Serotonin targets inhibitory synapses to induce modulation of network functions

Till Manzke¹,²,³, Mathias Dutschmann¹,⁴, Gerald Schlaf⁵, Michael Mörschel¹,⁴, Uwe R. Koch¹, Evgeni Ponimaskin¹,³, Olivier Bidon¹, Peter M. Lalley⁶ and Diethelm W. Richter¹,³,*

¹Department of Neuro- and Sensory Physiology, and ²Department of Child and Adolescent Psychiatry, University of Göttingen, 37073 Göttingen, Germany
³DFG Research Center Molecular Physiology of the Brain (CMPB), 37073 Göttingen, Germany
⁴Bernstein Center for Computational Neuroscience, 37073 Göttingen, Germany
⁵Department of Medical Immunology, University of Halle-Wittenberg, 06097 Halle, Germany
⁶Department of Physiology, University of Wisconsin, Madison, WI 53706, USA

The cellular effects of serotonin (5-HT), a neuromodulator with widespread influences in the central nervous system, have been investigated. Despite detailed knowledge about the molecular biology of cellular signalling, it is not possible to anticipate the responses of neuronal networks to a global action of 5-HT. Heterogeneous expression of various subtypes of serotonin receptors (5-HTR) in a variety of neurons differently equipped with cell-specific transmitter receptors and ion channel assemblies can provoke diverse cellular reactions resulting in various forms of network adjustment and, hence, motor behaviour.

Using the respiratory network as a model for reciprocal synaptic inhibition, we demonstrate that 5-HT₁A-R modulation primarily affects inhibition through glycinergic synapses. Potentiation of glycinergic inhibition of both excitatory and inhibitory neurons induces a functional reorganization of the network leading to a characteristic change of motor output. The changes in network operation are robust and help to overcome opiate-induced respiratory depression. Hence, 5-HT₁A-R activation stabilizes the rhythmicity of breathing during opiate medication of pain.

Keywords: glycinergic synapses; serotonin modulation; network operation; disinhibition; rescue breathing

1. INTRODUCTION

Despite detailed knowledge about the molecular processes controlling G protein-coupled receptor (GPCR)-induced modulation of neuronal excitability, we cannot predict the responses of neuronal networks to a global release of neuromodulators. This is due to a diversity of neurons that express cell-specific patterns of GPCRs targeting different membrane conductances, which induce distinct operations of the network. Even more difficult to predict is the endogenous capacity of neuronal networks to adapt to variable conditions while preserving or adjusting elementary processes, such as rhythmic burst generation, activity synchronization and pattern formation. To study such integrated processes of network modulation, it is necessary to know the principles of network architecture, identify cell-specific transmitter receptor/ion channel expression profiles and develop appropriate pharmacological tools to dissect GPCR-specific responses. In such studies of integrated network physiology, the respiratory network is a suitable model system, because it produces an ongoing oscillatory activity and a highly integrated synaptic drive that generates augmenting burst activity. Eupnoic breathing movements also depend on the network to accurately shape firing patterns and precisely control burst termination and phase transitions (Richter & Spyer 2001). All these processes are effectively adjusted by serotonergic neurons of the Raphe nuclei, which exhibit ongoing discharges (Ballantyne et al. 2004) to continuously release serotonin (5-HT; Richter et al. 1999).

Most serotonin receptors (5-HTRs) are GPCRs operating through signalling pathways that are shared targets of other transmitters, e.g. noradrenaline, dopamine and opiates. All these GPCRs are abundantly expressed in the pre-Bötzinger complex (pre-BötC; Gray et al. 1999; Manzke et al. 2003; Viemari & Ramirez 2006), which is an essential part of the respiratory rhythm generator in the medullary brainstem of mammals (Smith et al. 1991; Pierrefiche et al. 1998; Tan et al. 2008). Signalling via most of these GPCRs converges at the shared molecular target adenyl cyclase (AC), which regulates the formation of cellular cyclic adenosine 3′,5′-monophosphate (cAMP). This has inspired several investigations of the mechanisms of the dynamic adjustment of respiratory network function through regulation of intracellular cAMP levels (Ballantyne et al. 1997; Lalley et al. 1997; Richter et al. 1999; Bocchiaro & Feldman 2004).
Figure 1. Expression patterns of 5-HT$_{1AR}$, 5-HT$_{7R}$ and GlyT2 in the pre-BöötC of rat. (a) Whole cell recording in current-clamp mode from an identified respiratory pre-BöötC neuron discharging in synchrony with integrated hypoglossal nerve (XIIa) activity (lower trace). The cytosol of this cell was used for single-cell RT–PCR analysis. (b) Single-cell RT–PCR analysis of inspiratory neurons. (i) Agarose gel electrophoresis carried out for RT–PCR product amplified from four identified inspiratory neurons (Ins-1–4) with specific primers for Htr1a (5-HT$_{1AR}$). A control reaction without reverse transcription (w/o RT) is shown in the last lane. (ii) RT–PCR products from inspiratory neuron 3 amplified with specific primers for Actb (β-actin), Oprm1 (µ-OR) and Htr7 (5-HT$_{7R}$). (c) A novel monoclonal antibody against 5-HT$_{7R}$ was produced by selecting part of the rat 5-HT$_{7R}$ TM5-TM6 intracellular loop (Phe$^{281}$–Gly$^{294}$) for immunization. The epitope is 100 per cent conserved in mouse and rat, and mismatches in the human 5-HT$_{7R}$ sequence are indicated by red lettering. (d) The specific signal of approximately 48 kDa in the immunoblot corresponds with the predicted molecular mass of 5-HT$_{7R}$. (e) Triple labelling with the guinea pig anti-5-HT$_{1AR}$ antibody (Cy3, red), hamster anti-5-HT$_{7R}$ antibody (Cy5, blue) and rabbit anti-GlyT2 antibody (Cy2, green). The data reveal a predominant expression of 5-HT$_{1AR}$ and 5-HT$_{7R}$ in GlyT2-positive inhibitory neurons. (f) Double labelling of pre-BöötC neurons with the rabbit anti-GlyT2 antibody (Cy2, green) and the guinea pig µ-OR antibody (Cy3, red) revealing abundant expression of µ-OR expression in glycinergic neurons. In a recent study on the reduced brainstem slice preparation (Winter et al. 2009), a 20 per cent population of these GlyT2-eGFP-labelled neurons revealed spontaneous respiratory activity.
An unanswered key question of clinical significance concerns the consequences of convergent signalling via serotonin receptors (5-HT$_{1A}$Rs) and $\mu$-opioid receptors ($\mu$-ORs). The latter are well-known pharmacological targets for anaesthesia and the treatment of pain. Clinically useful $\mu$-OR agonists, however, depress central respiratory activity, causing cessation of rhythmic breathing movements (Mellen et al. 2003; Lalley 2006). An averting medical approach against opioid-induced apnoea is possible with 5-HT$_{1A}$R agonists that re-activate cellular AC (Manzke et al. 2003), but such medication is so far not useful in men because

