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Research Paper

Interaction of morphine but not fentanyl with cerebral α_2-adrenoceptors in α_2-adrenoceptor knockout mice

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Abstract

Objectives α_2-Adrenergic and μ-opioid receptors belong to the rhodopsin family of G-protein coupled receptors and mediate antinociceptive effects via similar signal transduction pathways. Previous studies have revealed direct functional interactions between both receptor systems including synergistic and additive effects. To evaluate underlying mechanisms, we have studied whether morphine and fentanyl interacted with α_2-adrenoceptor-subtypes in mice lacking one individual α_2-adrenoceptor-subtype (α_2-adrenoceptor knockout).

Methods Opioid interaction with α_2-adrenoceptors was investigated by quantitative receptor autoradiography in brain slices of α_{2A}-, α_{2B}- or α_{2C}-adrenoceptor deficient mice. Displacement of the radiolabelled α_2-adrenoceptor agonist [^{125}I]paraiodoclonidine from α_2-adrenoceptors in different brain regions by increasing concentrations of morphine, fentanyl and naloxone was analysed. The binding affinity of both opioids to α_2-adrenoceptor subtypes in different brain regions was quantified.

Key findings Morphine but not fentanyl or naloxone provoked dose-dependent displacement of [^{125}I]paraiodoclonidine from all α_2-adrenoceptor subtypes in the brain regions analysed. Binding affinity was highest in cortex, medulla oblongata and pons of α_{2A}-adrenoceptor knockout mice.

Conclusions Our results indicated that morphine interacted with α_2-adrenoceptors showing higher affinity for the α_{2B} and α_{2C} than for the α_{2A} subtype. In contrast, fentanyl and naloxone did not show any relevant affinity to α_2-adrenoceptors. This effect may have an impact on the pharmacological actions of morphine.

Keywords α_2-adrenergic receptors; α_2-adrenoceptor knockout mice; binding affinity; morphine; μ-opioid receptors

Introduction

Agonists of α_2-adrenoceptors, such as clonidine and dexmedetomidine, are increasingly used in pain therapy as well as in modern clinical anaesthesia. They are characterised by their potent anaesthetic/sedative, sympatholytic, antinociceptive and anti-shivering properties.[1,2] An additional advantage of α_2-adrenoceptor agonists is their ability to potentiate the analgesic effects of opioids, including both a reduction of opioid requirement to reach an equivalent level of analgesia and a longer duration of analgesia.[3]

α_2-Adrenergic and μ-opioid receptors belong to the rhodopsin family of G-protein coupled receptors and mediate antinociceptive effects via similar signal transduction pathways. Previous studies have revealed functional interactions between these two receptor systems.[4,5] Stone *et al.*[6] demonstrated a decreasing analgesic potency of spinally administered morphine in mice lacking functional α_{2A}-adrenoceptors (α_{2A}-adrenoceptor knockout) compared with wild-type mice, suggesting a direct interaction between opioid and α-adrenergic receptors. The molecular mechanisms of these synergistic effects are still poorly defined. However, several studies demonstrated interactions of α_2-adrenoceptor agonists and morphine due to synergistic actions of both receptor systems involving N-type voltage-dependent calcium channels, G-proteins and protein kinase C.[7–9]

Furthermore, there is evidence for physical associations of μ-opioid receptors and α_2-adrenoceptors modulating receptor functions.[10,11] Jordan *et al.*[12] have shown that

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α_{2A}-adrenergic and μ-opioid receptors reside in direct proximity in several regions of the brain (e.g. in hippocampal neurons) and were coexpressed as μ-opioid/α_{2A}-adrenergic receptor complexes from heterologous cells or primary neurons. Further, Vilardaga *et al*.[13] demonstrated that in these receptor heterodimers a μ-opioid/α_{2A}-adrenergic receptor conformational 'cross-talk' led to direct inhibition of one receptor by the other, introducing a new mechanism in signal transduction. These findings suggested that both functional as well as physical interactions between μ- and α_2-adrenoceptors may have, at least in part, accounted for the clinically demonstrated synergistic or even additive effects. Focusing on functional interactions between both receptor systems, we were interested whether effects of morphine and fentanyl, the most widely used opioid analgesics in pain therapy, may be also mediated by actions via one or more α_2-adrenoceptor subtypes. To differentiate possible α_2-adrenoceptor subtype specific effects, we investigated the ability of morphine and fentanyl to displace [^{125}I]paraiodoclonidine in brain slices of mice lacking single α_2-adrenoceptor subtypes (α_2-adrenoceptor (AR) knockout mice) *in vitro*.

Materials and Methods

Animals

The generation of mouse lines lacking different single α_2-adrenoceptors (α_{2A}, α_{2B}, α_{2C}) has been described elsewhere.[14,15] The mice were maintained in a specified pathogen-free facility at our institution. Permission for this study was received from the local Institutional Review Board before the initiation of work. All experiments conformed to the guidelines of the European Communities Council Directive of 24th November 1986 (86/906/EEC). As a control group and for determination of nonsubtype-specific α_2-adrenoceptor binding and saturation binding assays wild-type mice without genetic modifications were analysed (C57 BL/6).

Tissue preparation

Ten male and eight female mice, six of each genotype, as well as five male wild-type mice were anaesthetised with dimethyl ether. After decapitation, brains were carefully removed, frozen by immersion into cold 2-methylbutane and stored at −70°C. Parasagittal brain sections (15 μm) were prepared on a cryostat (Slee, Mainz, Germany) at −18°C and immediately mounted onto coated slides. Until the start of the autoradiographic experiments, slides were stored at −70°C in sealed containers. Sections were brought to room temperature and air dried 30 min before use.

