Expression and Function of Serotonin 2A and 2B Receptors in the Mammalian Respiratory Network

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Abstract

Neurons of the respiratory network in the lower brainstem express a variety of serotonin receptors (5-HTRs) that act primarily through adenylyl cyclase. However, there is one receptor family including 5-HT2A, 5-HT2B, and 5-HT2C receptors that are directed towards protein kinase C (PKC). In contrast to 5-HT2ARs, expression and function of 5-HT2BRs within the respiratory network are still unclear. 5-HT2BR utilizes a Gq-mediated signaling cascade involving calcium and leading to activation of phospholipase C and IP3/DAG pathways. Based on previous studies, this signal pathway appears to mediate excitatory actions on respiration. In the present study, we analyzed receptor expression in pontine and medullary regions of the respiratory network both at the transcriptional and translational level using quantitative RT-PCR and self-made as well as commercially available antibodies, respectively. In addition we measured effects of selective agonists and antagonists for 5-HT2ARs and 5-HT2BRs given intra-arterially on phrenic nerve discharges in juvenile rats using the perfused brainstem preparation. The drugs caused significant changes in discharge activity. Co-administration of both agonists revealed a dominance of the 5-HT2BR. Given the nature of the signaling pathways, we investigated whether intracellular calcium may explain effects observed in the respiratory network. Taken together, the results of this study suggest a significant role of both receptors in respiratory network modulation.

Introduction

Immunohistochemical and electrophysiological studies carried out over the previous twenty years have provided considerable evidence that serotonin (5-HT) released from caudal medullary raphé nuclei modulates respiratory network discharges in bulb and spinal regions [1–8]. Subsequent research set out to determine which subtypes of 5-HT receptors (5-HTRs) are operative as pharmacological targets for a potential therapy to treat centrally caused breathing disturbances [9–17]. Those studies revealed that 5-HT2ARs, 5-HT2AR/C, and 5-HT2C receptors modulate respiratory network discharge properties. These receptors represent only a fraction of the 5-HTR subtypes that modulate excitability of CNS neurons through various signaling pathways.

Amongst the 5-HTR family 5-HT2Rs include 5-HT2A, 5-HT2B, and 5-HT2C-R isoforms that couple preferentially to Gαq/11-proteins. The resulting activation of phospholipase C (PLC) increases hydrolysis of inositol phosphates and elevates cytosolic Ca2+ [18,19]. 5-HT2Rs are located post-synaptically [20–22], and there is evidence that they modulate neurotransmission at various central and peripheral synaptic sites [23,24].

5-HT2ARs stimulate PLC, leading to activation of protein kinase C (PKC), and increased excitability in bulbary respiratory neurons [25–27]. Earlier studies demonstrated PKC pathway-mediated modulation of the respiratory pattern [26] and excitation of respiratory neurons by activation of 5-HT2ARs [25,27]. Beside direct modulation of the respiratory motor pattern, 5-HT2ARs may have a key role in the induction of long-term facilitation of phrenic nerve activity in response to intermittent hypoxia [28–31].

5-HT2BRs have been implicated in anxiety, schizophrenia, autism, migraine, and spreading depression [32]. In addition, 5-HT2BR-dependent serotonin uptake influences the plasma serotonin level [33]. 5-HT2BRs are also important regulators of embryonic development; inactivation of the 5-HT2BR gene leads to partial embryonic and early neonatal death in mice [34]. In the respiratory network, it has been shown that 5-HT2BRs enhance rhythmic motor discharge activity recorded in neonatal mice in vivo [35].

Until now, there were no detailed descriptions published of 5-HT2BR distribution in the brainstem with expression data only available for the neocortex, cerebellum, dorsal hypothalamus, and medial amygdala [36].

In the present report, we provide a detailed account of the expression and distribution of 5-HT2ARs and 5-HT2BRs in the ponto-medullary respiratory network including respiratory motor population of the cervical spinal cord and brainstem. Using a monospecific anti-5-HT2AR-antibody prepared in our laboratory and a commercially available 5-HT2AR antibody, we show that 5-
HT2ARs and 5-HT2BRs are expressed in neurons of the pre-Botzinger complex (pre-BotC), an essential kernel of the respiratory network associated with the primary rhythmogenesis [37–40].

Furthermore, our study also demonstrates that both receptors affect discharge properties in the phrenic motor output.

### Materials and Methods

The experimental procedures were performed in accordance with European Community and National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The Ethics Committee of the Georg-August-University, Göttingen, Germany approved the study and assigned the approval ID T1108 to this work.

#### Antibody generation

The polyclonal antibodies against the rat 5-HT2BR were generated by immunizing three New Zealand White rabbits (Charles River) with a 16mer peptide derived from the second intracellular loop of the rat 5-HT2BR amino acid sequence (NH2-CaI SDLRl lA lK PKI Q-COOH; NCBI-Accession No.: NP_058946). For immunization purposes, peptides were coupled to keyhole limpet hemocyanin (KLH) according to standard protocols. The rabbits were immunized with 300 μg of KLH-coupled peptide in Hunter’s adjuvant (TierMax Gold, Sigma) five times (28-days-interval). The resulting antisera was affinity-purified against the immunizing peptide.

