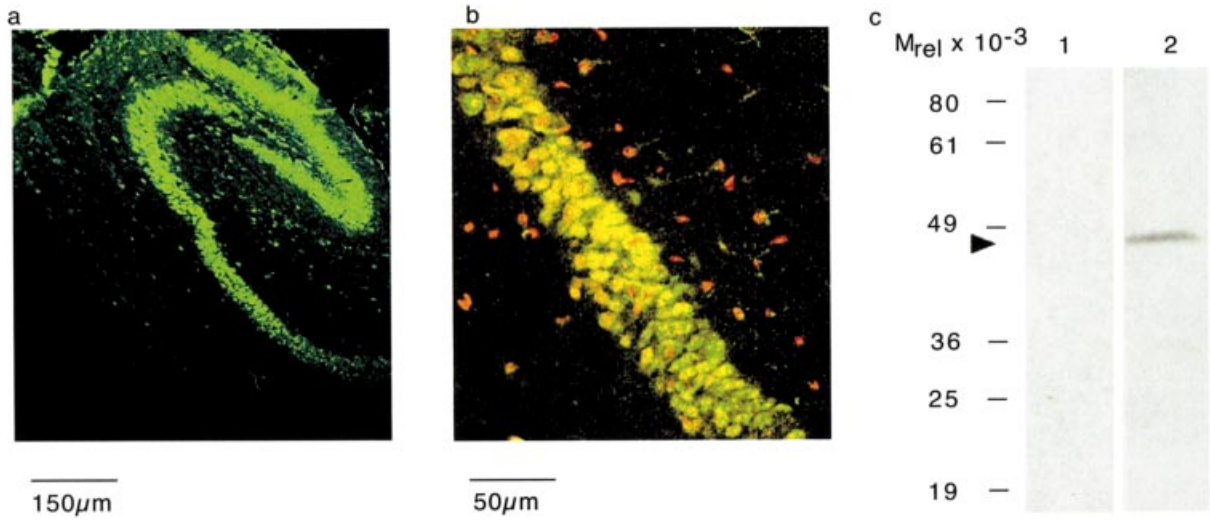


FIG. 6. (a) Fluorescence staining of the hippocampus. The 5-$HT_7$ antibody binds to the cell bodies and proximal dendrites. (b) Fluorescence staining of the stratum pyramidale of the CA1 region using the 5-$HT_7$ antibody (1 : 400) and propidium iodide (25 μg/mL) staining of cell nuclei in this area. Many cells are stained with propidium iodide but unstained by the antibody, indicating the absence of 5-$HT_7$ receptors from interneurons. (c) Immunoblot: test of activity of the polyclonal anti-5-$HT_7$ antibody. Cytosolic fraction (1) and membrane fraction (2) from lysed brain with anti 5-$HT_7$ antibody. Lines represent each 1.2 μL of fraction.

end of a hyperpolarizing voltage pulse. The resulting *I–V* curves for the corrected $I_h$ revealed that $I_h$ increased with application of the 5-$HT_4$ agonist BIMU8 (20 μM). The 20 μM BIMU8 also increased the leakage current but, in contrast to what is typical for $I_h$, the leakage current did not reveal any significant voltage dependence (Fig. 4). Activation of 5-$HT_4$ receptors increased inward currents significantly at potentials lower than –50 mV ($n = 10$, $P < 0.05$).

The leakage current was not as sensitive as $I_h$ to 8-OH-DPAT (20 μM), while the separated $I_h$ current (see above) revealed a clear dependency on 8-OH-DPAT (Fig. 4). Activation of 5-$HT_{1A}$ receptors by 8-OH-DPAT reduced the amplitude of $I_h$ significantly at potentials lower than –90 mV ($n = 9$, $P < 0.05$).

### *Receptor-specific modulation of tail current activation curves*

To verify our current-clamp data for the 8-OH-DPAT-evoked shift in $I_h$ activation towards negative voltages, we determined the $I_h$ activation curve by plotting normalized tail currents as measured at –60 mV against varying voltage prepulses (Fig. 5). The activation curve was fitted by a Boltzmann equation. This revealed that 20 μM 8-OH-DPAT actually induced a shift towards negative voltages. The voltage of half-maximal current activation ($V^1/_2$) changed from –94 mV (slope 12.5 $mV^{-1}$) to –99 mV (slope 11.4 $mV^{-1}$).