Ligand binding autoradiography

The technique of ligand binding autoradiography has been described elsewhere.[16] Briefly, mounted brain slices were incubated with the radioligand in 50 mM Tris buffer, pH 7.4 for 30 min at room temperature followed by five 5-min washes in the same buffer. Slides were dried under an airstream. For saturation binding assays, concentrations of [^{125}I]paraiodoclonidine ranged from 0.05 to 10 nM. Radioactivity was measured in a cobra auto-gamma counter (Packard, Meriden, CT, USA). For competition binding assays, [^{125}I]paraiodoclonidine was applied at approximately twice the equilibrium dissociation constant (K_d) determined in previous saturation binding assays at a final concentration of 1 nM. The concentration of the competing ligands ranged from 140 nM to 70 μM for morphine and from 120 nM to 1.2 mM for fentanyl. Nonspecific binding of the radioligand was determined by incubation of parallel slides with unlabelled clonidine at 200 μM. To evaluate the specificity of morphine binding to α_2-adrenoceptors we additionally administered increasing concentrations of the μ-opioid receptor antagonist naloxone (1 μM–1 mM) in α_{2A}-adrenoceptor knockout mice. In a second experiment, the incubation of brain slices with increasing concentrations of morphine was followed by a subsequent incubation with 100 mM naloxone. Radiolabelled dried slices were attached to a high-performance autoradiography film (Kodak Biomax MR, GE Healthcare Bio-Sciences, Piscataway, NJ, USA) for three days. Films were developed automatically by a standard procedure.

Image and data analysis

Densitometrical analysis of the resulting autoradiograms was performed by a computerised image analysis system (Umax Power Look III, Aida Scan Soft). Values for nonspecific binding were subtracted from total binding. For saturation binding, the anatomical structures were analysed throughout the whole brain. For competition binding, six different brain regions were analysed (cortex, cerebellum, medulla oblongata, thalamus, hippocampus and pons) by calculating relative optical density (ROD) values of three independent ellipses (defined as one group), which were positioned into the defined brain regions. The identification and nomenclature of brain regions was based on the mouse brain atlas of Franklin and Paxinos.[17] Each experiment was performed in triplicate. All saturation and competition binding results were analysed using a nonlinear regression program (GraphPad Prism 5, GraphPad Software, San Diego, CA, USA). A binding curve was fitted according to a one-site competition model. The equilibrium dissociation constant K_d and maximum binding capacity B_{max} values of [^{125}I]paraiodoclonidine were determined from saturation binding experiments, K_d and EC50 values were analysed according to the Cheng–Prusoff equation.

Pharmaceutical compounds

The α_2-adrenoceptor agonist [^{125}I]paraiodoclonidine was used as radioligand with a specific activity of 2000 Ci/mmol (GE Healthcare Bio-Sciences, Munich, Germany). Nonspecific binding was determined by addition of nonlabelled clonidine (Sigma-Aldrich, Taufkirchen, Germany). Morphine was purchased from Merck (Darmstadt, Germany) and fentanyl from Janssen-Cilag (Neuss, Germany).

Statistics

After negative testing for Gaussian distribution, nonparametric data (displacement of [^{125}I]paraiodoclonidine from α_2-adrenoceptors at varying concentrations of morphine and fentanyl in six different brain regions between different mouse types) were analysed with the Kruskal–Wallis test followed by Dunn's post test. P values < 0.05 were regarded

as statistically significant. Results are displayed as mean ± SEM, except if otherwise specified.

Results

Saturation experiments and determination of specific and nonspecific binding

Saturation experiments were performed with [^{125}I]paraiodoclonidine, 0.05–10 nM. Binding of [^{125}I]paraiodoclonidine to mice brain slices was found to be concentration-dependent and saturable with B_{max} values from 149 ± 10 (α_{2A}-AR knockout mice) to 207 ± 13 fmol/mg protein (wild-type mice) and K_d values from 0.49 ± 0.09 (α_{2A}-AR knockout mice) to 0.58 ± 0.08 nM (wild-type mice) (Figure 1a, b).

Binding of [^{125}I]paraiodoclonidine to α_2-adrenoreceptors was time-dependent, reaching equilibrium at 5 min and lasting at least for one hour (data not shown). Therefore, a [^{125}I]paraiodoclonidine concentration of 1 nM, which was approximately twice the value for K_d, and an incubation period of 30 min were chosen for subsequent competition binding assays.

Specific [^{125}I]paraiodoclonidine binding was found in most regions of the brain. Cortex, cerebellum, medulla oblongata, thalamus, hippocampus and pons were selected for analysis of competition binding studies (Figure 2a). Nonspecific binding was low in all brain regions (Figure 2b) and accounted for at maximum 3.4%.

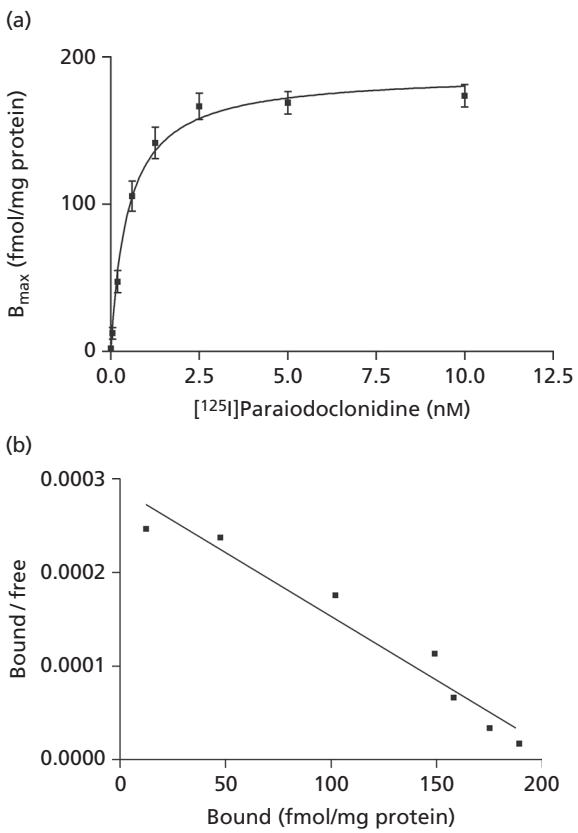

Figure 1 Specific binding of [^{125}I]paraiodoclonidine in wild-type mouse brain. Representative saturation plots of specific [^{125}I]paraiodoclonidine binding (a) in wild-type mouse brain with Rosenthal transformation (b). [^{125}I]Paraiodoclonidine concentration for competition binding experiments was subsequently adjusted to 1 nM.