#### Western Blot detection of 5-HT2BR protein

Brain stem tissue isolated from both male Sprague-Dawley rats and male C57BL/6J mice were resuspended in 200 μl cell lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2% w/v SDS, 1% NP40, 0.5% Na-Deoxycholate) supplemented with protease inhibitor cocktail (Sigma). Protein content was determined using a Lowry assay for high SDS concentration according to manufacturer’s instructions (BioRad). 40 μg of total protein of each sample were boiled in 5× Laemmli buffer (250 mM Tris/HCl, pH 6.8; 10 mM EDTA, 10% w/v SDS, 5% v/v 2-mercaptoethanol, 50% v/v glycerol, 0.5% w/v bromophenol blue) for 5 min at 95°C and then separated using a precast SDS-PAGE (Novex). Proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 4% w/v BSA/TBS/0.05%/Tween (pH 7.4) for 45 min at RT. 5-HT2BR protein was detected with a self-made monospecific polyclonal antibody (1:1,000 dilution) after incubation for 3 hours at RT. After extensive washing, appropriate secondary horseradish peroxidase (HRP)-conjugated antibodies (Dianova, Germany; 2% BSA/PBS, antibody dilution 1:400). Neuronal immunofluorescence was analyzed with a confocal laser-scanning microscope Meta-LSM 510 (Zeiss, Germany) using laser lines at 488 nm (Ar/Kr laser) and at 633 nm (He/Ne laser). Confocal images were recorded by using overlays of two channels with the LSM 510 software provided by Zeiss. Digital images were taken at 2,048×2,048 dpi and were imported into Adobe Photoshop CS4, were digitally adjusted if necessary for brightness and contrast and were assembled into plates. Subsequent imaging procedures (cell counting) were performed using ImageJ [http://rsb.info.nih.gov/ij/].

#### Molecular Biology

(a) **Generation of expression constructs.** Brain tissue from one rat male at P11 was explanted and used for total RNA isolation with the OLS RNA kit (OLS, Germany) according to manufacturer’s instructions. The total RNA was used in one-step RT-PCR (Invitrogen) using primer pairs for the 5-HT2AR gene [Htr2a, F (5’-attgaaactctggagaaacg-3’)/R (5’-actcacagatacactcttgc-3’)] and 5-HT2BR gene [Htr2b, F (5’-agttgcgtactatgaaatg-3’)/R (5’-ttcctatgtaagctgccctc-3’)], respectively. The cycling program used for RT-PCR comprised of: initial reverse transcription at 55°C for 30 min followed by denaturation at 94°C for 2 min. 40 cycles of denaturation at 94°C for 15 sec, annealing at 57°C for 30 sec, and elongation at 68°C for 90 sec were concluded with a final elongation step at 68°C for 5 min. The resulting RT-PCR fragment was purified from the gel and cloned into pTarget expression vector (Promega). Sequencing validated the correct insert identity.

(b) **Transfection of cell lines.** Murine neuroblastoma cell line N1E-115 was obtained from ATCC and maintained at 37°C in humid atmosphere with 5% CO2 and passaged every second day. For transfection, cells were seeded 24 hours prior to transfection at a density of 100,000 cells in 4-well-plates (Nunc) on acid-washed and poly-L-lysine coated 12 mm round glass cover
slips. Cells were transfected with 2 μg DNA and 2 μl Lipofectamine (Invitrogen) in 500 μl OptiMEM (Invitrogen) per well and kept under normal culture conditions for 20 hours, afterwards fresh OptiMEM replaced the medium.

(c) Detection of endogenous Htr2b in cell lines by RT-PCR. Total RNA from 10^5 non-transfected cells or cells transfected with 6 μg of the plasmid encoding 5-HT2BR was prepared using the OLS RNA kit. One μg of total RNA each was entered in the one-step-RT-PCR reaction, and the resulting PCR fragment was analyzed on an agarose gel. While the 5'-sequence of murine and rat Htr2b is identical, the 3'-sequences do differ. Therefore, for RT-PCR the rat forward primer was used, while the reverse primer for mouse was 5'-ctatgtatgctgcttcgtcttc-3'.

(d) RT-PCR analyses of Htr2a and Htr2b of rat brain tissue. The total RNA of the cortex, hypoglossal nucleus, and pre-Boëtzinger complex dissected from corresponding 300-μm-thick slices preparations was isolated using GenElute™ mammalian total RNA kit (Sigma). First strand cDNA was synthesized from 1 μg total RNA using SuperScript™ first-strand synthesis system with random hexamers according to manufacturer's instructions (Invitrogen). Samples without reverse transcription (w/o RT) served as negative controls for the following PCR to exclude amplification of genomic DNA. For the PCR, specific forward and reverse primers were derived from different exons of the 5-HT2AR and 5-HT2BR cDNA to avoid amplification of genomic DNA. The cDNA sequences were obtained from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). Specificity of selected primers was tested by partial sequencing of the amplification products for their identification by SeqLab company (Götingen, Germany). The following primer pairs were used for amplification:

5-HT2AR [Htr2a, NCBI-Accession No.: NC_005114.2; F (5'-acccctggagtgagcact-3')/R (5'-taggactcctatgtag-3')], 5-HT2BR [Htr2b, NC_005110.2; F (5'-cctgtgtacttggctgccttc-3')/R (5'-gacactcatacactcttatta-3')], and for β-actin [Actb, NC_005111.2; F (5'-gatactcctcgtgctg-3')/R (5'-cctcgggcatcggaacc-3')].

The PCR reaction mixture for one sample was composed of 1–2 μl cDNA, 1 μl forward primer, 1 μl reverse primer, 1 μl dNTPs (100 mM dNTP mix), 1 μl DMSO, 5 μl NH4 buffer (10x), 2 μl MgCl2 (50 mM solution), and 1 μl PANScript red DNA polymerase (PAN Biotech, Germany). The mixture was filled up to 50 μl with DEPC-treated water. The following program was used for the PCR reaction: initial denaturation at 94°C, 4 min/38×[denaturation 94°C, annealing 1 min/55°C, extension 1 min/72°C, 2 min]/final elongation 72°C, termination 10 min/4°C hold. Actb (β-Actin) was used as an internal standard for all PCR reactions.

