

RESEARCH PAPER

Melatonin inhibits tachykinin NK₂ receptor-triggered 5-HT release from guinea pig isolated colonic mucosa

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BACKGROUND AND PURPOSE

Melatonin is involved in the regulation of colonic motility, and sensation, but little is known about the influence of melatonin on 5-hydroxytryptamine (5-HT) release from colonic mucosa. A tachykinin NK₂ receptor-selective agonist, [β -Ala⁸]-neurokinin A₄₋₁₀ [β Ala-NKA-(4-10)] can induce 5-HT release from guinea pig colonic mucosa via NK₂ receptors on the mucosal layer. The present study was designed to determine the influence of melatonin on 5-HT release from guinea pig colonic mucosa, evoked by the NK₂ receptor agonist, β Ala-NKA-(4-10).

EXPERIMENTAL APPROACH

The effect of melatonin was investigated on the outflow of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) from muscle layer-free mucosal preparations of guinea pig colon, using high-performance liquid chromatography with electrochemical detection.

KEY RESULTS

Melatonin caused a sustained decline in the β Ala-NKA-(4-10)-evoked 5-HT outflow from the muscle layer-free mucosal preparations, but failed to affect its metabolite 5-HIAA outflow. The specific MT₃ receptor agonist, 5-methoxycarbonylamino-N-acetyltryptamine mimicked the inhibitory effect of melatonin on β Ala-NKA-(4-10)-evoked 5-HT outflow. A MT₃ receptor antagonist prazosin shifted the concentration-response curve of melatonin to the right in a concentration-dependent manner and depressed the maximum effect, but neither a combined MT₁/MT₂ receptor antagonist luzindole, nor a MT₂ receptor antagonist N-pentanoyl-2-benzyltryptamine modified the concentration-response curve to melatonin.

CONCLUSIONS AND IMPLICATIONS

Melatonin inhibits NK₂ receptor-triggered 5-HT release from guinea pig colonic mucosa by acting at a MT₃ melatonin receptor located directly on the mucosal layer, without affecting 5-HT degradation processes. Possible contributions of MT₁/MT₂ melatonin receptors to the inhibitory effect of melatonin appear to be negligible. Melatonin may act as a modulator of excess 5-HT release from colonic mucosa.

Abbreviations

5-HIAA, 5-hydroxyindoleacetic acid; 5-MCA-NAT, 5-methoxycarbonylamino-N-acetyltryptamine; β Ala-NKA-(4-10), [β -Ala⁸]-neurokinin A₄₋₁₀; DH-97, N-pentanoyl-2-benzyltryptamine; IBS, irritable bowel syndrome; NA, noradrenaline

Introduction

5-Hydroxytryptamine (serotonin, 5-HT) has long been recognized as an important messenger substance, which regulates colonic motility, secretion or sensation by acting via multiple receptor subtypes (Camilleri, 2002; Gershon and Tack, 2007). Most of the intestinal 5-HT is produced and stored in the mucosal enterochromaffin (EC) cells from which this amine is released into both the intestinal lumen and portal circulation (Racke and Schworer, 1991); therefore, alterations in the release of 5-HT from colonic EC cells affect the colonic function. As alterations of the colonic mucosa 5-HT have been also linked to functional bowel disorder, such as irritable bowel syndrome (IBS) (Miwa *et al.*, 2001; Coates *et al.*, 2004), a comprehensive understanding of the regulatory mechanism of 5-HT release from the colonic mucosa may offer a therapeutic strategy to help treat bowel disorders such as IBS.

Melatonin is a neurohormone derived from 5-HT, which is synthesized in the pineal gland as well as in the gastrointestinal tract (Bubenik, 2008). This indoleamine has been shown to be involved in the regulation of colonic motility and sensation (Lu *et al.*, 2005; 2009; Bubenik, 2008), and pharmacological doses have a beneficial effect on abdominal pain in IBS patients who suffer from disturbed sleep (Song *et al.*, 2005). However, the current knowledge on the role of melatonin in physiological and pathophysiological colonic functions is still poor.