**Figure 2.** Respiratory network responses to systemic 5-HT$_{1A}$R and 5-HT$_7$R ligand applications of **in situ** rats. (a–c) Action of 5-HT$_{1A}$R and 5-HT$_7$R ligands in the perfused brainstem–spinal cord preparation. (a) Application of the 5-HT$_7$R agonist 5-CT (0.3 $\mu$g ml$^{-1}$) depressed PNA and subsequent application of the 5-HT$_{1A}$R-antagonist WAY 100635 (2.7 $\mu$g ml$^{-1}$) exacerbated this effect. These data reveal constitutive 5-HT$_{1A}$R activity under normal conditions. (b) Blockade of 5-HT$_7$Rs by SB 269970 (2.0 $\mu$g ml$^{-1}$) did not change PNA. Subsequent administration of 8-OH-DPAT (1.6 $\mu$g ml$^{-1}$) increased PNA by augmenting both the amplitude and frequency of PNA. (c) The effect of 8-OH-DPAT is absent when glycine receptors are blocked with strychnine (1.0 $\mu$g ml$^{-1}$).

(d) Intravenous administration of 8-OH-DPAT (100 $\mu$g kg$^{-1}$) to anaesthetized rats **in vivo** increased rRMA (red trace). This effect was mainly due to an increase in breathing frequency (RA, respiratory activity; exp, expiration; insp, inspiration). A final injection of the 5-HT$_{1A}$R antagonist WAY 100635 (1 mg kg$^{-1}$) blocked these effects, and rRMA returned to below baseline levels (blue trace). Statistical analysis is denoted in the panels on the right side with asterisks indicating significance (*$p<0.05$; **$p<0.01$).
5-HT_1R agonists that permeate through the blood–brain barrier are not available for clinical practice (Lotsch et al. 2005).

In the present experiments, we focused on the convergent signalling of µ-OR and 5-HT_1A-Rs following the observation that fentanyl-induced depression of breathing is reversed by 5-HT_1A-agonists (Sahibzada et al. 2000). This finding is unexpected, because both µ-OR and 5-HT_1A-R agonists lower cAMP levels and decrease neuronal excitability (Richter et al. 1999). A straightforward explanation could be that most 5-HT_1A-R-agonists such as the applied 8-hydroxy-dipropyl-aminotetralin (8-OH-DPAT) also activate G_s-coupled 5-HT_7Rs (Hedlund et al. 2004) that might prevail over the inhibitory G_i/o–AC pathway, thus restoring intracellular cAMP levels (Hoyer et al. 2002). Alternatively, one has to search for more complex changes in network operation if this simplistic explanation is not supported.

2. METHODS

(a) In vivo cat experiments

Experiments were performed on 10 adult cats of either sex weighing 2.8–3.8 kg using procedures that have been described in detail in other published studies from this laboratory (Lalley et al. 1997; Richter et al. 1999). Briefly, cats were anaesthetized with IV pentobarbital sodium in doses sufficient to produce deep surgical anaesthesia. The initial dose was 40 mg kg^{-1} intraperitoneally, followed by supplemental maintenance doses of 4–8 mg kg^{-1} IV, as required. Animals were given atropine methyl nitrate (0.2 mg kg^{-1} intraperitoneally) to minimize airway fluid secretion. Surgical procedures included insertion of a catheter in a femoral artery to monitor blood pressure, and placement of catheters in both femoral veins to administer slow IV to maintain recording stability. Doses were increased in 5 μg kg^{-1} amounts to a final concentration of 10–50 μg kg^{-1}. The effects of 8-OH-DPAT on respiratory network rhythm were obtained from a population of 4 post-inspiratory and 12 expiratory neurons of the ventrolateral medulla.

(b) In vivo rat experiments

Sprague Dawley rats of either sex (250–350 g) were anaesthetized by intraperitoneal injection of pentobarbital (60 mg kg^{-1}). The depth of anaesthesia was tested with a forepaw pinch. In case of reflex responses, an additional dose of pentobarbital was applied (approx. one-tenth of the initial dose). The trachea and right femoral vein were cannulated with polyethylene tubing to record respiratory air flow and for drug and fluid injections, respectively. The inspiratory/expiratory airflow was recorded using a MacLab-linked pressure transducer connected to the tracheal tubing. Nociceptive responses were assessed by the tail-flick (TF) response. High-intensity light sufficient to stimulate nociceptors was applied to marked spots (1 cm from the tip of the tail, four spots at an interval of 1 cm), and the TF was quantified by measurement of the latency from ‘heat on’ to the evoked withdrawal response. The average TF response latency values of three consecutive trials before drug application were used as baseline. To avoid tissue damage, the heating was stopped when the TF latency exceeded 300 per cent of control, concluding TF response abolishment. To prevent central hypoxia, the animals were insufflated with oxygen during the whole experiment and ventilated artificially with room air at low frequency (10–15 breath min^{-1}) after opioid-induced apnoea. Artificial ventilation was immediately stopped after detection of the first signs of spontaneous breathing movements evoked by drug application. At the end of the experiments, all animals were sacrificed using an overdose of pentobarbital that produced irreversible cardiac arrest.