(e) Real-time RT-PCR. The relative quantification of Htr2a and Htr2b gene expression in specific rat tissues was done by real-time RT-PCR analysis. Spinal cord, inferior olive, pre-Boëtzinger complex, and parabrachial complex were dissected from corresponding 300-μm-thick cryostat sections [P32; n = 3 animals] under visual control. The total ribonucleic acid (RNA) of homogenized brain tissue was isolated using the Trizol® method according to manufacturer’s instructions (Gibco/BRL) and its concentration was determined using the NanoDrop ND-1000 spectrophotometer followed by its quality and integrity measurement by electrophoresis on RNA 6000 LabChip® kit (Agilent 2100 Bioanalyzer). The RNA was transcribed into the corresponding deoxynucleic acid (cDNA) using the iScript cDNA Synthesis Kit (BioRad). The following primer pairs were designed by using the Primer3 program (http://frodo.wi.mit.edu/primer3/):

Htr2a (NM_017254.1): F (5'-tgacgccagcaaatcaactcata-3')/R (5'-gcgggtctcctctctaagc-3'); Htr2b (NM_017250.1): F (5'-agcagccgagacccgagg-3')/R (5'-gggctctcctgcgggctcct-3'); Hprt1 (NM_012533.2): F (5'-gtcaagctgagcctctggatc-3')/R (5'-ttggctggttattccttgctg-3').

Gel electrophoresis revealed a single polymerase chain reaction (PCR) product, and the melting curve analysis showed a single peak for all amplification products. The PCR products were sequenced and blasted to confirm the correct identity of each amplicon. Ten-fold serial dilutions generated from cDNA of each sample were used as a reference for the standard curve calculation to determine primer efficiency. Triplicates of all real-time PCR reactions were performed in a 25 μl mixture containing 1/20 volume of the sample cDNA preparation from 250 ng total RNA, 400 nM of each primers, and 1× Fast-SYBR Green Master Mix (Applied Biosystems, USA).

The PCR-reactions were performed as follows: initial activation at 95°C for 60 s, 42 cycles of (denaturation 95°C/10 s, annealing and extension 60°C/30 s), and a final gradual increase of 0.5°C in temperature from 60°C to 90°C.

All real-time quantifications were performed using the iCycler IQ system (BioRad) and were adjusted by using the method according to Pfaffl [41].

Calcium imaging of cells recombinantly expressing 5-HT2AR or 5-HT2BR

The perfused brainstem preparation is, due to its thickness and need for constant perfusion not suited for microscopic analysis. Therefore, we opted to do the calcium imaging in murine neuroblastoma N1E-115 cells, where endogenous expression of 5-HT2Rs is negligible, but are known to signal via the PLC-DAG pathway [42,43]. Another advantage of transfection is the control over which receptors (5-HT2AR, 5-HT2BR or both) are expressed in individual cells, avoiding the need for antagonists and simplifying analysis. 12–16 hours post transfection, cells were transferred to calcium-free imaging medium (130 mM NaCl, 3.5 mM KCl, 1.25 mM NaH2PO4, 24 mM NaHCO3, 1.2 mM MgSO4, 10 mM Glucose) and incubated with Fluo-4-AM (Invitrogen) at a final concentration of 5 μM for 30 min at 37°C. The Fluo-4-AM stock solution was prepared as 2 mM using 10% pluronic acid F-127 in DMSO (Sigma) and was diluted just before use. After incubation, cells were washed with calcium-free medium and Fluo-4-AM was allowed to hydrolyze for another 30 min in the presence of probenecid to avoid leeching of fluorescent probe from the cell.

For calcium imaging, Fluo-4-AM loaded cells were transferred to a recording chamber equipped with an inverted Olympus microscope (IX71) with appropriate filters (515 nm beam splitter and a 535/50 band-pass filter) and a triggered LED light source (PreciseExcite, CoolLED) with 465 nm excitation. Images were taken for 300 μsec at 1 sec intervals. After recording baseline fluorescence, cells were stimulated with 1000 nM serotonin (Sigma) in calcium-free medium. This concentration was chosen based on a dose-response curve giving a linear response for serotonin stimulation between 500 and 1500 nM. For all experiments, a 10×, 1.0 NA objective (Olympus) was used.

To compare calcium measurements between experiments, we calculated the apparent fluorescent intensity F/F0 by dividing the fluorescent intensity (F) at every time point by the average fluorescence recorded before stimulation (F0). Data were statistically analyzed with Student’s t-test and presented as mean ± standard deviation (s. d.).

Perfused brainstem preparation of rats

(a) Perfused brainstem preparation of rats. The experiments on the perfused brainstem preparation [44] were performed on male Sprague-Dawley rats (P25–P32, 90–150 g).
that were housed under a 12 h light/dark cycle, with food and water provided ad libitum.

Animals were deeply anesthetized with halothane until they were unresponsive to a forepaw pinch, decerebrated at the precollicular level and cerebellotomized, bisected below the diaphragm, and the skin was removed. The upper body was placed into a recording chamber and perfused retrogradely via the thoracic aorta with ACSF (containing in mM: MgSO\(_4\) 1.25; KH\(_2\)PO\(_4\) 1.25; KCl 5; NaCl 125; CaCl\(_2\) 2.5; NaHCO\(_3\) 25; glucose 10, 1.25\% Ficoll and aerated with carbogen (5% CO\(_2\)/95\% O\(_2\); pH 7.35 at 30 °C). The perfusate was collected, filtered twice and re-circulated. Norcuronium-bromide (0.5 mg/ml) was added for muscle relaxation. The perfusion pressure was set to 45 to 55 mm Hg.