The guinea pig colonic mucosa is not innervated by intrinsic 5-HT-ergic neurones (Wardell *et al.*, 1994); therefore, the mucosal release of 5-HT is not contaminated by neuronal 5-HT. Our previous *in vitro* studies in the guinea pig colon have demonstrated that a tachykinin NK₂ receptor-selective agonist, [β -Ala⁸]-neurokinin A₄₋₁₀ [β Ala-NKA-(4-10)] is capable of inducing tetrodotoxin-resistant and loperamide-insensitive 5-HT release from the colonic mucosa, indicating that β Ala-NKA-(4-10) facilitates 5-HT release from the guinea pig colonic EC cells via the activation of tachykinin NK₂ receptors located on the mucosal layer (Kojima *et al.*, 2004; 2005). Consistent with this hypothesis, we have recently shown that this β Ala-NKA-(4-10)-evoked 5-HT release is sensitive to the L-type calcium channel blocker nifedipine or the syntaxin inhibitor botulinum toxin type C, indicating that the tachykinin NK₂ receptor-triggered 5-HT release is mediated by syntaxin-related exocytosis mechanisms (Kojima *et al.*, 2009). Also, strong NK₂ receptor immunoreactivity has been observed on the surfaces of enterocytes at the bases of crypts in the guinea pig proximal colon (Portbury *et al.*, 1996). Overall, these results demonstrate that the guinea pig-isolated colonic mucosa is a useful *in vitro* preparation for studying non-neuronal regulatory mechanisms involved in the control of 5-HT release from colonic EC cells. In addition, daily melatonin supplementation has been recently shown to decrease the availability of 5-HT at the colonic mucosal surface of older mice: this indicates that melatonin can inhibit 5-HT release (Bertrand *et al.*, 2010). Therefore, we investigated the possibility that melatonin affects the NK₂ receptor-triggered 5-HT release from guinea pig colonic mucosa. To accomplish this, we determined whether melatonin affects the release of 5-HT from guinea pig colonic mucosa evoked by the selective NK₂ receptor agonist, β Ala-NKA-(4-10), using isolated muscle layer-free mucosal preparations.

Methods

Tissue preparation

All procedures were performed in accordance with the Dokkyo University School of Medicine animal care guidelines, which conform to the Guide for the Care and Use of Laboratory animal (NIH publication no. 85-23, revised 1985). Male Dunkin-Hartley guinea pigs (250–500 g body weight) were purchased from the Shizuoka Laboratory Animal Center, Inc. (Shizuoka). Guinea pigs were anaesthetized with enflurane and bled via the femoral artery. A segment of the proximal colon, 3–8 cm distal from the caecum was removed, and the luminal contents were washed out with a modified Tyrode's solution (136.8 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl₂, 1.05 mM MgCl₂, 0.42 mM NaH₂PO₄, 11.9 mM NaHCO₃, 5.56 mM glucose and 0.06 mM ethylenediaminetetraacetic acid Na₂). Muscle layer (longitudinal/circular muscle layer)-free colonic mucosal preparations (1.5 cm in length) from the proximal colon were prepared as described in a previous study (Kojima *et al.*, 2004). The tissue preparations were suspended in a longitudinal direction under a 4.9 mN load in 2-mL tissue baths filled with modified Tyrode's solution at 37°C and were aerated with 95% O₂/5% CO₂. The tissue preparations were allowed to equilibrate for 90 min with fresh replacement of the bathing medium every 10 min. Following the equilibration period, the experiments were conducted by collecting the bathing medium every 10 min. The medium obtained during the first 90–100 min was discarded. β Ala-NKA-(4-10) and melatonin was added to the incubation medium from 120 to 140 min or from 120 to 160 min respectively. At the end of the collection period, the tissue preparations were blotted and weighed.

Measurement of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and noradrenaline (NA)

The collected medium was lyophilized, dissolved in 0.4 M perchloric acid (200 μ L) and passed through a 0.45- μ m filter (Dismic-13CP; Advantec, Tokyo, Japan). 5-HT, 5-HIAA and NA levels in the filtrate were measured by a high-performance liquid chromatography (HPLC) with electrochemical detection (ECD-300; Eicom, Tokyo, Japan) and a pen rectifier (SS250F; Sekonic, Tokyo, Japan), as described previously (Kojima *et al.*, 2004). Known concentrations of 5-HT, 5-HIAA and NA (Sigma, St Louis, MO, USA) were used as standards. The separation of 5-HT, 5-HIAA and NA was achieved by a reverse-phase column [length of 100 mm, inner diameter of 4.6 mm, C-18 (3 μ m); Shiseido, Tokyo, Japan], using a mobile phase consisting of 0.1 M monochloroacetic acid, 1 mM ethylenediaminetetraacetic acid, 55 mg·L⁻¹ sodium octylsulphate and 5–10% acetonitrile (pH 3.2) at a flow rate of 0.5 mL·min⁻¹. Aliquots (20 μ L) of the filtrate were injected directly into the HPLC column. The limit of detection was between 142 and 283 fmol for 5-HT, between 131 and 261 fmol for 5-HIAA, and between 75 and 148 fmol for NA per injection. The levels of 5-HT, 5-HIAA and NA in the incubation medium are expressed in units or pmol·g⁻¹·10 min⁻¹. The results are expressed as a percentage of the mean outflow observed during the first two collection samples (100–120 min of incubation) of the individual experiments.