(c) Perfused brainstem–spinal cord preparation of rat or mouse

The experiments on the in vivo-like in situ brainstem–spinal cord preparation were performed on Sprague-Dawley rats (P22–P32) or C57BL mice (P20–P25) as described originally (Paton 1996). For isolating the in situ brainstem–spinal cord from higher brain areas, animals were deeply anaesthetized with halothane until apnoea occurred and they were unresponsive to a forepaw pinch. Animals were then decerebrated at the pre-collicular level and cerebellarized, bisected below the diaphragm and the skin was removed. The upper body was placed in a recording chamber and perfused retrogradely via the thoracic aorta with artificial cerebrospinal fluid (ACSF; containing in mM: 1.25 MgSO_4; 1.25 KH_2PO_4; 5 KCl; 125 NaCl; 2.5 CaCl_2; 25 NaHCO_3; 10 glucose, 70 Ficoll (0.1785 mM)), and gassed with carbogen (5% CO_2/95% O_2, pH 7.35). The perfusate was warmed to 30 °C as measured in the skull base, filtered twice and recirculated. Norcuronium-bromide at 0.5 mg 200 ml^{-1} was added for immobilization. The perfusion pressure was set to 45–65 mm Hg. Using a glass suction electrode,
phrenic nerve discharges were recorded as an index of central respiratory rhythm. Drugs were added to the perfusate for specific pharmacological manipulation of 5-HT7R and glycine receptors.

**(d) Rhythmic brainstem slice preparation of rat**

Inspiratory neurons in the pre-Bötzinger complex (pre-Bo) were recorded in whole-cell mode before harvesting the cytosol for single-cell RT-PCR analysis. Transverse slices (600 μm) were cut from the caudal medulla at the level of the pre-Bötzinger complex. Nerve fibers were covered with a mixture of agarose and ACSF at room temperature (20–23°C) for at least 30 min before experiments were started. They were then transferred to a recording chamber and kept submerged by nylon fibres for mechanical stabilization. The chamber was mounted on an upright microscope (Axioskope FS; Zeiss, Oberkochen, Germany) and perfused continuously with ACSF (20–23°C) at a flow rate of 5–10 ml min⁻¹. Whole-cell voltage-clamp recordings were obtained with a Multiclamp 700A amplifier (Axon Instruments). Patch electrodes were pulled from borosilicate glass capillaries (Biomedical Instruments, Loughborough, UK) and stored under vacuum conditions for mechanical stabilization. The pipettes were filled with a ‘patch solution’ containing (in mM): 125 KCl, 1 CaCl₂, 2 MgCl₂, 4 Na₂ATP, 10 EGTA, 10 HEPES (pH adjusted to 7.2 with KOH). Patch electrodes had DC impedances ranging between 2 and 6 MΩ.

**(e) Pharmacology**

The following pharmaceutical substances were administered in *in vivo* cat experiments: 8-hydroxy-2-(di-N-propylamino)-tetralin hydrobromide (8-OH-DPAT; 5-HT1A receptor antagonist; Tocris), buspirone (5-HT1B receptor antagonist; Sigma-Aldrich), WAY 100635 (5-HT1D receptor antagonist; Sigma-Aldrich), 5-carboxamidotryptamine maleate (5-HT1D, 5-HT1F receptor antagonists; Tocris), SB 269970 hydrochloride (5-HT1B, 5-HT1F receptor antagonists; Tocris), strychnine hydrochloride (glycine receptors antagonist; Sigma-Aldrich), fentanyl citrate salt (μ-OR antagonist; Tocris), SB 269970 hydrochloride (5-HT1B, 5-HT1F receptor antagonists; Tocris), strychnine hydrochloride (glycine receptors antagonist; Sigma-Aldrich), fentanyl citrate salt (μ-OR antagonist; Sigma-Aldrich). In the *in vitro* rat experiments, the antagonists WAY 100635, SB 269970 and naltrexone were used at a concentration of 1 mg l⁻¹, whereas 8-OH-DPAT was given at 100 μg kg⁻¹ and fentanyl at 10–15 μg kg⁻¹. In the perfused brainstem–spinal cord preparations, 8-OH-DPAT, WAY 100635 and SB 269970 were applied in 5–20 μM concentrations; buspirone, 20–40 μM and 5-HT1D receptor antagonist, 1 μM. Fentanyl was given at a concentration of 10–30 nM and strychnine at 0.4 μM.

**(f) Monoclonal antibody against 5-HT7R**

Since 5-HT7Rs show a high amino acid sequence identity in mouse, rat and man, we used Syrian hamsters (Charles River Laboratories), which are evolutionarily more distant from these species, to raise monoclonal 5-HT7R antibodies. Five Syrian hamsters were immunized with a 14mer peptide derived from the TM5–TM6 intracellular loop of the rat 5-HT7R (NH2-PRVQDPSVISLNG-COOH; National Center for Biotechnology Information, NCBI). Monoclonal antibodies against 5-HT7R were generated by fusion of hamster spleen cells with P3X63Ag8.6.5.3 mouse myeloma cells and purified using polyethylene glycol (PEG4000; Sigma) according to standard protocols. First screenings of mouse-hamster heterohybridoma supernatants to identify peptide-binding antibodies were performed by enzyme-linked immunosorbent assay (ELISA) with solid phase-coated peptide. After isotype characterization using sandwich-ELISA procedure (clone 5HTR7/220; immunoglobulin class: IgG, light chains: λ-type), the antibodies were purified on a protein A-Sepharose column with the ‘high-salt’ method according to standard protocols. The efficiency of purification was tested with both Ponceau staining of the membrane and Coomassie blue staining of SDS–PAGE gels containing the separated IgG molecules. Under reducing conditions, only the heavy (55 kDa) and light chains (25 kDa) of the IgG molecules were detected.