(b) Phrenic nerve signal processing. A silver wire immersed in bath solution within a capillary suction electrode picked up phrenic nerve activity representing the respiratory motor output to the diaphragm and inspiratory rib cage muscles. Phrenic nerve signals were amplified 2,000–5,000 (time constant, 100 ms). The processed signals were digitized by a PowerLab 8/30 microprocessor and stored using LabChart 7 software (ADInstruments, Australia).

Drugs added to the perfusate for specific pharmacological manipulation of 5-HT\(_2R\)s were purchased from Tocris Bioscience, Ellisville, USA: 5-HT\(_2A\)R agonist TCB-2 and 5-HT\(_2A\)R antagonist Altanserin hydrochloride, 5-HT\(_2B\)R agonist BW 723C86 and 5-HT\(_2B\)R antagonist LY 272015.

(c) Analysis of phrenic nerve discharge properties. Discharges of a representative one-minute duration were measured in the absence of (control) and after intra-arterial perfusion with ACSF containing a 5-HT\(_2A\) or 5-HT\(_2B\) receptor agonist or antagonist. Measurements of drug effect were made at 5-minute intervals. The peak of the integrated discharge (mV) was used as an estimator of discharge intensity and normalized to the control, which was set to 100\%. Discharge frequency (bursts per minute) was calculated from the integrated signals. Values (mean ± standard error of the mean) for amplitude and frequency were calculated from consecutive discharges that occurred over one minute during control and when drug effects were maximal.

All statistical tests (paired t-test) for pharmacological experiments were performed using GraphPad Prism version 5.0d for MacOS X.

Test drugs
5-HT receptor ligands tested for effects on phrenic nerve discharge properties were purchased from Tocris Bioscience, USA: TCB-2 (5-HT\(_2A\)R agonist), Altanserin hydrochloride (5-HT\(_2A\)R antagonist), BW 723C86 (5-HT\(_2B\)R agonist), LY 272015 (5-HT\(_2B\)R antagonist).

Results
Production and characterization of monospecific anti-5-HT\(_{2B}\)R antibodies
The peptide for immunization was derived from the second intracellular loop of the rat 5-HT\(_{2B}\)R-sequence (NH\(_2\)-CAILQ-DRTRAIEEEKPLQ-COOH; fig. 1Aa). The specificity of the monospecific polyclonal anti-5-HT\(_{2B}\)R antibody was tested in three different test systems: Immunoblot analysis (n = 3) of both mouse and rat brainstem lysate revealed a specific band at 48 kDa, which is in accordance with the predicted relative molecular mass of the receptor (fig. 1Ab). Murine neuroblastoma cells recombinitely expressing rat 5-HT\(_{2B}\)R (fig. 1B) were used for specificity testing of the antibody, while the neocortex and the hypoglossal nucleus (XII) was selected to test immunohistochemistry in tissue (fig. 1C) based on previous positive results reported by Duxon [36]. The control cells faintly expressed the mouse 5-HT\(_{2B}\)R that is also recognized by the antibody because of sequence homology. After transfection with the rat receptor the antibody labeling revealed a strong fluorescent signal. Also, both brain regions selected showed strong 5-HT\(_{2B}\)R reactivity (fig. 1Bc).

The anti-5-HT\(_{2B}\)R antibody immunoreactivity on cells as well as on neurons of both regions was effectively blocked after pre-incubation of the primary antibody with a 50-fold molar excess of the peptide that was used for immunization indicating specificity. As a control, RT-PCR analysis confirmed 5-HT\(_{2B}\)R-specific mRNA expression in cells within both regions (fig. 1Cc).

Expression analysis of 5-HT\(_{2A}\) and 5-HT\(_{2B}\)Rs in the respiratory network
Prior to analysis of 5-HT\(_{2A}\)R and 5-HT\(_{2B}\)R expression at the protein level, we confirmed their expression at the RNA level. For this, we dissected specific brain stem regions (fig. 2Aa) from corresponding frozen cryostat sections of juvenile rats (P30; n = 3 in which patches of both sides of each section were combined for one sample). Conventional RT-PCR analysis revealed gene expression for 5-HT\(_{2A}\) (Ht2a) and 5-HT\(_{2B}\)R (Ht2b) in the pre-BotC at the RNA level (fig. 2Ab). However, relative quantification of gene expression using real-time RT-PCR analysis revealed a 5.3-fold stronger expression of the Ht2a compared to the Ht2b gene in the spinal cord (0.0562 ± 0.0061 vs. 0.0110 ± 0.0036), 11.8-fold in the inferior olive (0.1875 ± 0.0196 vs. 0.0159 ± 0.0016), 6.8-fold in the pre-BotC (0.0617 ± 0.0046 vs. 0.0091 ± 0.0024), and a 4.1-fold stronger one in the parabrachial complex (0.0671 ± 0.0162 vs. 0.0188 ± 0.0061) (fig. 2C).

To analyze receptor expression at the protein level within the ponto-medullary respiratory network we applied our self-made monospecific polyclonal anti-5-HT\(_{2B}\)R antibody in combination with a commercially available monoclonal anti-5-HT\(_{2A}\)R antibody (BD Bioscience, San Diego, USA). Both receptor subtypes were expressed in crucial parts of the respiratory network such as the pre-BotC and the pontine Kölliker-Fuse nucleus (fig. 3, 4). A detailed analysis of the pre-BotC, the supposed kernel essential for the generation of the primary respiratory rhythm (Smith et al., 1991), showed a strong co-expression of 5-HT\(_{2A}\)R and 5-HT\(_{2B}\)R.