Drugs and solutions

The following drugs were used: β Ala-NKA-(4-10), melatonin, noradrenaline bitartrate salt hydrate, prazosin hydrochloride (Sigma); N-pentanoyl-2-benzyltryptamine (DH-97), luzindole, 5-methoxycarbonylamino-N-acetyltryptamine (5-MCA-NAT, Tocris, Bristol, UK). All drugs were dissolved in 10–70% dimethylsulphoxide with the following exceptions: β -Ala-NKA-(4-10) (100 μ M), noradrenaline bitartrate salt hydrate (100 μ M) and prazosin hydrochloride (10 μ M) were dissolved in distilled water. All subsequent dilutions of the drugs were made with distilled water. The vehicles had no effects on β Ala-NKA-(4-10)-evoked 5-HT/5-HIAA outflow or basal 5-HT/5-HIAA outflow.

Presentation of results and statistical analysis

Data are expressed as the means \pm SEM from n experiments. In all cases, n = the number of colonic mucosal preparations from different animals. The significance of differences was evaluated by one-way analysis of variance (ANOVA) followed by unpaired Student's t -test, by the computer programme Prism, where necessary multiple comparisons were conducted using one-way ANOVA with Newman–Keuls post hoc testing. A value of $P < 0.05$ was considered statistically significant. In some experiments, the inhibitory effect of melatonin or 5-MCA-NAT on β Ala-NKA-(4-10)-evoked 5-HT outflow was expressed as the per cent change from the control response. The negative logarithm of the molar concentration of melatonin or 5-MCA-NAT causing 50% of the maximal inhibitory effect (pIC_{50}) was calculated from the concentration–response curves for the inhibitory effect of the agonists, according to Van Rossum's method (Van Rossum, 1963).

Results

Effects of β Ala-NKA-(4-10)

The mean spontaneous outflow of 5-HT and 5-HIAA from the muscle layer-free mucosal preparations incubated in modified

Tyrodé's solution in the absence of test compounds (determined between 100 and 120 min of incubation) amounted to 112.8 ± 21.2 and 214.2 ± 18.6 $\text{pmol}\cdot\text{g}^{-1}\cdot 10\text{ min}^{-1}$ respectively ($n = 10$). In control experiments, the spontaneous outflow of 5-HT/5-HIAA from the muscle layer-free mucosal preparations did not change significantly during the period of observation up to 160 min (Figure 1A,B). As in previous studies (Kojima *et al.*, 2004; 2005; 2009), addition of the NK_2 receptor-selective agonist β Ala-NKA-(4-10) to the incubation medium (1 μ M, the maximally effective concentration, from 120 to 140 min of incubation) caused a sustained increase in the outflow of 5-HT: 5-HT outflow was enhanced to 195.9 ± 31.5 $\text{pmol}\cdot\text{g}^{-1}\cdot 10\text{ min}^{-1}$ ($n = 10$, $188.5 \pm 15.1\%$, compared with the initial outflow, $P < 0.01$) and then remained significantly elevated compared with the initial outflow after washout of the NK_2 agonist ($P < 0.05$; Figure 1A), but caused a marginal increase in the outflow of 5-HIAA; 5-HIAA outflow was enhanced to 250.6 ± 23.7 $\text{pmol}\cdot\text{g}^{-1}\cdot 10\text{ min}^{-1}$ ($n = 10$, $116.3 \pm 2.9\%$, compared with the initial outflow, $P < 0.05$; Figure 1B).

Effects of melatonin or 5-MCA-NAT

In the next series of experiments, we attempted to characterize the effect of melatonin or 5-MCA-NAT on the β Ala-NKA-(4-10)-evoked 5-HT/5-HIAA outflow. Addition of melatonin to the incubation medium (1 μ M, the maximally effective concentration, from 120 to 160 min of incubation) did not significantly affect the basal outflow of 5-HT and its metabolite 5-HIAA, but caused a sustained decline in the β Ala-NKA-(4-10)-evoked 5-HT outflow (67.4 \pm 5.3% inhibition, $n = 7$, $P < 0.01$; Figure 2A,B). The inhibitory effect of melatonin (10–1000 nM) on the β Ala-NKA-(4-10)-evoked 5-HT outflow was concentration-dependent with a pIC_{50} of 7.78 (Figure 3), but melatonin (10–1000 nM) failed to affect the β Ala-NKA-(4-10)-evoked 5-HIAA outflow (Figures 2B and 3). Pre-incubation with melatonin (1 μ M, from 100 to 160 min of incubation) also inhibited the β Ala-NKA-(4-10)-evoked 5-HT outflow (71.3 \pm 7.7% inhibition, $n = 4$, $P < 0.05$).