SDS–PAGE and subsequent immunoblot analysis were performed according to standard protocols. In brief, 150 μg total protein from membrane fractions of Triton X-114-extracted brainstem lysates was boiled in SDS sample buffer under reducing conditions for 5 min. Proteins were separated using a 12 per cent SDS–PAGE and transferred onto a nitrocellulose membrane by electroblotting, with a constant current of 150 mA for 2 h in a semi-dry transfer chamber. The efficacy of protein transfer was tested with both Ponceau staining of the membrane and Coomassie blue staining of the remaining protein in the gel. The membrane was blocked with 2 per cent (w/v) bovine serum albumin (BSA) for 30 min at room temperature and incubated with 5HT7R/220 antibodies at a concentration of 2 μg ml⁻¹ for 2 h at room temperature (RT). The secondary horseradish peroxidase-conjugated goat anti-hamster IgG (Dianova, Hamburg, Germany) was used at a dilution of 1:4000 for 2 h at RT. Visualization of the antigen–antibody reaction was achieved with diaminobenzidine (Sigma) and hydrogen peroxide. The colour reaction was terminated with TBS/0.05 per cent (v/v) TWEEN 20. Finally, the membrane was air-dried and photographed.

**(g) Immunohistochemistry**

Juvenile rats (P28–P32) were deeply anaesthetized with isoflurane (1-chloro-2,2,2-trifluoroethyl ether; Abbott, Wiesbaden, Germany) and perfused with 0.9 per cent sodium chloride followed by 200 ml 4 per cent phosphate-buffered formaldehyde. The brainstem and spinal cord were removed and post-
fixed for 4 h and then cryoprotected with 30 per cent sucrose. Series of 20 and 40 μm thick transverse brainstem sections were cut using a cryostat (Frigocut, Reichert-Jung, Germany).

The following primary antibodies were diluted (1–5 μg ml⁻¹) in a solution containing 2 per cent (w/v) BSA in phosphate-buffered saline (PBS) and applied for 48–72 h at 4°C: affinity-purified hamster monoclonal antibody against 5-HT₁AR (clone 5HTR7/220), guinea pig polyclonal antibody against 5-HT₁AR (AB5406; Millipore), rabbit polyclonal GlyT2 antibody (G8168-21; US Biologicals) and goat polyclonal antibody against μ-OR (sc-7488; Santa Cruz), goat polyclonal antibody against 5-HT₇R (clone 5HTR7/220), guinea pig polyclonal antibody against 5-HT₁AR (AB15810; Chemicon). After incubation, the sections were rinsed three times in PBS and subsequently incubated for 4 h in 2 per cent (w/v) BSA in PBS containing species-specific secondary antibodies conjugated with Cy2, Cy3, or Cy5 (Dianova) at a dilution of 1:500. The analysis of the neuronal immunofluorescence was performed with a confocal laser-scanning microscope LSM 510 Meta (Zeiss, Jena, Germany). For data acquisition and analysis of the confocal images, we used the LSM 510 META software (Zeiss, Germany). Subsequent imaging procedures (cell counting) were performed using ImageJ (http://rsb.info.nih.gov/ij/).

For real-time RT–PCR analysis, we dissected the pre-Bötzinger center that was identified by the appearance of the principal nucleus of the inferior olive from corresponding cryostat sections cut in a rostro-caudal extension of 300 μm (n = 3, two patches from both sides of three juvenile Sprague-Dawley rats, P30). Total RNA was isolated using the Trizol method (Invitrogen) according to manufacturer’s instructions and quantified using a nanodrop ND-1000 spectrophotometer. RNA quality and integrity was assessed by electrophoresis using an RNA 6000 LabChip kit and an Agilent 2100 Bioanalyzer. First-strand cDNA was synthesized using the iScript cDNA Synthesis Kit (BioRad). Primer pairs were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/prime3/primer3_www.cgi): hypoxanthine guanine phosphoribosyltransferase (NM_012583, 135 bp; Hprt-F: 5′-gtcaagcagtcaccgcacccaaagt-3′, Hprt-R: 5′-gtcgaaggcatactccaaacaac-3′), 5-HT₁AR (NM_012585, 161 bp, Htr1a-F: 5′-caagcgtacacatcctctc-3′, Htr1a-R: 5′-gcaagttctcagcccagct-3′), 5-HT₁AR (NM_022938, 193 bp, Htr7-F: 5′-tgcaactctcccttcagct-3′, Htr7-R: 5′-gtgctagggttgctctcagc-3′), GlyT2 (NM_203334, 210 bp, Slc6a5-F: 5′-atgagccgagctggctgcagttc-3′, Slc6a5-R: 5′-agacaaaaagagacacaagctg-3′) and μ-OR (NM_013071, 208 bp, Oprm1-F: 5′-ggtgtagctgctggtataac-3′, Oprm1-R:

**Figure 3.** Modulation of inhibitory synaptic interactions within the respiratory network of anesthetized *in vivo* cats. (a and b) Two examples for the response of post-inspiratory (post-I) neurons with a mean membrane potential of −65 and −70 mV recorded within the rostral portion (3.5–4.0 mm rostral to obex) of the ventral respiratory group to systemic application of the 5-HT₁AR agonist 8-OH-DPAT. Post-I neurons normally receive the most powerful synaptic inhibition during inspiration, when phrenic nerves (PN) reveal an augmenting burst discharge (blue time periods). Applying 8-OH-DPAT systemically at increasing concentrations (5 μg kg⁻¹ amounts) induces the release of post-I neurons from inspiratory synaptic inhibition enabling them to discharge action potentials not only post-inspiration, but also during inspiration (pink time periods). At higher concentrations of 8-OH-DPAT (10–50 μg kg⁻¹), post-I neurons hyperpolarize and their action potential firing is limited to the inspiratory phase (pink time window). The release from inspiratory inhibition and activity shifting of post-I discharge into inspiration is illustrated by superposed traces at a time scale that is normalized to inspiratory duration in (b). (c) *In vivo* single-cell voltage-clamp analyses of post-I neurons show an inhibitory outward current during early inspiration that turns to a clear inward shift later in inspiration, which indicates the integration of inhibitory outward and excitatory inward currents. (d) The existence of an excitatory synaptic current during inspiration becomes apparent when the membrane potential is held close to the presumed chloride equilibrium potential, which exposes a clear inward current which begins at the start of inspiration (e).

