The potency of different serotonergic agonists in counteracting opioid evoked cardiorespiratory disturbances


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Serotonin receptor (5-HTR) agonists that target 5-HT4(a)R and 5-HT1AR can reverse μ-opioid receptor (μ-OR)-evoked respiratory depression. Here, we have tested whether such rescuing by serotonin agonists also applies to the cardiovascular system. In working heart–brainstem preparations in situ, we have recorded phrenic nerve activity, thoracic sympathetic chain activity (SCA), vascular resistance and heart rate (HR) and in conscious rats, diaphragmatic electromyogram, arterial blood pressure (BP) and HR via radio-telemetry. In addition, the distribution of 5-HT4(a)R and 5-HT1AR in ponto-medullary cardiorespiratory networks was identified using histochemistry. Systemic administration of the μ-OR agonist fentanyl in situ decreased HR, vascular resistance, SCA and phrenic nerve activity. Subsequent application of the 5-HT1AR agonist 8-OH-DPAT further enhanced bradycardia, but partially compensated the decrease in vascular resistance, sympathetic activity and restored breathing. By contrast, the 5-HT4(a)R agonist RS67333 further decreased vascular resistance, HR and sympathetic activity, but partially rescued breathing. In conscious rats, administration of remifentanil caused severe respiratory depression, a decrease in mean BP accompanied by pronounced bradycardia, but partially compensated the decrease in vascular resistance, sympathetic activity and restored breathing. By contrast, the 5-HT4(a)R agonist RS67333 further decreased vascular resistance, HR and sympathetic activity, but partially rescued breathing. In conscious rats, administration of remifentanil caused severe respiratory depression, a decrease in mean BP accompanied by pronounced bradycardia, but partially compensated the decrease in vascular resistance, sympathetic activity and restored breathing. By contrast, the 5-HT4(a)R agonist RS67333 further decreased vascular resistance, HR and sympathetic activity, but partially rescued breathing. In conscious rats, administration of remifentanil caused severe respiratory depression, a decrease in mean BP accompanied by pronounced bradycardia, but partially compensated the decrease in vascular resistance, sympathetic activity and restored breathing. By contrast, the 5-HT4(a)R agonist RS67333 further decreased vascular resistance, HR and sympathetic activity, but partially rescued breathing. In conscious rats, administration of remifentanil caused severe respiratory depression, a decrease in mean BP accompanied by pronounced bradycardia, but partially compensated the decrease in vascular resistance, sympathetic activity and restored breathing. By contrast, the 5-HT4(a)R agonist RS67333 further decreased vascular resistance, HR and sympathetic activity, but partially rescued breathing. In conscious rats, administration of remifentanil caused severe respiratory depression, a decrease in mean BP accompanied by pronounced bradycardia, but partially compensated the decrease in vascular resistance, sympathetic activity and restored breathing. By contrast, the 5-HT4(a)R agonist RS67333 further decreased vascular resistance, HR and sympathetic activity, but partially rescued breathing.
respiratory disturbances including apneusis or apnoeic periods (Lalley et al. 1994; Wilken et al. 1997; Sahibzada et al. 2000; El-Khatib et al. 2003; Manzke et al. 2003, 2009; Stettner et al. 2008; Yamauchi et al. 2008a,b). Although such neurological conditions causing central respiratory depression are relatively uncommon, these serotonergic agents may have a broader applicability in the treatment of opioid-mediated respiratory depression. Opioids are the most powerful analgesics available to treat pain in man, but their use is limited by side effects including the risk of fatal apnoea (Etches 1994; Goetz et al. 2003). Thus, inhibition of these respiratory glycinergic interneurons leads to network disinhibition, which paradoxically elevates intracellular levels of cAMP, hence restoring AC activity and thus a recovery of cardiovascular function (Manzke et al. 2003; Goméz et al. 2003). Importantly, 5-HT1A receptors were not expressed at the level of the spinal dorsal horn, which is the first relay for nociceptive afferents. Therefore, the beneficial analgesic effect of opioids can be maintained while breathing is protected by selective 5-HT receptor stimulation (Manzke et al. 2003). The mechanism underlying reactivation of breathing using 5-HT1A agonists is described in detail by Manzke and co-workers in this issue (Manzke et al. 2009). In brief, both 5-HT1A and μ-OR inhibit AC activity and thus a recovery of μ-OR-evoked respiratory depression using a 5-HT1A agonist appears paradoxical. However, it now appears that 5-HT1A is expressed predominantly on inhibitory glycinergic interneurons of the respiratory network, which have an essential function for respiratory rhythm generation and coordination of the pattern of cranial and spinal respiratory motor activity (Schmid et al. 1996; Pierrefiche et al. 1998; Büsselberg et al. 2001, 2003; Dutschmann & Paton 2002a,b; Ezure et al. 2003; Gomez et al. 2003). Thus, inhibition of these respiratory glycinergic interneurons leads to network disinhibition, which reliably restores breathing after opioid-induced depression (Manzke et al. 2009). Since 5-HT1A agonists per se have analgesic actions when applied at concentrations sufficient to compensate opioid-evoked respiratory depression (Güntner et al. 2009), opioid analgesia is maintained or even enhanced by 5-HT1A agonists (Güntner et al. 2009; Manzke et al. 2009).