Addition of a specific MT_3 melatonin receptor agonist, 5-MCA-NAT to the incubation medium (100 nM, the

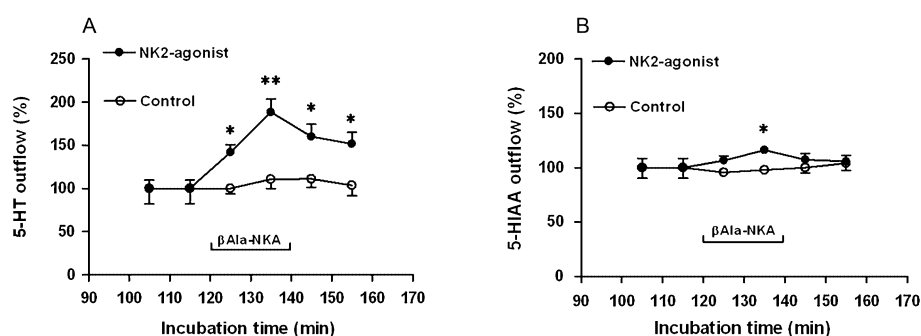


Figure 1

The outflow of 5-HT (A) and 5-HIAA (B) from muscle layer-free mucosal preparations of guinea pig colon in the absence (control) or presence of 1 μ M β Ala-NKA-(4-10) (NK_2 -agonist). β Ala-NKA-(4-10) was present from 120 to 140 min of incubation, as indicated by the horizontal bar. Ordinates: outflow of 5-HT and 5-HIAA, expressed as % of the mean outflow of first two collections (100–120 min of incubation). Each point represents the means \pm SEM from 10 experiments. Abscissae: time after onset of collection of the incubation medium. One-way analysis of variance followed by Newman–Keuls post hoc test, $*P < 0.05$, $**P < 0.01$, significantly different from the initial outflow. 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; β Ala-NKA-(4-10), [β -Ala⁸]-neurokinin A₄₋₁₀.

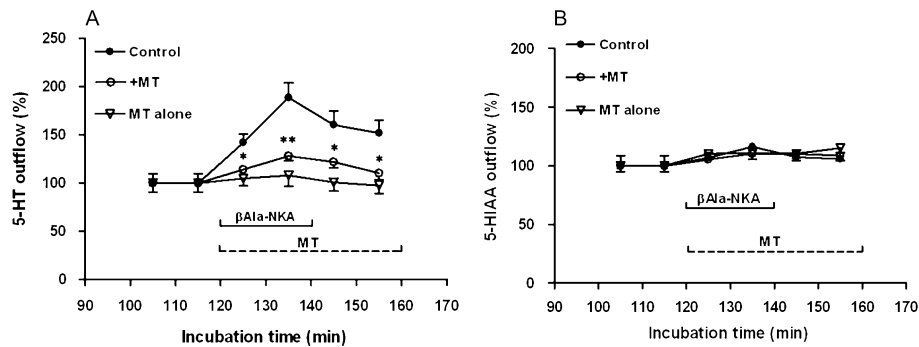


Figure 2

Effect of 1 μM melatonin (MT) on the outflow of 5-HT (A) and 5-HIAA (B) from the muscle layer-free mucosal preparations in the absence (MT alone) or presence (+MT) of 1 μM $\beta\text{Ala-NKA-(4-10)}$. $\beta\text{Ala-NKA-(4-10)}$ was present from 120 to 140 min of incubation, as indicated by the horizontal bar. Melatonin was present from 120 to 160 min of incubation, as indicated by the horizontal dotted line. Ordinates: outflow of 5-HT (A) and 5-HIAA (B), expressed as per cent of the mean outflow of first two collections (100–120 min). Each point represents the means \pm SEM from 6 to 10 experiments. * $P < 0.05$, ** $P < 0.01$, Significance of difference from the paired control. 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; $\beta\text{Ala-NKA-(4-10)}$, [$\beta\text{-Ala}^8$]-neurokinin A_{4-10} .