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Gel electrophoresis revealed a single PCR product, and melting curve analysis showed a single peak for all amplification products. As an additional control, all PCR products were sequenced to confirm the identity of each amplicon. Ten-fold serial dilutions generated from pooled cDNA from all samples were used as a reference for the standard curve calculation to determine primer efficiency. Triplicates of all

Figure 4. Treatment of opioid-induced depression of respiratory activity with 5-HT1AR agonists in rats. Treatment of perfused brainstem–spinal cord preparation with 8-OH-DPAT (a) and the clinically approved 5-HT1AR agonist buspirone (Buspar; b). Both drugs overcome fentanyl-provoked depression of rhythmic PNA. (a) Systemic administration of fentanyl (6.7 ng ml⁻¹) significantly reduced or blocked PNA (blue trace). Subsequent application of 8-OH-DPAT (3.3 µg ml⁻¹) recovered PNA to control levels (red trace). (b) A comparable action is shown for Buspar. (c) Systemic treatment with 5-HT1AR agonist reverses opioid-provoked depression of breathing without affecting analgesia. Relative changes of rRMA were measured in anaesthetized, fully intact rats. Black trace shows spontaneous breathing and heat-provoked TFR under control conditions. Application of the μ-OR agonist fentanyl (10 µg kg⁻¹) induced apnoea and the TFR was completely blocked (blue trace). Application of 8-OH-DPAT (100 µg kg⁻¹) reinstated breathing, which increased steadily towards control levels (red trace). However, TFR remained abolished. The μ-OR antagonist naloxone (1 mg ml⁻¹) induced an increase in rRMA above control levels and restored the TFR after a short latency (black trace). Statistical analysis is denoted in the histograms in the panels on the right, with asterisks indicating significance (*p < 0.05; **p < 0.01; ***p < 0.001).
real-time PCR reactions were performed in 25 µl reactions containing 1/20 dilution of the sample cDNA preparation from 250 ng total RNA, 400 nM of each primer, and 1 x iQ-SYBR Green Supermix (BioRad Laboratories, Ltd). The PCR reactions were cycled as follows: initial denaturation at 95°C for 10 min, 40 cycles of (denaturation 95°C/15 s, annealing 55°C/15 s, extension 72°C/30 s) and a final gradual increase of 0.5°C in temperature from 55°C to 95°C. All real-time quantifications were performed with the iCycler iQ system (BioRad) and were analysed by using a two-tailed paired t-test following normalization to the Hprt control.

(i) Single-cell RT–PCR

To harvest cytosol for RT–PCR analysis, cells were patched and identified as inspiratory neurons. Patch pipettes were filled with internal solution containing (in mM): 120 KCl, 15 NaCl, 2 MgCl₂, 10 HEPES, 0.5 Na₂ATP, 1 CaCl₂, 3 1,2-bis-(2-amino-phenoxo)ethane-N₂N₂N₂N2-tetraacetic acid, pH 7.4 adjusted with KOH; solution osmolarity ranged from 285 to 290 mOsm. To allow a diffusional exchange of cytosol and pipette solution, cells were held in the whole-cell configuration for at least 15 min. Subsequently, the cytoplasm was aspirated under visual control, while the access resistance was monitored to verify intact gigaseal formation. After breaking the gigaseal, the pipette was immediately withdrawn from the slice. Reverse transcription was carried out for 10 min at 25°C followed by incubation for 50 min at 42°C (SuperScript II reverse transcriptase; Invitrogen). The PCR amplification was performed with specific primers. All primers were tested on plasmid (5-HT₁₇ and 5-HT₇ receptors) as well as on total brain cDNA (5-HT₁₇A, 5-HT₇ and µ-ORs). The primer concentration for all amplification reactions was 0.2 µM. The cycling conditions for the amplification with AmpliTaq Gold polymerase (Applied Biosystems) were a hot start at 95°C for 10 min, 40 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min and an elongation step at 72°C for 10 min. In parallel, PCR was also performed with the AccuPrime Taq DNA polymerase system (Invitrogen), 94°C for 2 min followed by 39 cycles denaturation (94°C for 30 s), annealing (55°C for 30 s) and elongation (68°C for 1 min). The following primer pairs were used for the specific amplification of 5-HT₁₇A (Htr1a-F: 5'-tcgccacaaagagctgccgctggtc-3' and Htr1a-R: 5'-aggagccagtttaagctagcc-3'), 5-HT₁₇ (Htr7-F: 5'-agtttgagcaaggttgtgtgagct-3' and Htr7-R: 5'-agtttgagcaaggttgtgtgagct-3'), µ-OR (Oprml1-F: 5'-ttctggacacccctgctggc-3' and Oprml1-R: 5'-ttctggacacccctgctggc-3') and β-actin (Actb-F: 5'-tgctggactgctgctggg-3' and Actb-R: 5'-tgctggactgctgctggg-3').

(j) Statistics

All statistical tests were performed with repeated ANOVA followed by a Fisher LSD post hoc test using SYSTAT 8 software (SPSS Inc., Chicago, IL, USA).

3. RESULTS

(a) 5-HT₇R expression on inhibitory interneurons

Single-cell RT-PCR analysis of the cytosol harvested from identified inspiratory neurons (figure 1a) in the brainstem slice preparation of rat revealed the presence of µ-OR, 5-HT₁₇A and 5-HT₁₇R transcripts (figure 1b). The expression levels related to the housekeeping gene Hprt of the two 5-HTR transcripts were similar (5-HT₁₇A, 0.99 ± 0.22; 5-HT₁₇R, 1.14 ± 0.17), and significantly lower for the neuronal glycine transporter GlyT2 (0.43 ± 0.07).