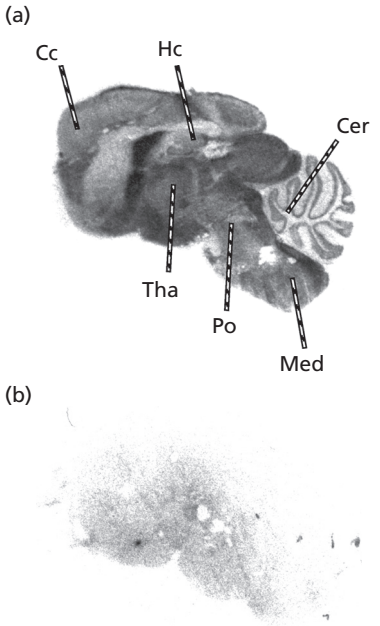

Figure 2 Binding site distribution of total and nonspecific binding of [^{125}I]paraiodoclonidine in wild-type mouse brain. Binding site distribution in wild-type mouse brain labelled with 1 nM [^{125}I]paraiodoclonidine (total binding) (a). For determination of nonspecific binding coincubation of 200 μM clonidine and 1 nM [^{125}I]paraiodoclonidine was performed (b). Nonspecific binding was subtracted from total binding after autoradiography. Cc, cerebral cortex; Hc, hippocampus; Cer, cerebellum; Tha, thalamus; Po, pons; Med, medulla. The selected autoradiographic images were digitised and resampled at a resolution of 600 × 600 dpi.

[^{125}I]Paraiodoclonidine displacement by morphine in wild-type, α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor knockout mouse brain

In α_{2A}-AR knockout mice, the lowest K_d values were found in pons, cerebral cortex, cerebellum and medulla oblongata, indicating efficient [^{125}I]paraiodoclonidine displacement by morphine (Table 1). In these regions, morphine concentrations of 7 μM led to substantial [^{125}I]paraiodoclonidine displacement (< 60% of the ROD) (Table 2; Figure 3a, b, Figure 4). At 14 μM morphine, [^{125}I]paraiodoclonidine displacement was almost complete (< 20% of the ROD) in all analysed α_{2A}-AR knockout mice brain regions except in the cerebellum and thalamus, where a concentration of 70 μM was required (Figure 3a, b, Figure 4).

In contrast, [^{125}I]paraiodoclonidine displacement in α_{2B}-AR knockout mice occurred at higher concentrations compared with α_{2A}-AR knockout mice. Values for K_d in cortex, pons, cerebellum, medulla oblongata and thalamus showed approximately a 4- to 6-fold increase compared with α_{2A}-AR knockout mice (Table 1). Partial [^{125}I]paraiodoclonidine displacement (< 80% of the ROD) occurred in most of these regions at 14 μM whereas almost complete displacement (< 20% of the ROD) in α_{2B}-AR knockout mice was observed

Table 1 Displacement of [^{125}I]paraiodoclonidine from different α_2-adrenoceptor subtypes by morphine

	α_{2A}-AR knockout (A)	α_{2B}-AR knockout (B)	α_{2C}-AR knockout (C)	Wild type (WT)
Cortex	7.6 (± 0.7)$^{***, \circ\circ\circ, \#\#}$	48.4 (± 7.4)	56.4 (± 7.4)§	44.3 (± 6.2)
Cerebellum	9.5 (± 3.6)$^{**, \circ\circ\circ, \#\#}$	43.8 (± 4.9) $^{++}$	76.8 (± 11.2)§	50.6 (± 4.1)
Medulla	8.2 (± 0.9)$^{**, \circ\circ\circ, \#}$	52.8 (± 12.3)	62.3 (± 10.6)	47.7 (± 7.9)
Thalamus	18.2 (± 1.8)$^{**, \circ\circ\circ, \#\#}$	58.7 (± 7.5)†	72.9 (± 6.9)§	42.6 (± 5.1)
Hippocampus	14.6 (± 1.5)$^{*, \circ\circ\circ, \#}$	31.7 (± 4.8)$^{++}$	77.3 (± 8.6)§	49.1 (± 5.3)
Pons	10.3 (± 1.3)$^{**, \circ\circ\circ, \#}$	40.2 (± 4.7)$^{++}$	70.1 (± 8.4)§	45.3 (± 7.8)

K_d values (μM) for displacement of [^{125}I]paraiodoclonidine from different α_2-adrenoceptor subtypes by morphine in different regions of mouse brain (mean ± SD). α_2-AR knockout, α_2-adrenoceptor knockout; (A) α_{2A}-AR knockout; (B) α_{2B}-AR knockout; (C) α_{2C}-AR knockout; (WT) wild type. ***, $^{\circ\circ\circ}P < 0.001$; **, $^{\#\#}$, $^{++}P < 0.01$; *, $^{\#}$, †, $^{\S}P < 0.05$; *A vs B, °A vs C, $^{\#}$A vs WT, $^{+}$B vs C, †B vs WT, §C vs WT.