It is also clinically observed that systemic administration of opioids can cause cardiovascular depression typically featuring hypotension and bradycardia (e.g. Elliott et al. 2000). While 5-HT1AR and 5-HT4R-mediated recovery of opioid-mediated respiratory suppression is relatively well understood (Manzke et al. 2003, 2009), the actions on the cardiovascular system are not yet investigated. Therefore, the present study was designed to investigate the opioid depression of the cardiovascular system and whether 5-HT4 activation is an effective rescue strategy. The studies reported herein have been performed in both the in situ-perfused working heart–brainstem preparation (WHBP) (Paton 1996) and conscious rats.

2. MATERIAL AND METHODS

Two different preparations were used according to the methods described previously: the WHBP (Paton 1996) and radio-telemetry recordings of cardiorespiratory activity in awake animals (Waki et al. 2003, 2007).

(a) Working heart–brainstem preparation

Rats (n = 10) were deeply anaesthetized with halothane (AstraZeneca) such that the withdrawal responses to noxious pinching of the tail and paw were absent. The animals were transected caudal to the diaphragm, exsanguinated and submerged in a cooled Ringer solution. They were decerebrated at the precollicular level to make them insentient and skinned. The descending aorta was isolated and the lungs removed. Preparations were then transferred to a recording chamber. The descending aorta was cannulated and perfused retrogradely with a Ringer solution (in mM: NaCl, 125; NaHCO3, 24; KCl, 5; CaCl2, 2.5; MgSO4, 1.25; KH2PO4, 1.25; dextrose, 10) containing 1.25 per cent Ficoll (an oncotic agent; Sigma, St Louis, MO, USA) and a neuromuscular blocker (vecuronium bromide, 3–4 μg ml\(^{-1}\)), using a roller pump (Watson-Marlow 502s) via a double-lumen cannula. The perfusion pressure (PP) was maintained in the range 50–70 mm Hg by adjusting the flow between 21 and 25 ml min\(^{-1}\) and by adding vasopressin (600–1200 pM, Sigma) to the perfusate, as described previously (Pickering & Paton 2006). The perfusate was gassed continuously with 5 per cent CO2 and 95 per cent O2, warmed to 31–32°C (temperature measured at the point of entry into the aorta) and filtered using a nylon mesh with 25 μm pore size.

(b) Phrenic and sympathetic chain recordings

Activity in the central end of the phrenic nerve (left) was recorded using a bipolar suction electrode. Its rhythmic ramping activity gave a continuous physiological index of preparation viability. The electrocardiogram was visible on the phrenic recording, and using a window discriminator, the R-wave was captured and the inter-R wave interval displayed as heart rate (HR). The efferent activity of the left thoracic sympathetic chain (xSNA) was recorded at the spinal level of T8–T12 via a bipolar suction electrode. All signals were amplified, bandpass filtered (0.5–5 kHz) and acquired in an A/D converter (CED 1401, Cambridge Electronic Design, CED, Cambridge, UK; 6 kHz sampling frequency) to a computer using SPIKE 2 software (CED). The frequency of phrenic discharge was determined by the time interval between consecutive phrenic bursts, and analysis of the thoracic sympathetic activity was carried out on

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the rectified and integrated signals (time constant of 100 ms). All the analyses were performed offline using SPIKE 2 software with custom-written scripts.

(c) Experimental protocols
Cardiorespiratory activity of the WHBP was recorded for at least 5 min during control and during the subsequent administration (to perfuse) of fentanyl (5–10 nM; µ-OR agonist) and either 8-OH-DPAT (1–5 µM; 5HT1aR agonist) or RS67333 (5–10 µM; 5HT4aR agonist). All preparations received naloxone (20–30 nM; µ-OR antagonist).

(d) Telemetry recordings in awake rats
We used a radio-telemetry system (Data Sciences International, USA) for recording blood pressure (BP). The system consists of three basic elements: (i) a transmitter for monitoring BP (TA11PA-C40); (ii) a receiver (RPC-1); and (iii) an adapter (R11CPA) with an ambient pressure monitor (APR-1) that produces analogue output signals of pulsatile BP. The system is calibrated relative to atmospheric pressure. Telemetered data were acquired by HEY-PRESTO (Mizuno Software, Miyagi, Japan) through an analogue input box (IP-810A, GigaTex Co., Ltd, Miyagi, Japan) and analogue I/O PC card (AD12-8(PM), CONTEC Co. Ltd, Japan), displayed on the computer screen and stored on a hard drive.