At the protein level, we studied the cellular expression patterns of 5-HT₁₇A and 5-HT₁₇R. A novel anti-peptide monoclonal antibody recognizing 5-HT₇R was developed using Phe 281–Gly294 (NH₂-FPRVQPSVISLNG-COOH) in the third intracellular loop of the rat 5-HT₇R as an antigen for immunization (figure 1c). This antibody was used in combination with commercially available antibodies recognizing 5-HT₁₇A and GlyT2, the neuronal glycine transporter, for specific labelling of inhibitory glycinergic neurons (figure 1e,f). To obtain a complete overview of receptor expression profiles in excitatory and inhibitory neurons, immunohistochemistry was performed using sections encompassing the entire 200 µm extension of the pre-Bötc (including five consecutive 40 µm thick transverse sections; n = 5 animals) counting in total 2438 neurons within the pre-Bötc (figure 1e). The analysis revealed that a large percentage (62.4%) of pre-Bötc neurons are glycinergic, as they show GlyT2 immunoreactivity. The majority of these glycinergic neurons also expressed 5-HT₁₇A (79.9%), while 73.9 per cent of neurons expressed 5-HT₁₇R. Only 18.7 per cent of cells solely expressed 5-HT₁₇A, and 12.7 per cent expressed only 5-HT₁₇R. A minority (7.4%) of cells lacked either 5-HT receptor. The remaining 37.6 per cent of pre-Bötc cells may comprise glutamatergic or GABAergic neurons. The expression of 5-HT₁₇A and 5-HT₁₇R was also high in the GlyT2-negative group of cells, 84.6 per cent of cells expressing 5-HT₁₇A and 74.8 per cent of cells expressing 5-HT₁₇R. A large portion of GlyT2-positive neurons also co-expressed µ-OR (figure 1f). The inference is that glycinergic neurons in the pre-Bötc network do not only transmit, but evidently also receive signals via glycine receptors.

(b) Network responses to 5-HT₇-specific signalling

Following the observation that breathing is highly sensitive to opioids as well as a variety of 5-HTR signalling pathways, we tested for pharmacological effects of selective 5-HT₁₇A and 5-HT₁₇R activation. The rationale was that agonists such as 8-OH-DPAT might also activate Gₛ-coupled 5-HT₇Rs (Hedlund et al. 2004) and coactivation of these 5-HT₇Rs might prevail over the inhibitory Gₛ-AC pathway to neutralize the 5-HT₁₇A-R-induced decline of intracellular cAMP levels (Hoyer et al. 2002).

We tested for 5-HT₁₇R- and 5-HT₁₇A-specific effects in the perfused rat brainstem–spinal cord
preparation, in which the respiratory network remains fully intact (Paton 1996). The network response was measured by recording phrenic nerve activity (PNA) and deriving minute respiratory activity (mPNA) online, before and after administering 5-HT_{1A}-R or 5-HT_{7}-R agonists or antagonists. Surprisingly, application of the 5-HT_{7}-R agonist 5-CT (0.3 μg ml^{-1}) did not activate, rather it significantly reduced, mPNA (from 10.42 ± 3.98 to 4.47 ± 1.77; n = 5). mPNA activity was suppressed further (to 0.96 ± 0.41) when 5-HT_{1A}-R were additionally blocked with the selective antagonist WAY 100635 (figure 2a). Conversely, mPNA was augmented when the agonist 8-OH-DPAT was administered after 5-HT_{7}-Rs had been blocked with SB 269970 (figure 2b). Augmentation of mPNA activity occurred by enhanced burst frequency (9.54 ± 0.20 V s; p < 0.05) rather than an increase in PNA burst amplitude. Notably, the 5-HT_{1A}-R-dependent enhancement of PNA frequency by 8-OH-DPAT was abolished when glycine receptors were blocked by systemic application of strychnine. Strychnine application itself provoked a significant increase of mPNA (from 13.33 ± 1.03 to 22.73 ± 4.44; p < 0.05; n = 5), which did not change further (21.40 ± 4.64) when 5-HT_{1A}-Rs were additionally activated by 8-OH-DPAT (figure 2c).

Results were quite similar when relative changes of respiratory minute airflow (rRMA) were measured in vivo in anaesthetized rats. Administration of 8-OH-DPAT (n = 5) enhanced rRMA to 187 per cent (from 58.2 ± 8.4 to 109.3 ± 9.2; p < 0.01), an effect that was blocked by the 5-HT_{1A}-R-antagonist WAY 100635 (n = 3; figure 2d).

Taken together, these data demonstrate that the 8-OH-DPAT-induced increase of mPNA originates from a 5-HT_{1A}-R-specific modulation and is not simply explained by a 5-HT_{7}-R antagonism. The surprising network responses to 5-HT_{1A}-R and 5-HT_{7}-R stimulation, together with their disappearance after strychnine application, suggests that there is a signal-reversing process mediated through modulation of glycinergic inhibition.

(c) Modulation of inhibitory synaptic processes during global 5-HT_{1A}-R modulation

To test for 5-HT_{1A}-R-specific modulation of inhibitory synaptic processes, single-electrode current- and voltage-clamp measurements of identified respiratory brainstem neurons were performed in pentobarbital-anaesthetized cats. In contrast to augmentation of synaptic inhibition by single-cell electrophoresis of 5-HT_{7}-R agonists (Laalley et al. 1994; Richter et al. 1997), global activation of 5-HT_{1A}-Rs by intravenous injection of 8-OH-DPAT provoked an increase in neuronal excitability owing to a diminution of synaptic inhibition as tested in expiratory (n = 12) and post-inspiratory (post-I; n = 4) neurons within brainstem coordinates that outline the localization of the pre-BoTc in cat (Schwarzacher et al. 1995). The reduction in synaptic inhibition was most obvious in post-I neurons (figure 3), which are known to be glycinergic (Schmid et al. 1991; Ezure et al. 2003). These post-I neurons exhibited a release from early-inspiratory synaptic inhibition, causing them to advance their action potential firing far into the preceding inspiratory phase of the oscillatory cycle (figure 3a,c). At higher concentrations of 8-OH-DPAT, this release from early-inspiratory inhibition was combined with a membrane hyperpolarization, which limited action potential discharge to the late-inspiratory period, when post-I neurons are normally effectively silenced (figure 3a,b). In vivo voltage-clamp measurements performed at holding voltages of −75 to −90 mV, which is close to the chloride (inhibitory post-synaptic current) equilibrium potential (Haji et al. 2000), uncovered a clear inward current component, revealing that the process enabling the 8-OH-DPAT-provoked activity shift is linked to an excitatory synaptic input during inspiration. Under control conditions such an excitatory drive seemed to be shunted by synchronous synaptic inhibition (figure 3c−e).