Table 2 Displacement of [^{125}I]paraiodoclonidine from different α_2-adrenoceptor subtypes by morphine in different brain regions

Brain region	Morphine concentration	α_{2A}-AR knockout (A)	α_{2B}-AR knockout (B)	α_{2C}-AR knockout (C)	Wild type (WT)
Cortex	140 nM	94 (± 8.6)	102 (± 8.3)	100 (± 6.5)	98 (± 7.0)
	1.4 μM	70 (± 6.5)	99 (± 9.8)	89 (± 8.5)	93 (± 10.5)
	7 μM	45 (± 7.2)$^{*,\circ,\#}$	91 (± 7.6)	84 (± 7.2)	82 (± 7.3)
	14 μM	19 (± 4.1)$^{***;\circ\circ,\#\#}$	78 (± 7.9)†	63 (± 3.1)	51 (± 7.1)
	70 μM	12 (± 2.6)°	16 (± 5.6)	29 (± 8.7)	19 (± 3.5)
Cerebellum	140 nM	98 (± 6.9)	101 (± 6.7)	92 (± 14.8)	95 (± 6.6)
	1.4 μM	79 (± 6.6)	98 (± 8.7)	91 (± 13.6)	85 (± 5.0)
	7 μM	55 (± 6.4)*	87 (± 11.6)	73 (± 8.3)	72 (± 4.6)
	14 μM	53 (± 3.9)*	74 (± 5.3)	71 (± 10.4)	63 (± 5.2)
	70 μM	17 (± 3.0)$^{\circ\circ,\#}$	27 (± 8.2)$^{++}$	53 (± 11.8)§	37 (± 6.6)
Medulla	140 nM	81 (± 8.8)	97 (± 6.8)	102 (± 5.2)	103 (± 8.6)
	1.4 μM	73 (± 6.7)	99 (± 9.2)	93 (± 7.3)	97 (± 5.9)
	7 μM	44 (± 4.9)$^{*,\circ,\#}$	98 (± 10.4)	88 (± 10.4)	86 (± 4.4)
	14 μM	16 (± 3.2)$^{***,\circ\circ,\#}$	82 (± 8.8)†	67 (± 6.5)	56 (± 7.1)
	70 μM	8 (± 3.6)$^{\circ\circ}$	11 (± 6.1)$^{++}$	33 (± 5.3)§	14 (± 4.2)
Thalamus	140 nM	84 (± 9.2)	102 (± 3.3)	93 (± 7.8)	95 (± 8.8)
	1.4 μM	78 (± 6.4)	99 (± 9.8)	94 (± 5.9)	91 (± 4.7)
	7 μM	70 (± 5.9)*	101 (± 6.6)	73 (± 8.4)	76 (± 3.3)
	14 μM	32 (± 4.0)$^{**,\circ\circ,\#\#}$	89 (± 9.2)	71 (± 9.2)	58 (± 6.4)
	70 μM	17 (± 4.5)$^{\circ\circ,\#}$	36 (± 4.7)$^{+++,\dagger}$	50 (± 8.9)§	23 (± 3.2)
Hippocampus	140 nM	103 (± 11.8)	101 (± 7.2)	91 (± 10.3)	94 (± 7.0)
	1.4 μM	95 (± 8.3)	94 (± 5.8)	89 (± 7.6)	88 (± 5.7)
	7 μM	67 (± 9.4)	83 (± 8.9)	85 (± 11.4)	81 (± 5.5)
	14 μM	15 (± 6.4)	59 (± 6.4)	82 (± 7.9)	69 (± 9.6)
	70 μM	9 (± 3.4)	22 (± 3.4)	53 (± 5.6)	26 (± 3.5)
Pons	140 nM	102 (± 6.6)	98 (± 4.8)	96 (± 7.2)	94 (± 6.7)
	1.4 μM	61 (± 7.4)	98 (± 6.3)	95 (± 6.0)	94 (± 5.4)
	7 μM	55 (± 6.9)	97 (± 8.3)	79 (± 5.9)	77 (± 8.8)
	14 μM	20 (± 5.2)	72 (± 6.8)	75 (± 3.6)	58 (± 3.6)
	70 μM	5 (± 4.1)	18 (± 3.6)	46 (± 6.0)	22 (± 5.3)

Values represent the relative mean optical density (ROD) in percent (mean ± SD). α_2-AR knockout, α_2-adrenoceptor knockout; (A) α_{2A}-AR knockout; (B) α_{2B}-AR knockout; (C) α_{2C}-AR knockout; (WT) wild type. ***, $^{+++}P < 0.001$; **, °°, $^{\#\#}$, $^{++}P < 0.01$; *, °, $^{\#}$, †, $^{\S}P < 0.05$; *A vs B, °A vs C, $^{\#}$A vs WT, $^{+}$B vs C, †B vs WT, §C vs WT.

at 70 μM morphine only in pons, medulla and cortex. Only in the hippocampus could substantial [^{125}I]paraiodoclonidine displacement (< 60% of the ROD) be observed already at 14 μM morphine (Table 2, Figure 3a, b).

In α_{2C}-AR knockout mice [^{125}I]paraiodoclonidine displacement in all analysed regions was decreased compared with α_{2A}-AR knockout mice. Morphine K_d values were above 60 μM for most α_{2C}-AR knockout brain regions, indicating poor competition (Table 1). Accordingly, even at the maximum concentration of 70 μM morphine, [^{125}I]paraiodoclonidine displacement < 40% of the ROD was only observed in cortex and medulla, whereas in all other investigated α_{2C}-AR knockout mice morphine displaced [^{125}I]paraiodoclonidine only to 50–60% of the ROD (Table 2, Figure 3a, b).