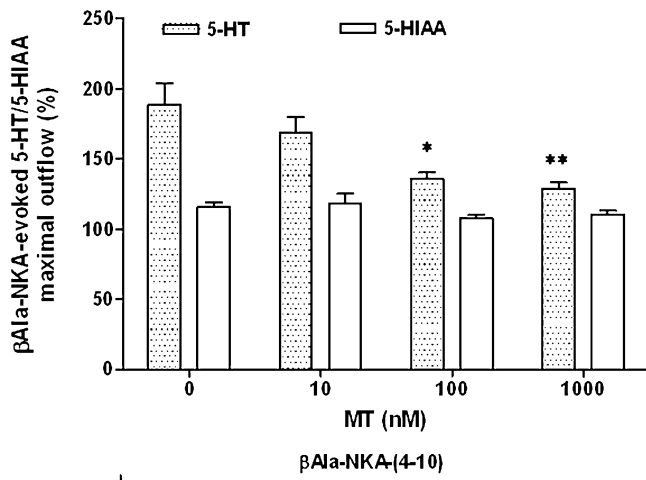


Figure 3

Effect of melatonin (10–1000 nM) on the maximal outflow of 5-HT and 5-HIAA from the muscle layer-free mucosal preparations evoked by 1 μM $\beta\text{Ala-NKA-(4-10)}$. Height of columns: $\beta\text{Ala-NKA-(4-10)}$ -evoked maximal 5-HT and 5-HIAA outflow, expressed as per cent of the mean outflow of first two collections (100–120 min). Results are the means \pm SEM from 6 to 10 experiments. One-way analysis of variance followed by Newman–Keuls post hoc test, * $P < 0.05$, ** $P < 0.01$, significantly different from the control. 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; $\beta\text{Ala-NKA-(4-10)}$, [$\beta\text{-Ala}^8$]-neurokinin A_{4-10} .

maximally effective concentration, from 120 to 160 min) did not significantly affect the basal outflow of 5-HT (Figure 4) and its metabolite 5-HIAA (data not shown), but caused a sustained decline in the $\beta\text{Ala-NKA-(4-10)}$ -evoked 5-HT outflow ($59.2 \pm 5.3\%$ inhibition, $n = 7$, $P < 0.05$; Figure 4). The inhibitory effect of 5-MCA-NAT (1–100 nM) was concentration-dependent with a pIC_{50} of 8.67 (Figure 5). 5-MCA-NAT (1–100 nM) also failed to affect the $\beta\text{Ala-NKA-(4-10)}$ -evoked 5-HIAA outflow (Figure 5).

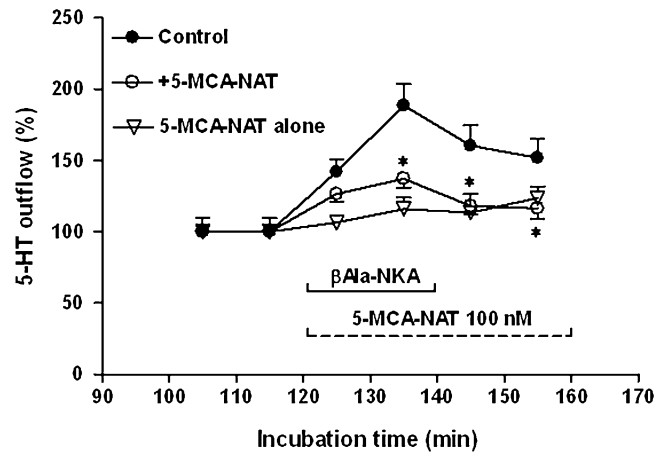


Figure 4

Effect of 100 nM 5-MCA-NAT on the outflow 5-HT from the muscle layer-free mucosa preparations in the absence (5-MCA-NAT alone) or presence (+5-MCA-NAT) of 1 μM $\beta\text{Ala-NKA-(4-10)}$. $\beta\text{Ala-NKA-(4-10)}$ was present from 120 to 140 min of incubation, as indicated by horizontal bar. 5-MCA-NAT was present from 120 to 160 min of incubation, as indicated by the horizontal dotted line. Ordinates: outflow of 5-HT, expressed as per cent of the mean outflow of first two collections (100–120 min). Each point represents the means \pm SEM from 6 to 10 experiments. * $P < 0.05$, Significance of difference from the paired control. 5-HT, 5-hydroxytryptamine; 5-MCA-NAT, 5-methoxycarbonylamino-N-acetyltryptamine; $\beta\text{Ala-NKA-(4-10)}$, [$\beta\text{-Ala}^8$]-neurokinin A_{4-10} .