(e) Implantation of transmitter
Transmitters were implanted at least 7 days before any experimental protocol began. Rats (n = 8) were anaesthetized with an intramuscular injection of ketamine (60 mg kg⁻¹) and medetomidine (250 µg kg⁻¹). The level of anaesthesia was checked frequently by assessing limb withdrawal reflexes to a noxious paw pinch. A midline incision of the abdominal wall was made in the supine position, and the intestines were retracted to allow visualization of the abdominal aorta. The tip of the catheter (diameter 0.7 mm, retracted to allow visualization of the abdominal phrenic (approx. 3 cm). The wire was secured by suturing to the ventral abdominal wall. The tip of the other wire was placed subcutaneously and secured at the level of the sternum. The opposite ends of these two wires were tunnelled subcutaneously and protruded through a small hole in the skin of the neck on the dorsal side. After the surgery, the anaesthesia was reversed with a subcutaneous injection of atipamezole (1 mg kg⁻¹) and the rat returned to its home cage for recovery. The electrical activity was amplified and filtered (500 to 5 kHz; Neurolog, Digitimer, UK). The EMG was acquired by HEY-PRESTO through an analogue input box and analogue I/O PC card as described earlier. Data were displayed on the computer screen and stored on the hard drive.

(f) Implantation of venous cannula
Forty-eight hours before any experimental protocol began, a double lumen catheter (inside diameter 0.28 mm, outside diameter 0.61 mm; Portex Limited, UK) was inserted into the right jugular vein under ketamine (60 mg kg⁻¹) and medetomidine (250 µg kg⁻¹) as described earlier. The opposite end was tunnelled subcutaneously and protruded through a small hole in the skin of the neck on the dorsal side.

(g) Recording of diaphragm electromyography (EMG)
After implantation of the venous cannula, the electrode for diaphragm EMG was implanted. The electrode consisted of two fine insulated stainless steel wires (diameter 0.25 mm, Unique Medical, Japan). The tips of the wire were bared of insulation (approx. 1 cm), and a small loop formed in one to avoid tissue damage. A midline incision (approx. 1 cm) of the abdominal wall was made just below the xyphoid process, and one wire inserted through this incision until the loop of the wire touched the diaphragm (approx. 3 cm). The wire was secured by suturing to the ventral abdominal wall. The tip of the other wire was placed subcutaneously and secured at the level of the sternum. The opposite ends of these two wires were tunnelled subcutaneously and protruded through a small hole in the skin of the neck on the dorsal side. After the surgery, the anaesthesia was reversed with a subcutaneous injection of atipamezole (1 mg kg⁻¹) and the rat returned to its home cage for recovery. The electrical activity was amplified and filtered (500 to 5 kHz; Neurolog, Digitimer, UK). The EMG was acquired by HEY-PRESTO through an analogue input box and analogue I/O PC card as described earlier. Data were displayed on the computer screen and stored on the hard drive.

(b) Experimental protocols
After recording cardiorespiratory parameters during control (quiet) conditions, remifentanil was infused intravenously (IV) using an infusion pump for all animals. Remifentanil infusion commenced at 0.5 µg kg⁻¹ min⁻¹, and at intervals, the perfusion rate was increased in 0.5 µg kg⁻¹ min⁻¹ steps until the animals showed substantial suppression of cardiorespiratory function. This was accompanied by the development of an abnormal posture and opioid-induced rigidity. Disturbed cardiorespiratory functions were recorded for 5 min, followed by a single bolus injection (IV via other lumen of double lumen cannula) of either 1 mg kg⁻¹ 8-OH-DPAT or 5 mg kg⁻¹ RS67333. In the rare case that the first injection of serotonergic agonists was ineffective in altering cardiorespiratory activity, a second bolus injection of the same dose was performed 5 min later. After recording the effects of 5-HT4aR and 5-HT1aR agonists, the infusion of remifentanil was stopped. One to three minutes after stopping the remifentanil infusion, animals regained normal posture and locomotor function. After 20–30 min, all animals showed full recovery of cardiorespiratory function and behaviour.

(i) Data analysis
HR and BP variability was analysed using the fast Fourier transform function of the HEY-PRESTO software (Waki et al. 2003). For each 5 min recording period, the systolic BP and beat-to-beat pulse intervals were converted into data points every 100 ms using a spline interpolation. According to Murasato et al. (1998), the magnitude of power was integrated in both the low-frequency (LF) band between 0.27 and 0.75 Hz and the high-frequency (HF) band.

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(0.75–3.3 Hz). In addition, the respiratory frequency and inspiratory duration were analysed from the diaphragm-EMG signal. The variability of the instantaneous pulse pressure and heart beat interval was analysed during control and pharmacological manipulations.

(j) **Histochemistry**

Juvenile rats (P28–P32) were deeply anaesthetized with isoflurane (1-chloro-2,2,2-trifluoroethanol-difluoromethylether; Abbott, Wiesbaden, Germany). The animals were transcardially perfused with 50 ml 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 with the same fixative at 4°C including 30 per cent sucrose. Later, series of 40 μm thick transverse brainstem sections were cut using a cryostat (Frigocut, Reichert-Jung, Germany).

(k) **Applied antisera**

The polyclonal anti-5-HT1AR antibody derived from guinea pigs was obtained from Chemicon (Catalogue no. AB5406, Temecula, USA). Specificity of the 5-HT4AR home-made affinity-purified polyclonal rabbit antibody was published previously (Manzke et al. 2003).