In wild-type mice, the lowest K_d values were detected in cerebral cortex and thalamus (Table 1). However, the differences between regions were relatively small. In most regions, morphine concentrations of 14 μM led to substantial [^{125}I]paraiodoclonidine displacement (< 60% of the ROD)

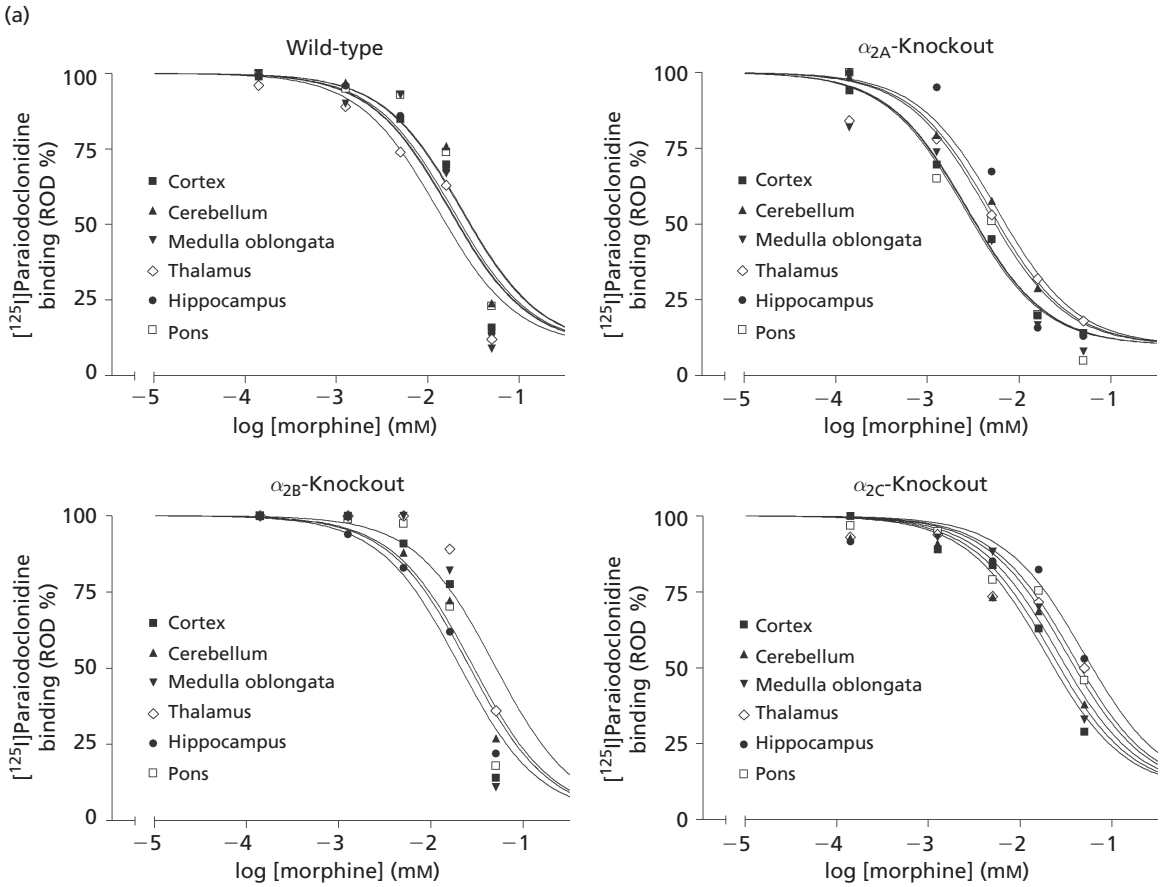

Figure 3 Displacement of [^{125}I]paraiodoclonidine by increasing concentrations of morphine in six different brain regions of wild-type, α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor knockout mice. Data are presented according to the different genotype (a) and the different brain regions (b). Displacement was measured by computer assisted densitometry of the autoradiograms. Values represent the relative optical density (ROD) in percent. Values were best fit by nonlinear regression.

except in cerebellum and hippocampus. Almost complete [^{125}I]paraiodoclonidine displacement (< 20% of the ROD) was observed at 70 μM only in cortex and medulla (Table 2, Figure 3a, b).

[^{125}I]Paraiodoclonidine displacement by fentanyl in wild-type, α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor knockout mouse brain

To determine [^{125}I]paraiodoclonidine displacement fentanyl was used at a concentration range from 120 nM to 1.2 mM. In contrast to morphine, fentanyl did not reveal any significant [^{125}I]paraiodoclonidine displacement even at the highest concentration of 1.2 mM in any of the analysed brain regions. No significant differences between wild-type, α_{2A}-, α_{2B}- and α_{2C}-AR knockout mice brains were detected (Figure 5).

[^{125}I]Paraiodoclonidine displacement by naloxone in α_{2A}-adrenoceptor knockout mouse brain

To rule out nonspecific morphine effects on [^{125}I]paraiodoclonidine displacement associated with μ-receptor agonism, we analysed the displacement of [^{125}I]paraiodoclonidine by naloxone in α_{2A}-AR knockout mouse brain. In contrast to morphine, increasing concentrations of naloxone did not result in a significant displacement of [^{125}I]paraiodoclonidine, indicating no relevant interaction with α_2-adrenoceptors (Figure 6a). Subsequent administration of 100 mM naloxone after incubation with morphine did not provoke an increase of [^{125}I]paraiodoclonidine-displacement in comparison with morphine alone (Figure 6b).

Discussion

Our results indicated that morphine interacted with α_2-adrenoceptors and displaced the α_2-adrenoceptor agonist [^{125}I]paraiodoclonidine from all α_2-adrenoceptor subtypes in a concentration dependent manner in all analysed regions of the mouse brain.

The displacement of [^{125}I]paraiodoclonidine from α_{2A}-adrenoceptors at defined morphine concentrations was up to 2–3-fold lower (calculated in percent of the ROD) compared with the displacement from α_{2B}- and α_{2C}-adrenoceptors. This indicated a higher affinity of morphine to α_{2B}- and α_{2C}-adrenoceptors compared with the α_{2A}-subtype.