Applying 50 μM 5-HT during blockade of 5-$HT_{1A}$ receptors by 1 μM WAY 100635, the $V^1/_2$ of the activation curve was shifted from –94 mV (slope 12.5 $mV^{-1}$) towards more depolarized potentials of –89 mV (slope 13.9 $mV^{-1}$). Activation of 5-$HT_4$ receptors with 20 μM BIMU8 alone did not induce a significant alteration of the activation curve ($V^1/_2$ –95 mV, slope 9.3 $mV^{-1}$ to $V^1/_2$ –93 mV, slope 10.1 $mV^{-1}$; *t*-test; Fig. 5d), but increased membrane conductance. After blockade of 5-$HT_{1A}$ receptors, the 5-HT effect therefore was most probably due to an activation of 5-$HT_7$ receptors. This interpretation was confirmed by the finding that 5-HT increased $I_h$, although 5-$HT_4$ receptors were blocked by 10 μM GR113808 and 5$HT_{1A}$ receptors by 1 μM WAY 100635. The membrane conductance at –50 mV was unchanged.

Considered for a long time to be 5-$HT_{1A}$ receptor specific, 8-OH-DPAT is now known to (in high concentrations) coactivate 5-$HT_7$ receptors, which are $G\alpha_s$-coupled (Lovenberg *et al.*, 1993; Vanhoenacker *et al.*, 2000). We verified this action of 8-OH-DPAT by blocking 5-$HT_{1A}$ receptors with the antagonist WAY 100635 (1 μM), which allowed separation of the 8-OH-DPAT-induced coactivation of 5-$HT_7$ receptors. Indeed, we observed that 50 μM 8-OH-DPAT increased $I_h$ in all four cells tested. These findings reveal effective blockade of 5-$HT_{1A}$ receptors by 1 μM WAY 100635 and the presence of 5-$HT_7$ receptors in CA1 pyramidal neurons.

### *Localization of 5-$HT_7$ receptors: immunohistochemistry*

The primary antibody (1 : 400) against a sequence of the third intracellular loop of the mouse 5-$HT_7$ receptor intensively labelled somata and, in some cases, parts of the proximal dendrites of CA1 pyramidal neurons (Fig. 6a). Controls with preimmune serum did not reveal any specific staining. Cell nuclei were stained with propidium iodide (25 μg/mL) to also localize those cells that were not labelled with the primary antibody. These controls clearly revealed that cells in the stratum radiatum, closely neighbouring CA1 pyramidal cells, were not labelled by the antibody and probably represent interneurons or glial cells not expressing any 5-$HT_7$ immunoreactivity (Fig. 6b). To test for the specificity of the antibody, we performed Western blots using standard procedures with brain homogenate and found a single band close to 48 kDa, which corresponds to the molecular weight of the receptor protein indicating receptor specificity (Fig. 6c).

### *Localization of 5-$HT_4$ receptors: electrophysiology and immunohistochemistry*

In order to test for a differential expression of 5-$HT_4$ receptor isoforms, we compared the effects of local and systemic bath application of the receptor-specific agonist BIMU8 (20 μM). We locally ejected 20 μM BIMU8 through a patch pipette directly on the cell body of CA1 neurons, while the flow of the bath solution was directed towards the axonal compartment, i.e. away from the remote dendritic compartments of the CA1 neurons in stratum radiatum. The 5-$HT_4$ receptor-induced effects were similar, regardless of whether the drug was applied at 20 μM in the bath or directly to the cell body