(l) **Peroxidase–anti-peroxidase (PAP) staining**

The intrinsic peroxidase activity was blocked with hydrogen peroxide–methanol (1:100) for 45 min at room temperature (RT) in the dark. After washing, sections were permeabilized with 0.2 per cent Triton X-100 for 30 min and directly transferred into phosphate buffered saline (PBS) containing 5 per cent bovine serum albumin for 1 h at RT to block non-specific binding sites. Sections were incubated overnight at 4°C in primary antibody solution at 1:1000 dilution. Secondary antibodies (diluted 1:100; DAKO, Denmark) were applied for 1 h at RT. After incubation in PAP solution (1:100; RT; DAKO) for 1 h, sections were pre-incubated with freshly prepared filtered dia-minobenzidine (DAB) solution (120 μl DAB (75 mg DAB dissolved in 1.5 ml 0.1 M phosphate buffer), 240 μl 1.25% CoCl2 and 240 μl 5% Ni(NH4)2SO4 diluted in 29.4 ml PBS) for 10 min at RT. The enzymatic reaction was started by adding 10 μl of 35 per cent H2O2 to 10 ml DAB solution and stopped with PBS after 1–5 min. DAB-stained sections were mounted onto gelatin-coated slides, dehydrated (2 x 50% ethanol, 2 x 80% ethanol and 2 x 99.9% ethanol, 5 min each) and coverslipped with mounting medium (DePeX from Serva, Heidelberg, Germany). Analysis was performed with the digital microscope Coolscope (Nikon, Melville, NY, USA). Images were taken at 2048 x 2048 dpi, imported into Adobe PHOTOSHOP CS3 and were digitally adjusted for brightness and contrast. The distribution of the labelled cells was plotted using a Neurolucida system (MBF Bioscience, Williston, VT, USA).

3. **RESULTS**

The effects of the 5-HT1A receptor and 5-HT4AR agonists, 8-OH-DPAT and RS67333, respectively, on the fentanyl-evoked cardiorespiratory disturbances were investigated in a total of 18 rats (n = 10 WHBP and n = 8 in vivo experiments).

(a) **Compensation of opioid-evoked cardiorespiratory disturbances in the working heart–brainstem preparation in situ**

(i) 5-HT1A receptor agonist 8-OH-DPAT (n = 5)

Systemic application of fentanyl (10 nM; figure 1a,b,c and table 1) caused significant prolongation of the duration of the inspiratory phase (Tc < 0.01), expiratory phase (Te < 0.01) and consequently of the respiratory cycle length (Ttot < 0.01), while PNA amplitude was reduced (p < 0.01). These changes were accompanied by bradycardia (figure 1a and table 1), and reductions in vascular resistance reflected by small falls in PP. Furthermore, fentanyl reduced sympathetic chain activity (SCA) (figure 1a,b,c and table 1) during both the inspiratory (SCAi < 0.05) and expiratory (SC Ae < 0.01) intervals. Subsequent application of 8-OH-DPAT (figure 1d,e) significantly recovered Tc (p < 0.01), Te (p < 0.01), Ttot (p < 0.01), PNA amplitude (p < 0.05) and SCAe (p < 0.05). However, PP and SCA showed trends in recovery only (table 1). By contrast, HR was further depressed after 8-OH-DPAT application (p < 0.05). Finally, subsequent application of naloxone to prove that the 8-OH-DPAT effects were not due to a time-dependent weakening action of fentanyl restored, at least partially, most of the recorded cardiovascular parameters to baseline values and increased breathing activity to levels above baseline (figure 1d,e and table 1).

(ii) Partial 5-HT4AR receptor agonist RS67333 (n = 5)

As mentioned earlier, systemic application of fentanyl (10 nM) caused significant prolongation of Ti (p < 0.01), Te (p < 0.01) and consequently of Ttot (p < 0.01), while PNA amplitude was reduced (p < 0.01; figure 2a,b,c and table 1). Accompanying cardiovascular changes (figure 2 and table 1) included bradycardia, and falls in vascular resistance and SCA (figure 2a,b,c and table 1). The subsequent application of the 5-HT4AR agonist partially recovered Ti (p < 0.05), Te (p < 0.01) and Ttot (p < 0.01). However, RS67333 had no effects on PNA amplitude, PP and inspiratory or expiratory SCA (table 1). In addition, HR was further suppressed (p < 0.05). Application of naloxone partially restored most of the recorded cardiovascular parameters to baseline values and increased breathing activity to levels above baseline (figure 2d,e and table 1).

(b) **Compensation of opioid-evoked cardiorespiratory disturbances in awake rats**

(i) 5-HT1A receptor agonist 8-OH-DPAT (n = 4)

Continuous IV infusion of remifentanil (0.5–3 μg kg−1 min−1) led to both a drop in respiratory rate (p < 0.01) and an increase in Ti (p < 0.01; figure 3 and table 1). The mean arterial blood pressure (MABP) showed a downward trend and HR dropped significantly (p < 0.05). Remifentanil also evoked bradycardia and large spontaneous fluctuations in MABP (figure 3a-c). Spontaneous baroreceptor