In contrast, fentanyl, another μ-receptor agonist of the 4-anilopiperidine group, as well as the μ-receptor antagonist

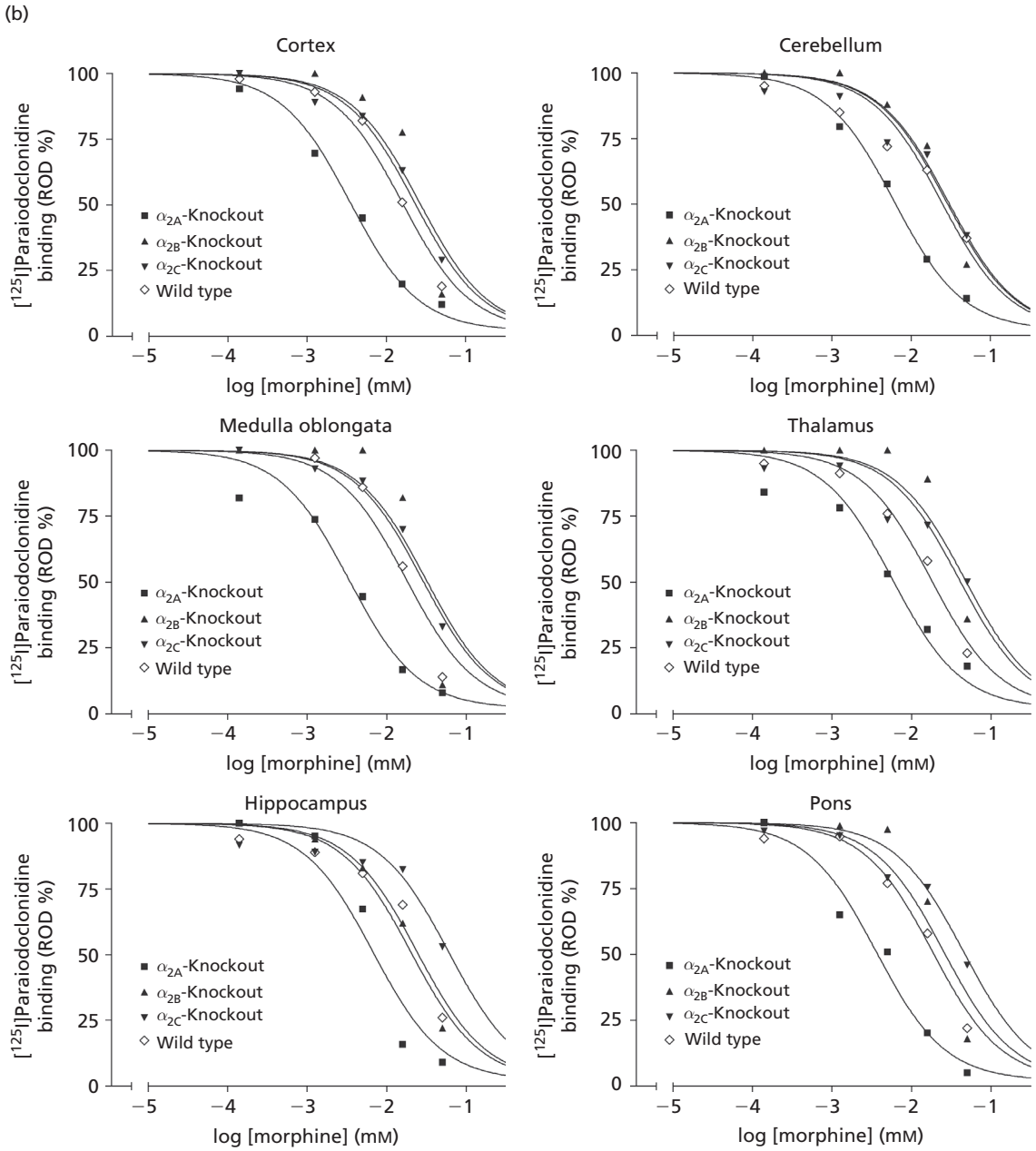

Figure 3 *(Continued)*

naloxone, did not interact with α_2-adrenoceptors, visualised by lack of [^{125}I]paraiodoclonidine displacement.

Receptor interactions of different classes of drugs (e.g. hypnotics, local anaesthetics and opioids) with α_2-adrenoceptors have been reported, hypothesising partial mediation of drug effects via α_2-adrenoceptors. Supported by clinical observations e.g. enhancement of epidurally administered opioids by α_2-adrenoceptor agonists and decreasing analgesic potency of spinally administered morphine in mice lacking functional α_{2A}-adrenoceptors, a mediation of the opioids' analgesic effects also via or in the presence of α_2-adrenoceptors seems to be conceivable.[6]

Different mechanisms of ligand/receptor interaction may be involved. Direct interaction of opioid ligands with α_2-adrenoceptors as well as recently detected mechanisms such as expression of μ-opioid/α_{2A}-adrenergic receptor heterodimers and conformational cross-talk between μ-opioid and α_{2A}-adrenergic receptors may have contributed to the observed effects.[12,13]

Although all subtypes of the α_2-adrenoceptor (α_{2A}, α_{2B} and α_{2C}) exhibit similar affinity for their natural ligands noradrenaline and adrenaline, they show noticeable differences in their pharmacological and biochemical properties.[18] The α_{2A}-subtype mediates most of the 'classic'