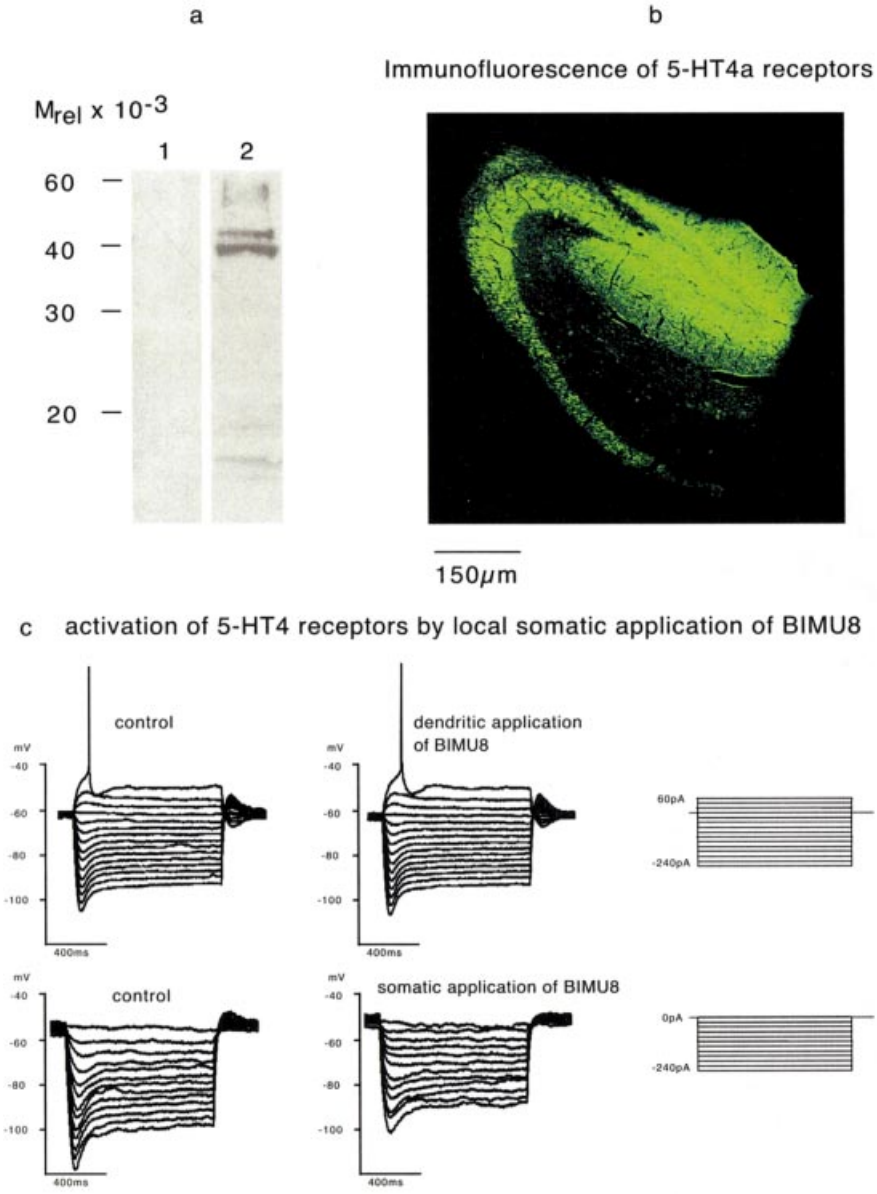


FIG. 7. (a) Immunoblot: test of activity of the polyclonal anti-5-HT4 antibody (1 : 400) indicated as in Fig. 6c. (b) Fluorescence staining of the hippocampus. The 5-HT4 receptor antibody bound to somata in the CA1 region. (c) Current-clamp recordings during local application of the 5-HT4 receptor agonist 20 μM BIMU8.

in the same concentration. The inward current was 64 ± 21 pA ($n$ = 5) during somatic application and 40 ± 19 pA ($n$ = 9) when applied in the bath. The slight differences are probably due to experimentally induced differences in receptor desensitization. Application of 20 μM BIMU8 to remote dendrites showed no effect ($n$ = 3, Fig. 7b). These data point to a preferential location of 5-$HT_4$ receptors on the cell bodies of CA1 pyramidal neurons.

The primary antibody AS9459 (Ponimaskin *et al*., 2001; Heine *et al*., 2002; Ponimaskin *et al*., 2002) directed against the C-terminus of the 5-$HT_{4A}$ receptor revealed a similar staining of pyramidal cells in the CA1 region. This indicates that 5-$HT_4$ receptors are coexpressed with 5-$HT_7$ receptors on the cell bodies of CA1 neurons. This finding confirms our electrophysiological data (Fig. 7).

## Discussion

5-HT is a very potent neuromodulator of the hippocampal circuitry, although specific serotonergic synapses have been identified only on GABAergic interneurons (Freund & Buszaki, 1996; Vizi & Kiss, 1998) but not on pyramidal cells. 5-HT acts presynaptically to modulate transmitter release (Schmitz *et al*., 1998) and postsynaptically to modify neuronal excitability of pyramidal CA1 cells (Andrade, 1998; Barnes & Sharp, 1999). Serotonin activates various 5-HT receptor isoforms that couple to different intracellular signalling pathways, e.g. $G\alpha_i$, $G\alpha_s$ (for review, see Barnes & Sharp, 1999). It is therefore necessary to analyse pyramidal CA1 neurons for the expression pattern of receptor isoforms and their spatial distribution.

In order to test for the functional significance of serotonergic modulation (Larkmann & Kelly, 1997; Gasparini & DiFrancesco, 1999), the hyperpolarization-activated nonselective ion channel generating the $I_h$ (Pape, 1996) is an adequate test tool because it is differentially modulated by 5-HT signal pathways (Bickmeyer *et al*., 2000). $I_h$ channels are expressed in dendrites and somata of CA1 neurons and contribute to the resting membrane potential and modulation of temporal summation of synaptic signals (Magee, 1998; Magee, 1999).