The 5-HT_{1A}-R-induced change in the inspiratory phase-terminating discharge of inhibitory post-I neurons (Richter et al. 1987) is consistent with the corresponding network response to 8-OH-DPAT recorded from the phrenic nerve, consisting of shortened inspiratory burst duration and increased burst frequency (figure 3a). It is noteworthy that a similar effect has been described after strychnine blockade of glycinergic synaptic transmission (Busselberg et al. 2003).

(d) Network responses to parallel 5-HT_{1A}-R and opiate receptor signalling

The perception that the activation of rhythmic network output is accomplished by disinhibition of glycinergic neurons that could counteract opioid-induced depression of respiratory activity was confirmed with a sequential strategy of pharmacological treatment in the in situ perfused rat brainstem preparation. Opiate treatment was used to depress respiratory PNA, followed by the systemic administration of 8-OH-DPAT or buspirone, which is a clinically approved partial 5-HT_{1A}-R agonist (figure 4). After fentanyl had significantly reduced or arrested mPNA (from 6.76 ± 1.41 to 2.11 ± 0.58; p < 0.01), systemic administration of 8-OH-DPAT recovered mPNA to approximately 70 per cent of normal activity levels (4.95 ± 0.87 compared with 6.76 ± 1.41; p < 0.05; n = 6; figure 4a). The fentanyl-induced depression of mPNA (1.93 ± 0.73 versus 6.17 ± 1.55 V s; p < 0.01) was effectively re-activated with successive administration of buspirone (3.88 ± 0.88; p < 0.05; n = 4; figure 4b).

The recovery from opioid-depressed respiratory activity as seen in wild-type rodents was also verified in vivo. The μ-OR-agonist fentanyl, which is sufficient to completely block the nociceptive TF reflex (TFR; defined as a threefold prolongation of the 7.35 ± 1.11 s TFR at control), induced apnoea in rats (figure 4c). Subsequent administration of 8-OH-DPAT restored breathing within 2−3 min (figure 4c). The relative rRMA reappeared with 0.21 ± 0.4 (p < 0.001) and increased steadily within the following 3 min (0.60 ± 0.03 compared with control values of 0.68 ± 0.06; p < 0.001), while the TFR was not restored. This effect was μ-OR specific, because the

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antagonist naloxone induced an increase in rRNA above control levels (1.03 ± 0.12 versus 0.68 ± 0.06) and also fully restored the TFR (figure 4c).

(e) Receptor expression in the nociceptive dorsal horn network

Real-time PCR analysis from total cervical spinal cord tissue of the segments C2–C4 revealed relative transcript levels of 1.09 ± 0.16 for 5-HT1AR, 1.04 ± 0.029 for 5-HT2R and 0.88 ± 0.012 for µ-OR. Immunohistochemical analysis of protein expression (five sections from five animals each) showed that 95.1% of the µ-OR-immunoreactive dorsal horn neurons are also positive for the 5-HT1AR, whilst 5-HT2R was detected only in 53.4% of cells coexpressed 5-HT2R. The 5-HT1AR, 5-HT2R and µ-ORs were strongly coexpressed in neurons of the ventral horn (vh, c).

Figure 5. 5-HT1A, 5-HT7, and µ-OR expression in dorsal horn neurons of the rat spinal cord. Triple-labelling of 5-HT1A, 5-HT7 and µ-ORs in 40 µm thick sections from the cervical spinal cord (spinal segment C3/C4; overview in a) revealed with a goat anti-µ-OR antibody, a guinea pig anti-5-HT1AR antibody and a hamster anti-5-HT2R antibody (clone SHTR7/220) and appropriate fluorochrome-conjugated secondary antibodies: µ-OR (Cv2, green), 5-HT1AR (Cv3, red) and 5-HT2R (Cv5, blue). Within the dorsal horn (dh, b) the majority (95.1%) of µ-OR-immunoreactive neurons expressed 5-HT1AR, while only 53.4% coexpressed 5-HT2R. The 5-HT1AR, 5-HT7 and µ-ORs were strongly coexpressed in neurons of the ventral horn (vh, c).

4. DISCUSSION

Global administration of cAMP-depressing modulators into a neuronal network that operates by synaptic interactions between excitatory and inhibitory neurons can be expected to induce depression of neuronal excitability that also provokes particular forms of disinhibition. These synaptic interaction-dependent effects are evident in the respiratory network, which operates with a reciprocal organization of antagonistic groups of glutamatergic, GABAergic and glycineric interneurons (Richter, 1996). The outcome of synaptic disinhibition depends on the specific action of these inhibitory interneurons (figure 6) whose contribution is revealed by the glycinic and GABAA receptor antagonists—strychnine and bicuculline (Schmid et al., 1996; Busselberg et al., 2001). Modulation of glycineric inhibition seems particularly effective, because it not only controls patterning of the steadily augmenting inspiratory (aug-I) and expiratory (aug-E) burst discharges, but also regulates the efficiency of respiratory phase control by early-inspiratory (early-I) and post-inspiratory (post-I) neurons (Richter, 1982; Busselberg et al., 2001; Ezure et al., 2003; figure 6a). Glycinergic control of respiratory phase termination is vital (Pierrefiche et al., 1998) and, therefore, it is not surprising that more than 60 per cent of neurons within the pre-Bötzinger complex are glycineric, as identified by the neuronal glycine transporter GlyT2 (Gomeza et al., 2003; Tanaka et al., 2003). Some of these GlyT2-labelled inhibitory neurons were identified as respiratory in whole cell recordings (Winter et al., 2009). The GlyT2-positive inhibitory neurons also express 5-HT1AR and 5-HT2R (figure 1) and, therefore, are subjected to ongoing modulatory control by serotonin that is continuously released from raphe neurons (Connelly et al., 1989; Mason et al., 2007). Another important finding is that inhibitory glycineric neurons themselves receive glycineric inhibitory inputs (figure 1). This makes disinhibition a critical process in network adjustment.