We analyzed a total amount of 2136 5-HT\(_{2B}\)R-immunoreactive cells (5 sections from each animal, n = 5 animals), 1345 cells (63\%) of these cells also expressed the 5-HT\(_{2B}\)R indicating strong co-expression of 5-HT\(_{2A}\)R and 5-HT\(_{2B}\)R. In the dorsolateral pons expression of 5-HT\(_{2B}\)R within the parabrachial complex (PB) and Kölliker-Fuse (KF) nucleus was weak, compared to the 5-HT\(_{2A}\)R (fig. 4D – F). Contrary, the 5-HT\(_{2A}\)R showed dense expression in the KF and lateral crescent nucleus of the PB (fig. 4A – C). Both nuclei are closely linked with respiratory control. In addition more modest expression was observed in the external lateral, central, and dorsal nuclei of the PB. Curiously, both 5-HT\(_{2A}\)R and 5-HT\(_{2B}\)R showed dense expression in the internal lateral nucleus (il) of the PB (fig. 4B, E), a subnucleus of the PB-complex that is still undefined in its physiological functions.

Analysis of systemic effects of 5-HT\(_{2A}\)Rs and 5-HT\(_{2B}\)Rs on respiratory activity
The effects of systemic application of specific antagonists for 5-HT\(_{2A}\)R and 5-HT\(_{2B}\)R (Altanserin hydrochloride and LY 272015, respectively) and specific agonists for 5-HT\(_{2A}\)R and 5-HT\(_{2B}\)R.
Figure 1. Verification of the anti-5-HT_{2B}R antibody. (A) (a) The 5-HT_{2B}R belongs to the family of seven-transmembrane-domain receptors that are coupled to hetero-trimeric guanine-nucleotide-binding protein q (G_{q}). The transmembrane-domains are indicated by red cylinders (I–VII). We produced a novel monospecific polyclonal antibody against the 5-HT_{2B}R by selecting a specific amino acid sequence (Cys^{146} - Gln^{161}; NH_{2}-\text{CAISLDRYAIKKPICOOH}) of the second intracellular loop of the rat 5-HT_{2B}R-sequence. The peptide exhibits 100% homology in mouse. Red letters indicate mismatches in the human-sequence (L^{150}R). (b) Immunoblot analysis of mouse (1) or rat (2) brainstem lysate revealed a specific band at about 48 kDa that corresponds with the predicted relative molecular mass of the 5-HT_{2B}R. (B) 5-HT_{2B}R expression in non-transfected (a) and 5-HT_{2A} and 2B Receptors in the Respiratory Network
transfected N1E-115 cells (b). The anti-5-HT_{2A}R antibody-dependent staining indicated a strong labeling of N1E-115 cells that had been transiently transfected with the rat 5-HT_{2A}R (b). Non-transfected cells expressing the mouse 5-HT_{2B}R showed a weak neuronal immunofluorescent signal that corresponded with a weak PCR signal (amplicon size 114 bp) for the mouse 5-HT_{2B}R mRNA (Htr2b) (c). Samples without reverse transcription (w/o RT) served as negative controls. (C) a, b Immunohistochemistry. Both pyramidal neurons of the cortex and motoneurons of the hypoglossal nucleus (XII) revealed a strong 5-HT_{2B}R immunoreactivity (-IR) (a) that was effectively blocked after pre-incubation of the antibody with a 50-fold molar excess of the peptide CAISLDRYIAIKKPIQ (+ peptide) that was used for immunization (b). Insets in (a) show labeled neurons at a higher magnification. Immunolabeling was performed using the PAP-method with diaminobenzidine as chromogen. (e) RT-PCR analysis of the rat cortex and hypoglossal nucleus. The 5-HT_{2B}R-specific mRNA (Htr2b) was detectable in neurons within both the rat cortex and the hypoglossal nucleus (XII) (amplicon size 380 bp).

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(TCB-2 and BW 723C86, respectively) on spontaneous breathing activity were investigated in male Sprague-Dawley rats using the perfused brainstem preparation [44]. We found that pharmacological manipulation of 5-HT_{2}Rs can either change the amplitude or the frequency of the phrenic nerve activity (PNA).

Application of the 5-HT_{2A}R-antagonist Altanserin hydrochloride (3.0 μg/ml, n = 3) [32,45] significantly reduced the amplitude from control of 100% to 32.94±4.72% (p<0.01), while breathing frequency increased from control of 10.25±0.63 to 14.50±3.39 bursts/min; n.s. (fig. 5A). Blockade of 5-HT_{2B}R with the antagonist LY 272015 (2.87 μg/ml, n = 3) [35,46] did not significantly change both the amplitude (100% of control vs. 96.54±2.36%; n.s.) and frequency (13.50±1.32 bursts/min vs. control: 14.50±1.32 bursts/min; n.s.) (fig. 5B). Application of the 5-HT_{2A}R-agonist TCB-2 (0.67 μg/ml, n = 5) [47] caused an increase of the amplitude (100% of control vs. 11.67±1.45 bursts/min of control vs. 11.00±1.12 bursts/min; n.s.). Administration of the 5-HT_{2B}R-antagonist Altanserin caused a further decrease of frequency to 8.33±0.88; p<0.05; (fig. 6A). Application of the 5-HT_{2B}R-agonist BW 723C86 (0.67 μg/ml, n = 5) [32] only caused an increase in frequency (13.33±1.45 bursts/min of control vs. 21.00±1.73 bursts/min; p<0.05), while the amplitude was not affected (from 100% of control to 95.67±3.76%; n.s.). Blockade of the 5-HT_{2B}R using LY 272015 diminished respiratory frequency nearly to baseline level (14.00±2.08 bursts/min; p<0.01) (fig. 6B). Interestingly, the simultaneous application of both agonists together resulted only in an increase of frequency (from 12.33±2.03 to 17.00±2.31 bursts/min; p<0.01), whereas no increment of amplitude could be observed (100% of control vs. 97.33±0.67%; n.s.; fig. 6C).