Immunofluorescence staining clearly demonstrated that 5-$HT_4$ and 5-$HT_7$ receptors are expressed in CA1 cells and preferentially localized on the cell body, while 5-$HT_{1A}$ receptors are located preferentially on dendrites, but also on the cell body (Azmitia *et al*., 1996; Kia *et al*., 1996). This corresponds to a specific subcellular distribution and differential modulation of $I_h$ currents by 5-$HT_{1A}$, 5-$HT_4$ and 5-$HT_7$ receptors as determined electrophysiologically in the present paper. Such differential modulation of $I_h$ by 5-HT receptor isoforms was verified by demonstrating that activation of 5-$HT_{1A}$ receptors induces a negative shift in the activation curve, while activation of 5-$HT_4$ and 5-$HT_7$ (5-HT application with blocked 5-$HT_{1A}$ receptors) induces a positive shift in the activation curve. 5-$HT_4$ receptors also increase another as yet unidentified conductance that obviously contributes to this effect (Cardenas *et al*., 1999). This conductance was insensitive to 10 mM tetraethylammonium, 2 mM 4-aminopyridine, 3 mM Cs and low doses of Cd (0.1 mM) and appears as an inward current at the resting membrane potential in physiological buffer (ACSF) using standard pipette solution. It is therefore not a potassium conductance. We assume that it derives from a cyclic nucleotide-gated (CNG) channel, which obviously is expressed in CA1 cells (Bradley *et al*., 1997). CNG channels show a voltage-independent conductance increase by cAMP in the micromolar range and are permeable to monovalent and divalent cations.

In addition, we verified that 5-$HT_{1A}$ receptors depress the persistently active $I_h$, which induces an increase in the electrotonic length of dendritic segments and therefore increases EPSP time constants (Magee, 1999). However, cell bodies and proximal dendrites are equipped with 5-$HT_4$ and 5-$HT_7$ receptors, which couple to $G\alpha_s$ to increase $I_h$, leading to augmentation of the inward currents to depolarize neurons and facilitate action potential discharge.

In conclusion, the $I_h$ current is active at the resting membrane potential of CA1 neurons and thus is capable of modifying temporal integration of synaptic currents (Magee, 1998) and thus differential adjustment of EPSP amplitudes arising at dendrites and/or the soma (Magee, 1999). $I_h$ also plays a functional role in cellular bursting of thalamic neurons (McCormick & Pape, 1990; Pape, 1996) and respiratory neurons (Mironov *et al*., 2000; Thoby-Bresson *et al*., 2000), where $I_h$ contributes in varying degrees to rhythm generation. Therefore, we assume that serotonergic modulation also exerts a differential control on periodic bursting behaviour of CA1 neurons.

### *Variability of responses*

It seems worthwhile to mention that not all CA1 pyramidal neurons responded uniformly to 5-HT. After application of 8-OH-DPAT (20 μM), 83% of neurons responded with a reduction in $I_h$, 11% showed no response and 6% even showed a slight increase. This might indicate an interference with other 5-HT receptor isoforms, e.g. 5-$HT_7$ receptors (Vanhoenacker *et al*., 2000). Application of the 5-$HT_4$ receptor agonist BIMU8 (20 μM) resulted in a current increase in 64% of neurons; 36% showed no clear response. One might speculate therefore that not all CA1 neurons express this 5-HT receptor isoform. Another explanation might be the high constitutive activity (Clayesen *et al*., 1999; Ponimaskin *et al*., 2002) of the 5-$HT_4$ receptor, which is so high in some neurons as to mask the effect of an agonist. Additionally, 5-HT might be spontaneously released in brain slices, leading to rapid desensitization of 5-$HT_4$ receptors, which reduces the effect of 5-$HT_4$ receptor agonists as shown in a heterologous expression system (Heine *et al*., 2002).

In conclusion, 5-$HT_{1A}$, 5-$HT_4$ and 5-$HT_7$ receptor subtypes are differentially expressed in CA1 neurons, which allows space-specific modulation of the $I_h$ current, which is known to adjust synaptic integration and repetitive discharge behaviour of CA1 neurons of the hippocampus.

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## Abbreviations

8-OH-DPAT, 8-hydroxy-2-(di-n-propylamino)tetralin; AC, adenylyl cyclase; ACSF, artificial cerebrospinal fluid; ATP, Na-adenosine 5′-triphosphate; BIMU8, 4-ethylphenylamino-1,2-dimethyl-6-((endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dehydro-2-oxo-3-(prop-2-yl)-ICH-benzimid-azole-1-carboxamide); BSA, bovine serum albumine; cAMP, 3′,5′-cyclic adenosine monophosphate; CNG, cyclic nucleotid-gated channels; EGTA, ethylene glycol-bis(β-aminoethylether)N,N,N′,N′-tetraacetic acid; GR113808, {1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl}methyl-1-methyl-1-H-indol-3-carboxylate, maleate salt; GTP, guanosine 5′-triphosphate; HEPES, (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonicacid]); IBMX, isobutylmethylxanthine; $I_h$, hyperpolarization-activated current; PBS, phosphate-buffered saline; WAY 100635, (N-[-(4-[2-Methoxyphenyl]-1-piperazinyl)ethyl]-N-2-pyridinylcyclohexanecarboxamide); ZD7288, methylaminopyrimidiumchloride.

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