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reflex gain (sBRG) increased \((p < 0.01)\), while both HF and HF–LF pulse interval variations, which are indicative of parasympathetic and sympathetic activity, respectively, increased after remifentanyl \((p < 0.001; p < 0.05)\). Subsequent IV bolus injection of 8-OH-DPAT \((1 \text{ mg kg}^{-1})\) with continuous remifentanyl infusion immediately compensated the remifentanyl-evoked bradycardia (figure 3—compare \(c,d\)) and stabilized the fluctuations in MABP as well as restoring breathing (figure 3 and table 1). Breathing frequency \((p < 0.05)\) and \(T_i\) \((p < 0.05)\) significantly recovered. Although HR, MABP, HF and HF–LF pulse interval

**Figure 1.** Cardiorespiratory and sympathetic activity recorded in a WHBP during control \((a)\) and subsequent application of fentanyl \((10 \text{ nM})\) \((b)\). The traces illustrate that fentanyl \((\mu\text{-OR agonist})\) caused severe respiratory depression accompanied by prolongation of the inspiratory phrenic burst (PNA), decreased tSNA activity (T12-symp.), fall in heart rate (HR) and decreased vascular resistance indicated by the drop in PP. The subsequent application of 8-OH-DPAT \((5 \text{ µM})\) \((5\text{-HT}_{1A}\text{R agonist})\) in the presence of fentanyl significantly increased all recorded cardiorespiratory parameters with the exception of HR \((c)\). Application of the \(\mu\text{-OR antagonist naloxone } (30 \text{ nM})\) \((d)\), increased PNA and T12-symp. above baseline levels and further recovered HR and PP. \((e)\) Group data and statistical significance \((*p < 0.05, **p < 0.01, \text{repeated-measures ANOVA, followed by Fisher's LSD post hoc})\) of subsequent drug applications. \(T_i\), time of inspiration; \(T_e\), time of expiration; \(T_{tot}\), total respiratory cycle length. Black bar, control; grey bar, remifentanyl; black and white checked bar, RS67333; white bar, naloxone.
of the remifentanyl infusion restored all cardiorespiratory variables measured.

### Table 1. Summary of numerical data of the in situ experiments and of 8-OH-DPAT effects in conscious rat. *p < 0.05; **p < 0.01; ***p < 0.001. Ttot, total respiratory cycle length; Tinspiration; Texpiration; PNA ampl., phrenic nerve discharge amplitude; SCAi, sympathetic chain activity during inspiration; SCAe, sympathetic chain activity during expiration; PP, perfusion pressure; HR, heart rate; RR, respiratory rate; MABP, mean arterial blood pressure; sBRG, spontaneous baroreceptor reflex gain; HF, high-frequency pulse interval variations of BP; PP, perfusion pressure; MABP, mean arterial blood pressure; sBRG, spontaneous baroreceptor reflex gain; HF, high-frequency pulse interval variations of BP; Var. HBI, variability of instantaneous heart beat interval; Var. PPF, variability of instantaneous pulse pressure fluctuation of BP.

<table>
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<td>Ttot (s)</td>
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<td>Texpiration (s)</td>
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<td>1.8 ± 0.4**</td>
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<td>PNA ampl. (µV)</td>
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<td>28.7 ± 12.3**</td>
<td>42 ± 13.7**</td>
<td>46.9 ± 15**</td>
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<td>SCAi (µV s)</td>
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<td>45.4 ± 9.8**</td>
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<td>SCAe (µV s)</td>
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<td>28.4 ± 4.9**</td>
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<td>42.0 ± 8.8**</td>
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<td>PP (mm Hg)</td>
<td>49.1 ± 3.1</td>
<td>45.2 ± 3.8*</td>
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<td>HR (b.p.m.)</td>
<td>299 ± 16.5</td>
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</tr>
<tr>
<td>Var. HBI (ms)</td>
<td>2.18 ± 0.2</td>
<td>103.8 ± 7.2***</td>
<td>55.8 ± 16.9*</td>
<td>10.5 ± 5***</td>
</tr>
<tr>
<td>Var. PPF (mm Hg)</td>
<td>5.3 ± 0.4</td>
<td>139.5 ± 14.3***</td>
<td>53.5 ± 19.1**</td>
<td>9.1 ± 3.2**</td>
</tr>
</tbody>
</table>

*Not significant.

variations showed no significant effects (figure S1, electronic supplementary material; table 1), a significant recovery of fentanyl-induced variability of the instantaneous mean arterial blood pressure and heart beat interval was observed after 8-OH-DPAT application (figure 3f and table 1: both p < 0.05). Termination of the remifentanyl infusion restored all cardiorespiratory parameters back to baseline levels (figure 3 and table 1; figure S1, electronic supplementary material).

(ii) Partial 5-HT₄ receptor agonist RS67333 (n = 4)
In this series, remifentanyl dropped respiratory frequency from 61 ± 5.4 to 40 ± 7.6 breaths min⁻¹ (p < 0.05). MABP decreased from 94 ± 4 to 63 ± 5 mm Hg (p < 0.01) and HR fell from 364 ± 25 to 231 ± 86 b.p.m. (p < 0.05). However, sBRG increased from 0.86 ± 0.4 to 4.3 ± 0.6 ms mm Hg⁻¹ (p < 0.01). HF and HF–LF pulse variation also increased after remifentanyl infusion (HF: from 16.1 ± 9 to 82 ± 30 ms², p < 0.05; HF–LF: from 0.49 ± 0.07 to 1.08 ± 0.49 ms², p < 0.05). Subsequent IV bolus injection of RS67333 (1 mg kg⁻¹) with continuous remifentanyl infusion had no positive effect on any of the cardiorespiratory parameters analysed (figure S1, electronic supplementary material). As in the previous experiments, termination of the remifentanyl infusion restored cardiorespiratory activity partially (figure S1, electronic supplementary material).