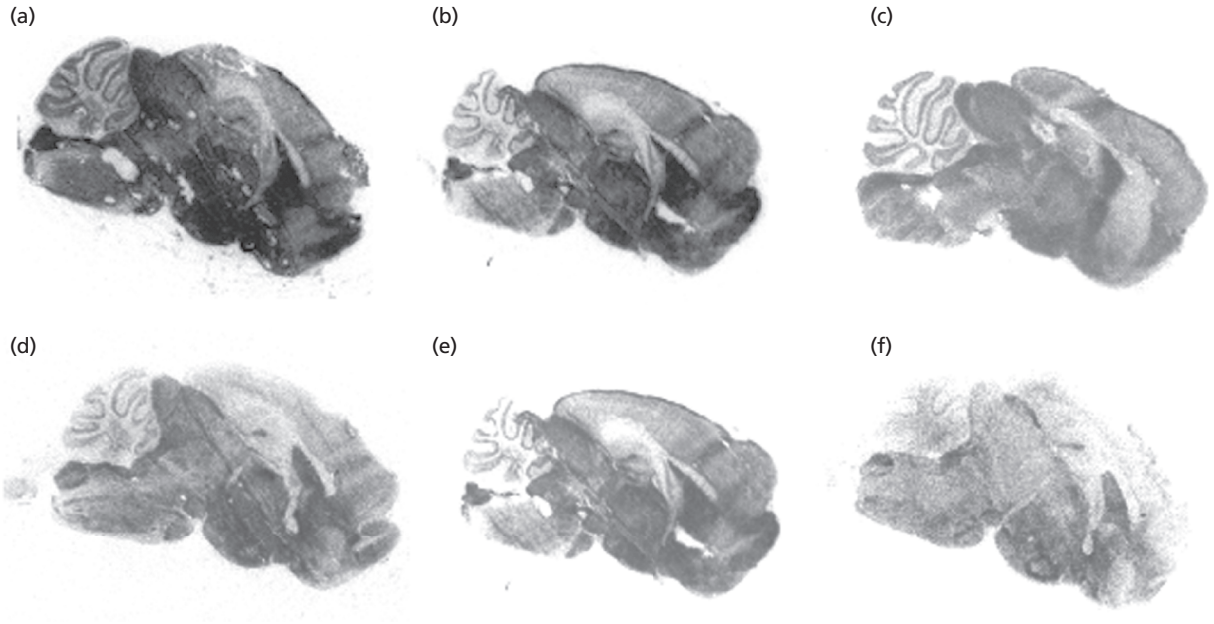

Figure 4 Ligand binding autoradiography: [^{125}I]paraiodoclonidine displacement from cerebral α_2-adrenoceptors in α_{2A}-adrenoceptor knockout mouse brain by morphine. Brain slices were incubated with increasing concentrations of morphine: 0 μM (a, control); 140 nM (b); 1.4 μM (c); 7 μM (d); 14 μM (e); 70 μM morphine (f).

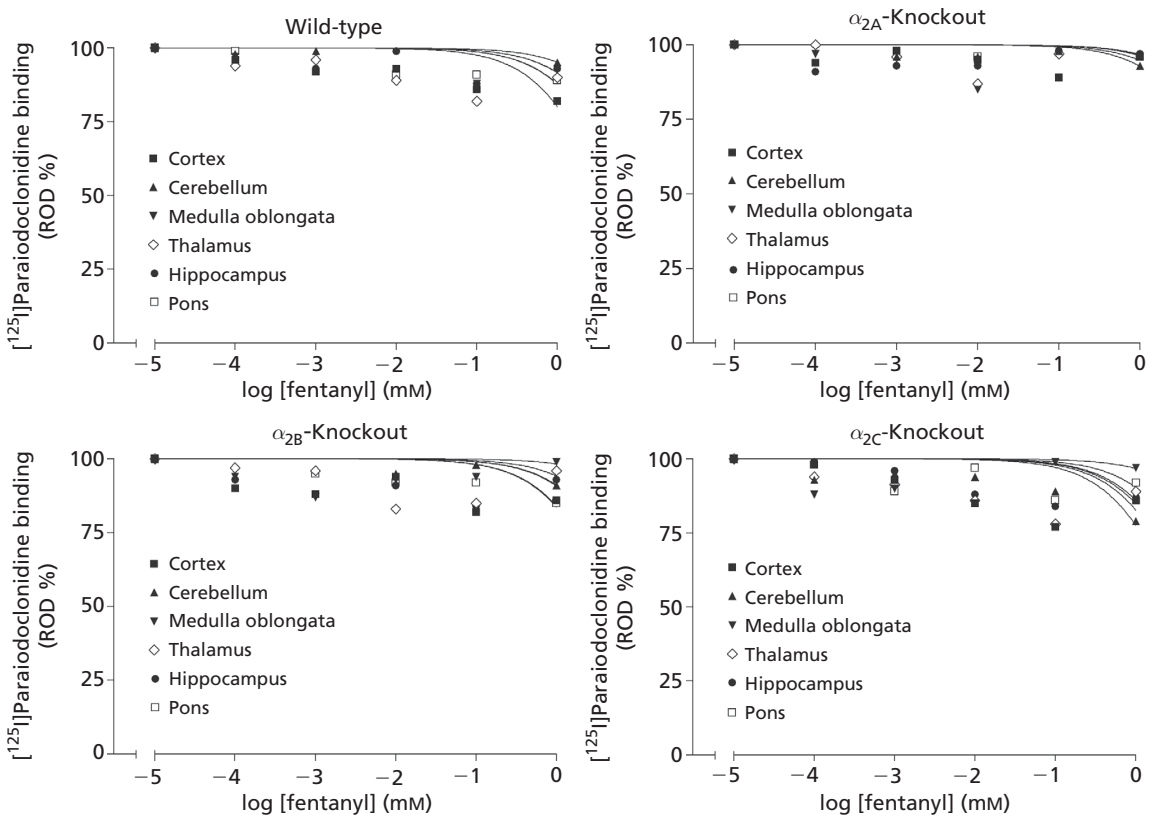

Figure 5 Displacement of [^{125}I]paraiodoclonidine by increasing concentrations of fentanyl in six different brain regions of wild-type, α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor knockout mice. Displacement was measured by computer assisted densitometry of the autoradiograms. Values represent the relative optical density (ROD) in percent. Values were best fit by nonlinear regression.