Calcium imaging of cells recombinantly expressing 5-HT_{2B}R or 5-HT_{2B}R

The electrophysiological recordings showed a frequency increase controlled by 5-HT_{2B}Rs when both receptors were stimulated concomitantly (fig. 5C), although both 5-HT_{2B}R and 5-HT_{2A}R are coupled to the same G_{q}-mediated signaling pathway (fig. 7A).

To analyze the calcium signaling of both receptors, we expressed them recombinantly either alone or together in neuroblastoma cells. To avoid artifacts, relatively low amounts of DNA were transfected to avoid overexpression.

The recombinant approach allowed us to image single cells and correlate resulting signals to a defined complement of receptors, which would not have been possible in perfused brainstem preparations.

In neuroblastoma cells expressing 5-HT_{2A}R and 5-HT_{2B}R alone, either agonist evoked a release of cytosolic Ca^{2+} from intracellular stores (see figure 7 and table 1) with a large initial calcium spike. While the reactions of individual cells varied slightly, the mean Ca^{2+} increase of cells expressing 5-HT_{2A}R (peak F/F_{0} of 2.59±0.8) was similar to those expressing 5-HT_{2B}R (peak

Figure 2. Quantification of expression levels and co-expression of 5-HT_{2A} and 5-HT_{2B}Rs within the pontine respiratory network. (A) (a) shows a schematic representation of the dissected pre-Bötz and its landmarks: pre-Bötzinger complex (pre-Bötz), nucleus of the solitary tract (Sol), nucleus ambiguus (NA), hypoglossal nucleus (XII), principal nucleus of the inferior olive (IO_{b}). (b) shows the specific mRNA of both receptors detected in the pre-Bötz. (B) Double labeling of 5-HT_{2A} and 5-HT_{2B}Rs. 5-HT_{2A} (Cy5, red) and 5-HT_{2B}Rs (Cy2, green) are strongly co-expressed in pre-BötzC-neurons. Immunohistochemical analysis does not reveal the ratio of co-expressed proteins. Therefore, we performed quantitative real-time RT-PCR on four selected nuclei of the respiratory network (C). The bar diagram represents results of quantitative real-time RT-PCR analysis of 5-HT_{2A}R genes (Htr2a, Htr2b) of spinal cord (SpC), inferior olive (IO), pre-Bötzinger complex (pre-BötzC), and parabrachial complex (PB). At the RNA level 5-HT_{2B}R is significantly stronger expressed compared to Htr2b in all regions analyzed. Asterisks indicate significance (** = p<0.001; ANOVA with Bonferroni’s post hoc test).

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In contrast, the calcium increase was significantly faster for 5-HT2AR (time to peak 30.5 ± 6.9 sec) than for 5-HT2BR (time to peak 57.7 ± 8.4 sec). The calcium level almost returned to baseline levels within ~35 seconds exhibiting no significant differences for both receptors, with a half-peak-width of 36.7 ± 6.1 sec for 5-HT2AR and 34.6 ± 3.2 sec for 5-HT2BR (fig. 7B).

Co-application of 5-HT2AR and 5-HT2BR agonists had unexpected effects. The Ca2+ signal (79.44 ± 8.9 sec) was significantly slower from onset to peak than the signals produced by either agonist alone (p<0.001), while the time of onset was similar to 5-HT2AR alone. In addition, the fluorescence signal at its peak (F/F0, 1.44 ± 0.17) was notably smaller (p<0.001). While the

Figure 3. Expression patterns of 5-HT2A and 5-HT2B Rs within the medullary respiratory network. (A) 5-HT2AR expression pattern in the pre-BötC. The plots (B, C) represent 5-HT2AR immunoreactivity within the pre-BötC (B) and BötC (C). (D–F) shows the corresponding expression pattern for the 5-HT2BR. The insets show labeled neurons at a higher magnification. Abbreviations: Bötzing complex (BötC), nucleus ambiguus (NA), pre-Bötzinger complex (pre-BötC), principal nucleus of the inferior olive (IOPr), interpolar spinal trigeminal nucleus (Sp5l).

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duration of the calcium peak by co-stimulation of both receptors was nearly doubled (half-peak-width of 86.32 ± 5.0 sec; p < 0.001), the amount of released calcium, measured as “area under the curve”, was very similar no matter if the receptors are expressed alone or together.

As our real-time PCR analysis showed that 5-HT_{2A}R and 5-HT_{2B}R are expressed in different amounts, with 5-HT_{2A}R being in 5- to 10-fold excess, we also transfected N1E cells with DNA ratios of 5-HT_{2A}R to 5-HT_{2B}R of 5:1 and 1:5, respectively. Regardless of the DNA ratios, the presence of 5-HT_{2B}R always

Figure 4. Expression patterns of 5-HT_{2A} and 5-HT_{2B}Rs within the pontine respiratory network. Both 5-HT_{2A} and 5-HT_{2B} receptors are abundantly expressed in neurons of the internal lateral nucleus of the parabrachial complex (B, E). Within the nucleus Kölliker-Fuse the 5-HT_{2B}R expression is weak compared to 5-HT_{2A}Rs (A, D). Abbreviations: internal lateral nucleus of the PB (il), lateral crescent nucleus of the PB (cr), nucleus Kölliker-Fuse (KF), parabrachial complex (PB), superior cerebellar peduncle (scp).

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produced slow but wide calcium transients (as shown for 5-HT2AR and 5-HT2BR in fig. 7C).

Discussion

This study reveals the locations of 5-HT2AR and 5-HT2BR in regions of the ponto-medullary respiratory network, including sites where the receptors are co-expressed. We demonstrate that agonist activation of the receptors evokes dramatic changes in discharge activity recorded from the phrenic motor output, and that activation of each type of receptor has distinctive effects on phrenic nerve discharge intensity and duration. Through the use of selective receptor antagonists, we found that only the 5-HT2AR constitutively modulates phrenic motor output. In neuroblastoma cells transfected with 5-HT2AR and 5-HT2BR, we discovered distinctly different calcium signal kinetics when each type of 5-HT receptor was activated. We also uncovered unexpected effects on signal amplitude and time course when 5-HT2AR and 5-HT2BR are co-activated.