Effects of antagonists

Several melatonin receptor antagonists were tested against the inhibitory effect of melatonin. None of the antagonists investigated had a significant influence on basal 5-HT/5-HIAA outflow. As shown in Figure 6, a MT_3 melatonin receptor antagonist prazosin (0.1 and 1 μM , from the onset of incubation) shifted the concentration–response curve of melatonin to the right in a concentration-dependent manner and

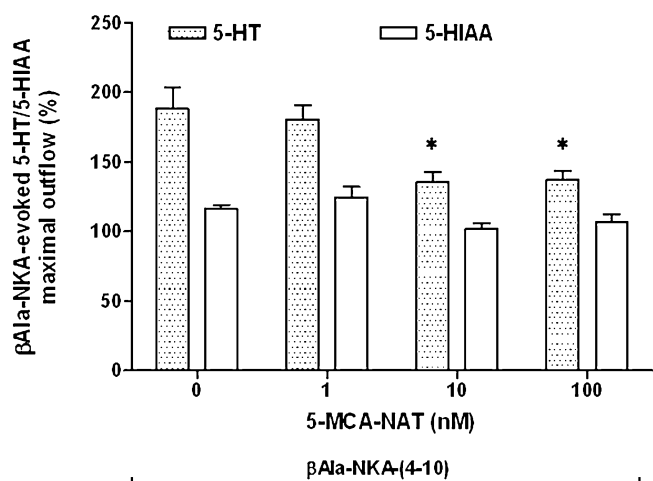


Figure 5

Effect of 5-MCA-NAT (1–100 nM) on the maximal outflow of 5-HT and 5-HIAA from the muscle layer-free mucosal preparations evoked by 1 μ M β Ala-NKA-(4-10). Height of columns: β Ala-NKA-(4-10)-evoked 5-HT and 5-HIAA maximal outflow, expressed as per cent of the mean outflow of first two collections (100–120 min). Results are the means \pm SEM from 6 to 10 experiments. One-way analysis of variance followed by Newman–Keuls post hoc test, * $P < 0.05$, significantly different from the control. 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-MCA-NAT, 5-methoxycarbonylamino-N-acetyltryptamine; β Ala-NKA-(4-10), [β -Ala⁸]-neurokinin A₄₋₁₀.

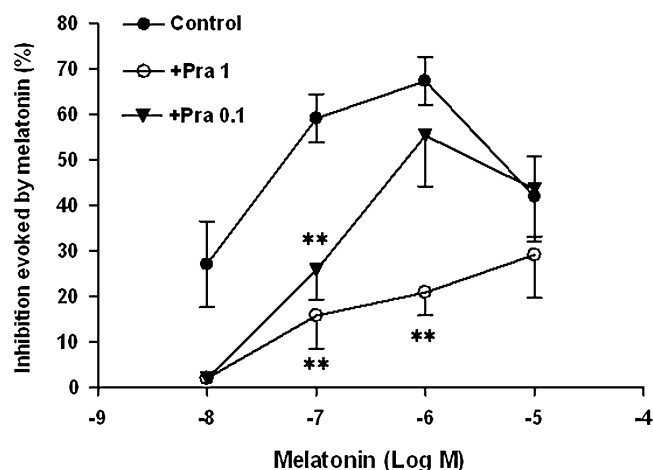


Figure 6

Effect of increasing concentrations of melatonin in the absence (control) or presence of prazosin (+Pra, 0.1 and 1 μ M) on the β Ala-NKA-(4-10)-evoked maximal 5-HT outflow from the muscle layer-free mucosal preparations. Ordinates: inhibition evoked by melatonin, expressed as the per cent change from the control response. Each point represents the means \pm SEM of 5 to 10 experiments. ** $P < 0.01$, significance of difference from the paired control. 5-HT, 5-hydroxytryptamine; β Ala-NKA-(4-10), [β -Ala⁸]-neurokinin A₄₋₁₀.