(c) Control experiments
We tested the effects of either 8-OH-DPAT (n = 4, 1 mg kg⁻¹) or RS67333 (n = 4, 5 mg kg⁻¹) alone on cardiorespiratory activity in the absence of remifentanyl in conscious rats. 8-OH-DPAT had no significant effect on breathing rate (74 ± 12 versus 65 ± 3.6 breaths min⁻¹), HR (388 ± 29 versus 329 ± 27 b.p.m.), HF of pulse interval (20.3 ± 11.9 versus 16.5 ± 2.6 ms⁻¹) and HF–LF of pulse interval (0.56 ± 0.2 versus 0.49 ± 0.1 ms²), but decreased MABP (from 95 ± 3 to 85 ± 7 mm Hg, p < 0.05) and increased sBRG (from 0.72 ± 0.4 to 1.2 ± 0.4 ms mm Hg⁻¹, p < 0.01). In stark contrast, RS67333 injections produced no measurable effects on any of the cardiorespiratory variables measured.

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Distribution of 5-HT1A and 5-HT4 receptors within the pontomedullary brainstem

The expression and distribution of 5-HT1A and 5-HT4 receptors at the level of nucleus tractus solitarii (NTS), pre-Bötzinger complex (pre-BötC), Bötzinger complex (BötC) including the C1 region, the pontine parabrachial complex (PB) and Kölliker–Fuse nucleus (KF) are summarized in figures 4–6. A comparative analysis of the expression profile revealed a denser expression of 5-HT1A in all key cardiorespiratory

Figure 2. Illustration of the cardiorespiratory and sympathetic activity recorded in a WHBP during control (a) and during subsequent application of fentanyl (10 nM) (b). Comparable to figure 1, fentanyl caused severe cardiorespiratory depression. The subsequent application of RS67333 (10 μM) (5-HT4(a)R agonist) in the presence of fentanyl restored PNA, but further significantly decreased T12-symp, HR and PP (c). Application of naloxone (30 nM) (d), increased PNA and T12-symp. activity above baseline and partially recovered HR and PP. For abbreviations, see figure 1. Importantly, the HR and PP clearly remained below baseline after blocking fentanyl effects with naloxone. This indicates negative peripheral effects of RS67333 in contrast to 8-OH-DPAT (cf.figure 1). (e) Group data and statistical significance of subsequent drug applications (*p < 0.05, **p < 0.01, repeated-measures ANOVA, followed by Fisher’s LSD post hoc). Black bar, control; grey bar, remifentanil; black and white checked bar, RS67333; white bar, naloxone.
control nuclei compared with 5-HT4(a)R. For example, at the level of the medial NTS (figure 4), a crucial relay for baro- and chemoreceptor afferents, 5-HT4(a)R expression is sparse while 5-HT1AR was abundant. In the pre-BötC and BötC including the C1 region, 5-HT4(a)R expression was strong (figure 5), but 5-HT1AR was more widely expressed (figure 6). Finally, in the pontine PB and, in particular, the KF region, 5-HT1ARs were relatively dense compared with those of 5-HT4(a)R.

4. DISCUSSION

Our findings indicate that activation of serotonergic receptor subtypes can rescue the cardiovascular and respiratory depression induced by μ-opioid activation. Our data indicate that 5-HT1AR appears more efficacious than 5-HT4(a)R in restoring central respiratory activity and disturbances in HR and BP. This may relate to the finding that 5-HT1AR was more densely expressed than 5-HT4(a)R in brainstem regions known to control cardiorespiratory function.

The potency of 5-HT1AR and 5-HT4(a)R agonists in recovery from opioid-induced respiratory depression was demonstrated previously in anaesthetized rodents in vivo (Sahibzada et al. 2000; Manzke et al. 2003). The present study (in vivo and in situ) confirms these findings for the 5-HT1A agonist, but not for the 5-HT4(a)R agonist used here. The discrepancy in the present data with that reported earlier for 5-HT4(a)R