In the paragraphs to follow, we discuss each of these aspects in turn, along with their physiological implications for respiratory motor output modulation.

Distribution of 5-HT2B and 5-HT2A receptors in regions of the ponto-medullary respiratory network

Within the medulla and pons, functionally defined respiratory regions provide input to cranial motoneurons controlling the airways, and to spinal motoneurons activating inspiratory and expiratory pump muscles. A variety of neurotransmitters and modulators involved in respiratory control have been identified in many of these respiratory related compartments [reviewed by Alheid and McCrimmon [48]]. In all these regions, 5-HT2AR and coupled protein kinase dependent signaling pathways have been identified functionally and anatomically [49]. Until now, however, the distribution of 5-HT2BRs had not been investigated, and nothing had been known about their functional importance to respiratory control. Our study shows that 5-HT2AR and 5-HT2BR are co-localized in the Kolliker-Fuse and Parabrachial regions of the Pons, and in the BotC and pre-BotC of the ventral medulla with an approximate 5-fold stronger expression of 5-HT2AR in all regions.

Respiratory neurons in the PB and KF constitute the pontine respiratory group. A variety of respiratory neuronal types are found in this region [50,51], which receives axonal projections from the ventral respiratory column, and from the nucleus of the solitary tract (NTS). The NTS itself is a receiving station for

Figure 5. Respiratory network responses to systemic 5-HT2A and 5-HT2B antagonist applications of in situ rats. Shown are representative traces of integrated phrenic nerve activity (PNA) of 2 different experimental conditions. Black traces represent the PNA under control conditions and blue traces indicate application of antagonists. The bar diagrams on the right give the averages of amplitude and frequency of least 3 independent experiments of each condition. Statistical analysis (paired t-test) is denoted in the panels on the right side with asterisks indicating significance (** = p<0.01). (A, B) Action of 5-HT2AR and 5-HT2BR antagonists in the perfused brainstem preparation of rat. (A) Application of the specific 5-HT2AR antagonist Altanserin (blue trace) significantly decreased phrenic nerve activity (PNA) by decreasing the amplitude. Also, a slight increase in frequency was observed. (B) Application of the 5-HT2BR antagonist LY 272015 had no discernable effect on either amplitude or frequency of PNA. We interpret these findings as constitutive activity of 5-HT2AR. doi:10.1371/journal.pone.0021395.g005
pulmonary afferents from the lungs and upper airways. Based on 5-HT2AR and 5-HT2BR co-expression patterns, our study would predict contributions by both types of receptors to respiratory modulation within the KF-PB complex, with a modulatory role played by 5-HT2BRs.

Based on both in vitro and in vivo [52,53] studies, the pre-Bo¨tC was identified as a medullary region essential for respiratory rhythm generation. The region may play a prominent role in inspiratory phase control, although the pre-Bo¨tC contains populations of neurons that exhibit a variety of respiratory related discharge patterns [Schwarzacher et al., 1995]. Previously, 5-HT1A, 5-HT2A, 5-HT4, and 5-HT7 receptors have been identified in the pre-Bo¨tC by immuno-labeling [8,12,17]. The Bo¨tC houses a prominent population of expiratory neurons that provide widespread inhibitory projections within the ventral respiratory column (VRC), targeting both inspiratory and expiratory bulbospinal neurons as well as respiratory-related cranial motoneurons [54]. Some expiratory Bo¨tC neurons also send axon collaterals to the spinal cord, reaching at least as far as the phrenic motor nucleus [48,55]. Our present study of 5-HT2AR and 5-HT2BR co-expression in the pre-Bo¨tC and Bo¨tC suggests that, as in the pontine respiratory group, 5-HT2AR modulation is predominant.

Differential effects of 5-HT2A and 5-HT2B receptor agonists and antagonists on phrenic nerve discharge properties

Activation of 5-HT2A Rs by the selective, CNS-permeable agonist TCB-2 [47,56] increased PNA discharge amplitude but not frequency, whereas the 5-HT2A antagonist Altanserin decreased both amplitude and frequency; in fact, discharge frequency decreased below control level. Although speculative, we suggest that 5-HT2A agonists target two functionally different populations of respiratory neurons: bulbospinal inspiratory neurons, leading to increased phrenic motor output, and propriobulbar inspiratory phase-regulating neurons that determine discharge frequency.
Altanserin’s capacity to reduce discharge frequency below control levels indicates that constitutive 5-HT2AR activation is substantial in inspiratory phase regulating neurons.

The 5-HT2BR agonist BW 723C68 increased only frequency, indicating that only neurons involved in discharge rate regulation were affected. However, the antagonist LY 272015 changed neither amplitude nor frequency. This suggests that in our in situ perfused brainstem preparation, 5-HT2BRs though present and activated by BW 723C68 were not constitutively activated.

When 5-HT2AR and 5-HT2BR agonists were given concurrently, a frequency increase that exceeded the singular effects of either agonist occurred. This is an expected outcome if frequency-controlling neurons are preferred targets for 5-HT2BR modulation in the ponto-medullary respiratory compartment.

5-HT2A and 5-HT2B receptors effects on Ca2+ signaling

Because 5-HT2A and 5-HT2B receptors both utilize an intracellular signal pathway that leads to a buildup of cytoplasmic Ca2+, the effects of these receptors on Ca2+ signaling were investigated.