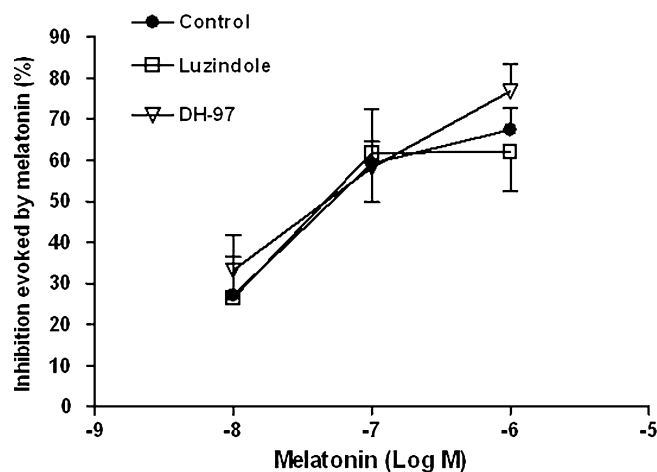


Figure 7

Effect of increasing concentrations of melatonin in the absence (control) or presence of DH-97 (1 μ M) or luzindole (0.3 μ M) on the β Ala-NKA-(4-10)-evoked maximal 5-HT outflow from the muscle layer-free mucosal preparations. Ordinates: inhibition evoked by melatonin, expressed as the per cent change from the control response. Each point represents the mean \pm SEM of 5 to 10 experiments. 5-HT, 5-hydroxytryptamine; β Ala-NKA-(4-10), [β -Ala⁸]-neurokinin A₄₋₁₀; DH-97, N-pentanoyl-2-benzyltryptamine.

depressed the maximum effect at 1 μ M. As prazosin is a α_1/α_{2B} adrenoceptor antagonist as well as a MT₃ receptor antagonist, we also investigated the effect of NA on the β Ala-NKA-(4-10)-evoked 5-HT outflow; NA (1 μ M, from 120 to 160 min of incubation) had no effect on the β Ala-NKA-(4-10)-evoked 5-HT outflow ($n = 5$, $186.1 \pm 18.1\%$, compared with the initial outflow). Also, no spontaneous outflow of NA from the muscle layer-free mucosal preparations was detected ($n = 4$).

Neither the combined MT₁/MT₂ melatonin receptor antagonist, luzindole (0.3 μ M, from the onset of incubation), nor the MT₂ melatonin receptor-selective antagonist DH-97 (1 μ M, from the onset of incubation) affected the concentration–response curve to melatonin (Figure 7).

Discussion and conclusion

Our previous *in vitro* studies have repeatedly indicated that the tachykinin NK₂ receptor-selective agonist β Ala-NKA-(4-10) is capable of inducing 5-HT release from EC cells of the guinea pig colonic mucosa via NK₂ receptors on the mucosal layer (Kojima *et al.*, 2004; 2005; 2009). This agrees with previous findings showing the presence of tachykinin NK₂ receptor immunoreactivity on the surfaces of enterocytes at the bases of crypts in the guinea pig proximal colon (Portbury *et al.*, 1996).

The present study deals with the question of whether melatonin affects the NK₂ receptor-triggered 5-HT release from guinea pig colonic mucosa.

In our initial experiments, we showed that melatonin inhibits the β Ala-NKA-(4-10)-evoked 5-HT outflow with a pIC₅₀ of 7.78, but failed to affect the outflow of 5-HT's

metabolite 5-HIAA. This indicates that melatonin inhibits the β Ala-NKA-(4-10)-evoked 5-HT outflow via direct interaction with the mucosal layer, without affecting the 5-HT degradation processes. Consistent with this observation, a recent study has shown that daily melatonin supplementation decreases the availability of 5-HT at the colonic mucosal surface of older mice (Bertrand *et al.*, 2010). Direct interaction with the mucosal layer has also been reported in rat distal colon, where melatonin inhibits prostaglandin E_2 -induced ion secretion (Mrnka *et al.*, 2008). The IC_{50} value (16.5 nM) obtained from the present study might seem to be higher than physiological levels, as the plasma concentrations of melatonin vary from the picomolar to low nanomolar range. However, the colonic mucosa seems to be capable of releasing melatonin to the lumen and its concentration at the mucosal surface in old mice reaches 6.6 μ M (Bertrand *et al.*, 2010), which is higher than the IC_{50} value reported here. Hence, it is possible that melatonin acts as a physiological modulator of excess 5-HT release from colonic mucosa.