Figure 3. Radio-telemetry recordings of electrocardiogram (ECG), diaphragm EMG (diaEMG) and arterial BP in a conscious rat during control (a). Initial response to remifentanil infusion (b) and cardiorespiratory activity 3 min after the start of remifentanil infusion (c). Cardiorespiratory activity 2 min after a bolus injection of 8-OH-DPAT during continuous remifentanil infusion (d). Note the recovery of diaEMG activity and the decrease in the severity of the bradycardia illustrated in the BP trace (cf. c, d). Full recovery of cardiorespiratory activity 5 min after remifentanil infusion (e). Group data of beneficial effects of 8-OH-DPAT and breathing frequency and variability of instantaneous mean pulse pressure and heart beat interval are illustrated in (f). *p < 0.05, **p < 0.01, ***p < 0.001, repeated-measures ANOVA, followed by Fisher’s LSD post hoc. Black bar, control; grey bar, 2 min after remifentanil; black and white checked bar, 2 min after 8-OH-DPAT; white bar, 5 min after remifentanil stop.
may relate to the fact that we used a different agonist. Previously, the 5-HT4R agonist, BIMU-8, stabilized breathing in the anaesthetized rat (Manzke et al. 2003), whereas RS67333 used in the present study failed to recover breathing following opioid-induced depression. These differences may be owing to different pharmacological profiles of the agonists potentially targeting cell-specific splice variants of 5-HT4R expressed in different tissues (Mine et al. 1997; De Maeyer et al. 2008). Additionally, RS67333 may not penetrate the blood–brain barrier optimally under in vivo conditions. Finally, the preparations used in the present study were both unanaesthetized, whereas those used previously were anaesthetized (Manzke et al. 2003). Interestingly, in humans, both the 5-HT1A agonist, buspirone, and the 5-HT4R agonist, mosapride, were found to be ineffective in compensating opioid-evoked respiratory depression (Lötsch et al. 2005; Oertel et al. 2007). The ineffectiveness of buspirone in humans is surprising and disappointing: first, in humans, buspirone has been used to reverse major respiratory arrhythmias including apneusis (Wilken et al. 1997; Saito et al. 1999; El-Khatib et al. 2003; Richter et al. 2003; O’Sullivan et al. 2008). Second, in the rat, both recent evidence (Sahibzada et al. 2000; Guenther et al. 2009; Manzke et al. 2009) and data from the present study show a potent recovery from opioid-evoked respiratory and, in part, cardiovascular suppression with the administration of 8-OH-DPAT. The reason(s) for this remains unresolved, but may be species related in terms of receptor subunit differences and/or their distribution on cardiorespiratory neurons. Finally, BIMU-8, which stabilized breathing in anaesthetized rats (Manzke et al. 2003), has not been tried in humans.

**Figure 4.** Expression pattern of (a) 5-HT1A and (b) 5-HT4R in the nucleus of the solitary tract (Sol) at the level of the area postrema (AP). The insets in (a) and (b) show labelled neurons at a higher magnification. XII, hypoglossal motor nucleus; IR, immunoreactivity.

**Figure 5.** (a) 5-HT1A expression pattern in the pre-BötC. The schematics (b–f) illustrate 5-HT4R-IR within the pre-BötC (e) and BötC (f). The insets in (a) and (d) show labelled neurons at a higher magnification. BötC, Bötzinger complex; NA, nucleus ambiguus; pre-BötC, pre-Bötzinger complex; IOpx, principal nucleus of the inferior olive; Sp5l, interpolar spinal trigeminal nucleus.

**Cardiovascular effects following 5-HT1A and 5-HT4R receptor activation**

The serotonergic system exerts powerful influences on both the central (Jordan 2005) and peripheral cardiovascular systems (Kaumann & Levy 2006). Thus, a major concern for the use of serotonergic agonists in the treatment of centrally mediated respiratory dysfunction is the possible augmentation of the deleterious effects on cardiovascular activity. Here, we showed that the 5-HT1A agonist administered alone lowered arterial pressure and greatly enhanced baroreceptor reflex gain (suggesting increased excitability of cardiac vagal motoneurons, Gilbey et al. 1984), while 5-HT4R activation failed to alter baseline parameters. Indeed, the application of RS67333 showed additional deleterious effects on the opioid-evoked cardiovascular disturbance both in situ and in vivo. Also, the administration of 8-OH-DPAT enhanced the opioid-evoked bradycardia, which is consistent with the notion that the serotonergic system is involved in the control of cardiovascular function.
with previous data indicating activation of cardiac vagal motor neurons by this serotonin receptor subtype (for review, see Jordan 2005). However, in conscious animals, 8-OH-DPAT helped to reduce the occurrence of the opioid-evoked bradycardia and arterial pressure instability, although HR and BP levels remained below control (figure 4). In line with previous reports (Jordan 2005), we observed that injection of 8-OH-DPAT caused a mild bradycardia, increased baroreceptor gain and decreased BP, indicating that centrally acting 8-OH-DPAT can increase tonic cardiac vagal tone. In turn, increased tonic vagal tone and improved HR variability prevent cardiac arrhythmias and sudden cardiac death (La Rovere et al. 1998; Villareal et al. 2002). Moreover, as this agonist rescued breathing, this may have re-enforced respiratory–autonomic coupling to stabilize HR; the latter is supported from our in situ experiments in which phrenic-triggered coupling to sympathetic activity was increased.