Table 1. Effects of 5-HT2A and 5-HT2B agonists on Ca2+ fluorescence signals.

| Ca2+ Signal Properties   | 5-HT2A-agonist | 5-HT2B-agonist | both agonists | Unit | T-test* | T-test** |
|--------------------------|----------------|----------------|--------------|------|---------|---------|
| Onset time               | 10.1 ± 1.7     | 40.3 ± 1.5     | 11.9 ± 2.3   | [sec] | p, 0.001| p, 0.01*|
| Time to peak             | 30.5 ± 9.6     | 57.5 ± 8.4     | 79.44 ± 8.9  | [sec] | p, 0.01 | p, 0.01 |
| Half peak width          | 36.7 ± 1.7     | 34.6 ± 3.2     | 86.32 ± 5.0  | [sec] | p, 0.500| p, 0.001|
| Height of peak (F/F0)    | 2.59 ± 0.8     | 2.23 ± 1.1     | 1.44 ± 0.17  | [AU]  | p, 0.500| p, 0.001|
| Area under the curve     | 366.38 ± 93.64 | 319.47 ± 74.86| 307.43 ± 24.43| [AU]  | p, 0.500| p, 0.500|

*comparing results from single transfected cells stimulated with 5-HT2A-agonist or with 5-HT2B agonist.

**comparing results from both single transfected/stimulated cells against double transfected/double stimulated cells.

*only when compared to single transfected cells stimulated with 5-HT2A-agonist.

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5-HT2A and 5-HT2B receptors effects on Ca2+ imaging

Figure 7. Signal transduction pathways of 5-HT2A and 5-HT2B receptors and calcium imaging. (A) The schematic diagram illustrates convergent signaling pathways for 5-HT2Rs. (B) shows summed calcium transients recorded in N1E-115 cells transfected with either 5-HT2AR (red line) or 5-HT2BR (black line). The curves were constructed from the means of 3–5 independent experiments, averaging at least 30 individual measurements. The arrow indicates the time of stimulation. Light and dark grey curves indicate control experiments of 5-HT2AR stimulated with Altanserin and 5-HT2BR stimulated with LY272015, respectively. (C) shows summed calcium transients recorded in N1E-115 cells transfected with a stoichiometric ratio of 5-HT2AR:5-HT2BR of 1:1 (green line), 5-HT2AR:5-HT2BR of 5:1 (black line) and 5-HT2AR:5-HT2BR of 1:5 (red line). The bar diagrams (D–G) show statistical analysis of (D) height of peak (F/F0), (E) time to peak (s), (F) half peak width (s) and (G) area under the curve (AU) for expression of 5-HT2AR alone (black), 5-HT2BR alone (red) or expression of 5-HT2AR and 5-HT2BR together at a stoichiometric ratio of 1:1 (green). Asterisks indicate significance (** = p < 0.01; *** = p < 0.001; paired t-test).

Abbreviations: serotonin 2A receptor (2A), serotonin 2B receptor (2B), phospholipase C (PLC), inositol 1, 4, 5-triphosphate (IP3), diacylglycerol (DAG), protein kinase C (PKC), hetero-trimeric guanine-nucleotide-binding protein q (Gq), adenylyl cyclase (AC), cyclic adenosine 5', 3'-monophosphate (cAMP), protein kinase A (PKA).

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Brainstem respiratory network, we interpret our findings as neuroblastoma cells can be equated with signaling in cells of the brainstem respiratory network. We acknowledge that there are limitations in relating Ca_{2+} signaling in intracellular organelles. A detailed interpretation of Ca_{2+} dynamics involves receptor activation, cell membrane Ca-channel openings as well as Ca_{2+} release and uptake processes in intracellular organelles. A detailed interpretation of Ca_{2+} signals recorded from the respiratory rhythm rodent slice preparation can be found in published studies by Keller and coworkers [57,58]. Those studies illustrate Ca_{2+} signals that are proportional to respiratory discharge intensity. Assuming that Ca_{2+} signaling in neuroblastoma cells can be equated with signaling in cells of the brainstem respiratory network, we interpret our findings as follows. The faster and larger Ca_{2+} signal may reflect more efficient 5-HT_{2A}R coupling to the PLC-DAG-PKC signaling pathway, or to the spatial arrangement of receptor, Ca_{2+}-channel, and organelles involved in Ca_{2+} release and uptake. We can offer no ready explanation for the slowing and diminution of the Ca_{2+} signal when 5-HT_{2A} and 5-HT_{2B} receptors co-expressed in neuroblastoma cells were coactivated. Perhaps different isoforms of DAG or PKC activated by 5-HT_{2A}R and 5-HT_{2B}R compete for phosphorylation sites on membrane calcium channels and storage sites and interact negatively.

Conclusion
Taking together the results from all sets of experiments (distribution, co-expression, phrenic nerve activity, and Ca_{2+} signaling), we formulate the following working hypothesis: 5-HT_{2A}R is the dominant receptor governing respiratory control as evidenced by its stronger expression and constitutive activity. 5-HT_{2B}R, being present in all respiratory nuclei analyzed and co-expressed with 5-HT_{2A}R in many cells, although at a much reduced level, may act as a dose-dependent modulator. If more serotonin is released than needed to activate all 5-HT_{2A}Rs, 5-HT_{2B}Rs become activated. This would allow the system to regulate the respiratory rhythm by controlling serotonin release. Activation of spare 5-HT_{2B}R has the strongest effect on respiratory frequency. This regulation could, at least in part, be due to Ca_{2+} signaling, as the presence of 5-HT_{2B}R changes the kinetics of the Ca_{2+} signaling observed for both receptors alone, without altering the overall amount of mobilized calcium.

Author Contributions
Conceived and designed the experiments: TM SV MN. Performed the experiments: TM SV MN URK AMB MK. Analyzed the data: TM SV MN. Wrote the paper: TM MN NB.

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