A predominant modulatory effect of 5-HT is exerted through 5-HT1AR on 80 per cent of all pre-Bötzinger cells. A commonly used drug to test for the functional consequences of 5-HT1AR modulation is the agonist 8-OH-DPAT. When respiratory neurons are tested individually with locally restricted ionophoretic application of 8-OH-DPAT or with drugs that inhibit cAMP-dependent protein kinase A (PKA), individual neurons respond equally well with a significant enhancement of inhibitory synaptic currents (Lalley et al., 1997). This is particularly clear for the glycineric processes mediated by post-I neurons (Richter et al., 1987, 1992) and such pronounced enhancement of glycineric synaptic currents (Lalley et al., 1997; Richter et al., 1997) must be expected to alter the operation of the whole respiratory network. We propose a reorganization of network operation as is schematically shown in figure 6b. Discharge of early-I neurons is evidently abolished,
because their inhibitory feedback to post-I neurons disappears (figure 3a). Disinhibition of post-I neurons causes a fundamental change in network operation, because it induces a notable shift of post-I neuronal firing into the preceding inspiratory phase (figure 6b, see also figure 3). Their time-shifted and prolonged discharge is supposed to be very efficient, because 5-HT_{1A}R-induced modulation potentiates inhibitory synaptic currents in the innervated neurons (Lalley et al. 1997). These effects are only partially reduced but not abolished at higher 8-OH-DPAT concentrations when post-I neurons undergo a gK^+-controlled membrane hyperpolarization (Lalley et al. 1994; Ballanyi et al. 1997) limiting their discharge to late-inspiratory periods.

The functional uncoupling of early-I neurons from the network, combined with phase-shifting of post-I firing into the inspiratory phase, leads to the disappearance of normal post-inspiratory activity and provokes a two-phased oscillation between inspiration and expiration. This is consistent with the observation that 5-HT_{1A}R-evoked modulation is greatly
reduced when glycine receptors were blocked by strychnine (figure 1d), while an identical ‘phase-shift‘ occurs in the discharge of post-I neurons (Busselberg et al. 2001; Dutschmann & Paton 2002). Post-I neurons originating from the pons (Dick et al. 1994; Dutschmann & Herbert 2006) should be modulated synergistically. A minor change in network excitability might occur by modulation of tonically active glycinergic inputs (Haji et al. 1992). In conclusion, the data suggest that glycinergic synapses are the critical target of 5-HT1AR-regulated modulation of network functions and that reinforcement of inhibitory glycinergic processes (Lalley et al. 1997) might protect against arrhythmic breathing disturbances.

μ-ORs are abundantly expressed in pre-BotC neurons (Manzke et al. 2003) including NK-1R-positive excitatory neurons (Gray et al. 1999) and GlyT2-positive inhibitory respiratory neurons (figure 1). When activated, μ-ORs depress neuronal excitability of all these cell types (Ballanyi et al. 1997; Mellen et al. 2003). At opioid concentrations sufficient to slow respiration, respiratory periods are prolonged, indicating that the onset of post-I neuronal discharge is delayed and burst duration is lengthened (Lalley 2006). Such suppressed post-I neuronal discharge seems to be insufficient for adequate termination of the inspiratory phase. Our finding of a 5-HT1AR-induced disinhibition of post-I neurons (figure 3), enabling them to shift their discharge into the preceding inspiratory phase and potentiation of their inhibitory postsynaptic currents, appears to significantly shorten the latency of inspiratory phase termination and thus the rescue of respiratory rhythmicity. The present experiments demonstrate that this effect is fairly robust and compensates for μ-OR-induced depression of neuronal bursting (Brunton & Charpak 1997; Wagner et al. 2000; Chieng et al. 2006). Further analyses are necessary to understand how the accompanying membrane hyperpolarization affects membrane properties necessary for endogenous and/or rebound bursting (Ramírez & Richter 1996; Ramírez et al. 1997; Butera et al. 1999; Del Negro et al. 2002).

(a) Anti-nociceptive effect of 5-HT1AR agonists

In the sensory network of spinal nociceptive dorsal horn neurons, our immunohistochemical analysis showed that 95.1 per cent of the μ-OR-immunoreactive neurons coexpress 5-HT1ARs, while only 53.4 per cent of neurons express 5-HT7Rs (see figure 5). Therefore, the effects of 5-HT3Rs must be weaker, because the synergistic actions of μ-ORs and 5-HT1ARs should effectively depress cellular cAMP levels in this population of neurons. This should diminish PKA-dependent phosphorylation of the glycine receptors (Harvey et al. 2004) and potentiate synaptic inhibition to further enhance anti-nociception (Sandkühler et al. 1987; Helmchen et al. 1995).

(b) Significance for translational medicine

The present analyses of molecular, cellular and systemic processes reveal that in the case of reciprocally wired networks, predominant modulation of inhibitory neurons can prevail over depression of excitatory neurons to initiate a functional reorganization of the operational network machinery (figure 6c). The significance of such self-reorganization is evident in the respiratory network and explains how fentanyl depression of respiratory rhythmicity can be overcome with 5-HT1AR agonists (Sahibzada et al. 2000; Meyer et al. 2006). An important inference can be drawn: 5-HT1AR agonists may effectively protect against opiate-induced apnoea or severe slowing of breathing, but the underlying processes also induce a depression of inspiratory peak activity that affects tidal volume. Therefore, there is the possibility that the treatment could result in shallow breathing, which would require additional medical treatment.

The animal experiments were performed in accordance with the European Community and National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Ethics Committee of the Georg-August-University, Göttingen, Germany.

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