(b) Differences in the expression profile of 5-HT_{1A} and 5-HT_{4(a)} receptors within cardiovascular-respiratory brainstem regions

The μ-OR is abundantly expressed in the entire pontomedullary respiratory network (Mansour et al. 1994; Haji et al. 2003; Lonergan et al. 2003; Manzke et al. 2003). Therefore, the respiratory depression that accompanies opioid administration (which includes μ-OR activation) in the treatment of severe pain can involve actions within the brainstem respiratory network. Our comparative immunohistochemical analyses of the expression pattern of 5-HT_{1A}R and 5-HT_{4(a)}R revealed obvious differences. In all parts of the ponto-medullary respiratory network investigated, which included the afferent integration nucleus (NTS) and the pontine PB/KF complex (figures 5 and 6), the 5-HT_{1A}R showed more dense expression. Both NTS and PB/KF have essential functions in the control of inspiratory duration (for review, see Kubin et al. 2006; Dutschmann et al. 2008). This is relevant since in both in vivo and in situ experiments, μ-OR activation depressed respiratory rate and caused apneusis, a pathological prolongation of the inspiratory phase (figures 1–3). From the lack of direct opioid-mediated effects on medullary respiratory neurons, it was concluded that suppressive actions of opioids essentially include neurons upstream to medullary respiratory neurons (Lalley 2006). We suggest that these upstream respiratory neurons involve those in the PB/KF. Consistent with this proposal is that blockade or lesion of PB/KF triggers apneusis, including the loss of post-inspiratory activity (Dutschmann & Herbert 2006; Smith et al. 2007).

Indeed, the proposed mechanism of 8-OH-DPAT-mediated recovery from opioid-evoked respiratory depression involves the re-instatement of post-inspiratory activity (Manzke et al. 2009), which is known to be present not only in medullary respiratory groups (Büsselberg et al. 2001, 2003; Dutschmann & Paton 2002a,b; Ezure et al. 2003; Smith et al. 2007) but also in the PB/KF (Dick et al. 1994; Mörschel & Dutschmann 2009). Furthermore, a recent publication suggested that the pons, including the PB/KF, is crucial for the coupling of both cardiac vagal and sympathetic activity to centrally generated respiratory drive (Baekey et al. 2008). Taken together, these data could explain why activation of 5-HT_{1A}Rs stabilizes the μ-OR-evoked cardiorespiratory disturbance. Thus, we propose that activation of 5-HT_{1A}Rs, particularly those in the pons, reverses the μ-OR-mediated apneusis and cardiovascular dysfunction (bradyarrhythmia and arterial pressure instability) by reinstating post-inspiratory activity that underlies much of the respiratory-related coupling to autonomic motor outflows (figures 1–3; Bainton et al. 1985; Baekey et al. 2008; Zoccal et al. 2008; Simms et al. 2009).

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Based on the greater density of 5-HT1A receptors relative to 5-HT4 receptors in brainstem cardiovascular and respiratory control regions, we suggest that this explains the more efficacious recovery of the opioid-induced cardiorespiratory depression by 8-OH-DPAT than RS67333. The abundant expression of the 5-HT1A receptors in the ponto-medullary brainstem enables 8-OH-DPAT to produce a global network disinhibition, which was shown to be the underlying mechanism to recover breathing after opioid poisoning (for details, see Manzke et al. 2009). Indeed, in respiratory nuclei of the medulla oblongata, 5-HT1A receptors are predominantly expressed on small-sized glycinergic interneurons (Manzke et al. 2009). However, 5-HT4 receptor expression is confined to larger neurons (figure 4). As shown previously, 5-HT4 receptor-mediated recovery of opioid-induced respiratory depression is based on a mechanism different from that of 5-HT1A receptors. 5-HT4 receptors antagonize the μ-OR-mediated decrease in intracellular cAMP through activation of AC (Lalley et al. 1997; Manzke et al. 2003). The lower expression profile of 5-HT4 receptors in the brainstem cardiorespiratory network can limit the efficacy of 5-HT4 receptor agonists, particularly if partial agonists such as RS67333 are used, as is the case herein.

(c) Summary and clinical implications

The present study revealed that the 5-HT1A receptor agonist 8-OH-DPAT evoked a potent recovery of breathing, following opioid-induced depression in the unanaesthetized decerebrated in situ rat preparation and in conscious animals. In addition, stabilization of cardiovascular activity was observed, which we propose relates, in part, to a re-inforcement of pontine respiratory-cardiovascular autonomic coupling. Despite the finding that 8-OH-DPAT has undesired effects on both central and peripheral cardiovascular activity, the beneficial effects of 5-HT1A receptor activation may be a useful strategy to overcome opioid-evoked cardiorespiratory disturbances. Clinically, the 5-HT1A receptor agonist buspirone is available and broadly used as an anti-anxiolytic drug. Results from investigations in the rat demonstrate identical respiratory responses to 8-OH-DPAT and buspirone (Sahibzada et al. 2000; Manzke et al. 2009). Further, buspirone has been used effectively in the treatment of apneustic disturbances in man (Wilken et al. 1997; Saito et al. 1999; El-Khatib et al. 2003; Richter et al. 2003; O’Sullivan et al. 2008). We suggest that clinically the 5-HT1A receptor agonist such as buspirone could serve as a pharmacological intervention to avoid and/or recover μ-OR-evoked cardiorespiratory disturbances.

All procedures conformed to the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of Bristol Ethical Review Committee.

REFERENCES


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