Melatonin receptors are distributed throughout the gastrointestinal tract: effects of melatonin are mediated by specific high-affinity membrane MT_1 , MT_2 and MT_3 melatonin receptors (Dubocovich *et al.*, 2003). MT_1 and MT_2 melatonin receptors are negatively coupled to adenylate cyclase, while the MT_3 receptor has been identified as the enzyme quinone reductase 2 (QR2) in some mammalian species (Jockers *et al.*, 2008). Herein, we have also examined the effect of the specific MT_3 melatonin receptor agonist 5-MCA-NAT (Pintor *et al.*, 2003), as previous pharmacological data suggested the presence of the MT_3 melatonin receptors in guinea pig proximal colon (Santagostino-Barbone *et al.*, 2000). As the second main result of the present study, the specific MT_3 receptor agonist 5-MCA-NAT was found to mimic the inhibitory effect of melatonin on the β Ala-NKA-(4-10)-evoked 5-HT outflow with an IC_{50} of 2.1 nM, which is close to the K_i value (6.6 nM) reported for the MT_3 binding site in hamster intestinal membranes (Paul *et al.*, 1999). This indicates that melatonin inhibits the β Ala-NKA-(4-10)-evoked 5-HT outflow via MT_3 melatonin receptors on the mucosal layer. Moreover, we have made use of a selective MT_3 melatonin receptor antagonist, prazosin, to define a role for MT_3 receptors, because prazosin, familiarly known as an α_1/α_{2B} -adrenoceptor antagonist, has been used as a MT_3 melatonin receptor antagonist in the guinea pig proximal colon (Lucchelli *et al.*, 1997; Santagostino-Barbone *et al.*, 2000). Hence, the third finding of the present study was that prazosin shifted the concentration–response curve of melatonin to the right in a concentration-dependent manner and depressed the maximum effect. This indicates that prazosin acts non-competitively. Prazosin was also found to be a non-competitive MT_3 receptor antagonist in the guinea pig proximal colon (Lucchelli *et al.*, 1997; Santagostino-Barbone *et al.*, 2000). It was further observed that NA failed to affect the β Ala-NKA-(4-10)-evoked 5-HT outflow, indicating that α_1/α_{2B} -adrenoceptor-mediated effects do not play a role in the inhibitory action of melatonin. Thus, the melatonin receptor that mediates the inhibitory effect of melatonin appears to be a mucosal MT_3 melatonin receptor. Also, the possibility that the MT_3 melatonin receptor-mediated inhibition of basal 5-HT release is evoked via endogenously released melatonin is unlikely, because prazosin alone had no effect on the basal

5-HT release. Although the MT_3 receptor has been identified as the enzyme QR2 in some mammalian species, the relationship between the pharmacological actions of melatonin and the MT_3 receptor/QR2 protein is not yet well understood. Thus, the relationship between the inhibitory action of melatonin and the enzyme QR2 remains to be established.

We also made use of the selective MT_2 receptor antagonist DH-97 (Ting *et al.*, 1999) to define a role for MT_2 melatonin receptors in our experiments, as a recent immunohistochemical study has shown MT_2 melatonin receptor immunoreactivity in rat colonic mucosa (Stebelova *et al.*, 2010). However, the concentration–response curve to melatonin was not affected by the presence of the selective MT_2 receptor antagonist DH-97; therefore, the role of MT_2 melatonin receptors in the inhibitory effect of melatonin is questionable. Furthermore, the lack of effect of a combined MT_1/MT_2 receptor antagonist, luzindole ($pA_2 = 7.7$ in rabbit retina, Dubocovich, 1988) suggests that mechanisms unrelated to MT_1/MT_2 receptors contribute predominantly to the inhibitory action of melatonin. This further confirms our findings with 5-MCA-NAT as 5-MCA-NAT has been shown to have only micromolar affinity for MT_1/MT_2 receptors, whereas it has nanomolar affinity for MT_3 receptors (Jockers *et al.*, 2008).

Given that colonic mucosal 5-HT may play an important role in the regulation of gut function in IBS patients (Miwa *et al.*, 2001; Coates *et al.*, 2004), it is important to elucidate the role of melatonin in the regulatory mechanism of 5-HT release from the colonic mucosa in order to understand the pathophysiology of IBS. Our observation that melatonin has an inhibitory effect on NK_2 receptor-triggered 5-HT release from colonic mucosa may be important from a clinical perspective, as oral melatonin has the potential to be used in the treatment of IBS (Lu *et al.*, 2005; Song *et al.*, 2005).

In conclusion, we demonstrated that melatonin inhibits tachykinin NK_2 receptor-triggered 5-HT release from guinea pig colonic mucosa by acting at a MT_3 melatonin receptor located directly on the mucosal layer without affecting 5-HT degradation processes. Possible contributions of MT_1/MT_2 melatonin receptors to the inhibitory effect of melatonin appear to be negligible. Melatonin may act as a modulator of excess 5-HT release from colonic mucosa.

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Conflicts of interest

None.

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