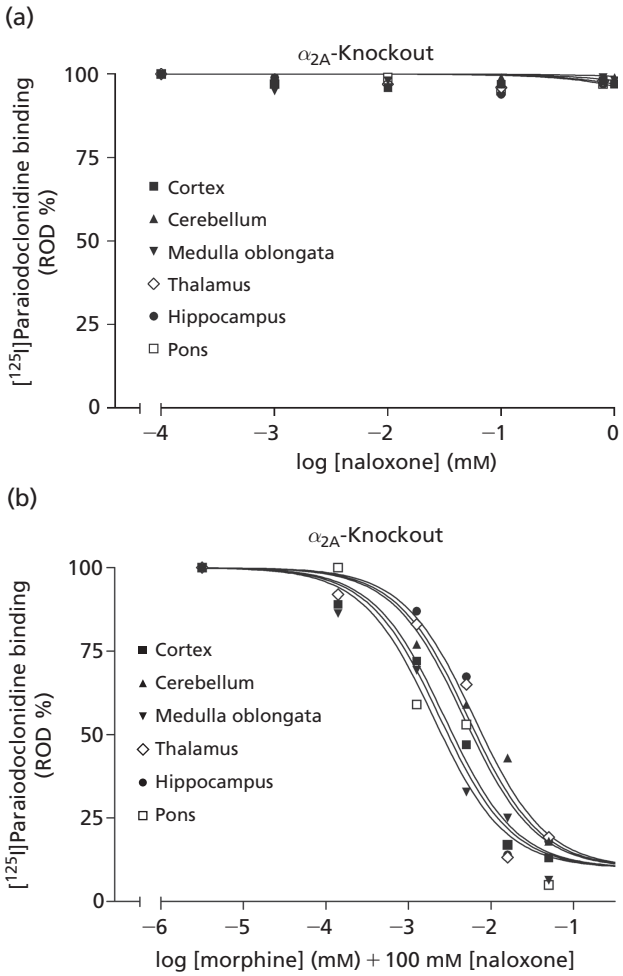

Figure 6 Displacement of [^{125}I]paraiodoclonidine by increasing concentrations of naloxone (a) and morphine plus 100 mM naloxone (b) in six different brain regions of α_{2A}-adrenoceptor knockout mice. Displacement was measured by computer assisted densitometry of the autoradiograms. Values represent the relative optical density (ROD) in percent. Values were best fit by nonlinear regression.

effects such as hypotension, hypothermia and sedation, and is still the primary mediator for analgesic properties of α_2-adrenoceptor agonists.[19–22] Earlier pharmacological studies suggested only the α_{2A}-adrenoceptor mediated analgesia.[23] However, a contribution of the α_{2B}- and α_{2C}-subtype seems likely including modulation of spinal antinociception via α_{2C}-adrenoceptors and mediation of antinociceptive effects of nitrous oxide also via α_{2B}-adrenoceptors.[19,24–26]

The mediation of α_2-adrenoceptor subtype-specific or subtype-preferring effects is closely related to the regionally different receptor distribution in the central nervous system (CNS). The α_{2A}-adrenoceptor is the most predominant subtype and is widely distributed in cerebral cortex, septum, amygdala, brain stem, hypothalamus, hippocampus, nucleus of the solitary tract and in locus coeruleus. Contrarily, α_{2B}-adrenoceptor mRNA has been visualised in thalamic nuclei but not in other CNS areas.[27,28] α_{2C}-Adrenoceptors were demonstrated in the basal ganglia, olfactory tubercle, hippocampus, amygdala and cerebral cortex and also widely distributed throughout the mouse brain, but at relatively low density.[27,29] Furthermore, high densities of α_2-adrenoceptors have been detected at various sites of the human spinal cord as well as in the spinal cord of rats and mice.[27,29,30–32]

Coexistence of μ-opioid- and α_2-receptors or even coexpression as μ-/α_2-adrenoceptor complexes has been shown further in several regions of the rat spinal cord and in single neurons of different cerebral nuclei and the hippocampus.[12,33] Therefore, analgesic properties of α_2-agonists have been attributed, at least in part, to interactions of both receptor systems.[12,27]

Our study confirmed regional distribution patterns of α_2-adrenoceptor subtypes, emphasising the dominant role of the α_{2A}-adrenoceptor subtype, according to previous autoradiographic, in-situ hybridisation and immunolabelling studies.[29,32,34–36] Moreover, we found a higher affinity of morphine to α_{2B}- and α_{2C}-adrenoceptors compared with the α_{2A} subtype, which is rather the primary mediator of analgesic effects of α_2-adrenoceptor agonists.[19] In agreement with our results for morphine, the μ-receptor agonist pethidine (meperidine) has been demonstrated to act as an agonist on α_2-adrenoceptors showing higher affinity for the α_{2B}- and α_{2C}-adrenoceptor compared with the α_{2A}-subtype.[16,37] In contrast with our studies, Takada *et al.*[37] investigated pethidine binding to COS-7-cells transiently transfected with α_2-adrenoceptor subtypes. Under these conditions, however, morphine did not interact with α_2-AR detected by absent displacement of the radioligand [^{3}H] RX821002, though administered concentrations were comparable when compared with our experiments. Different experimental methodology, such as incubation of brain tissue slices versus transfected cells, in combination with unequal binding affinity of both radioligands may account for these differing results.

In fact, the interactions of opioidergic and α_2-adrenergic analgesic mechanisms are very complex and there are some partly conflicting reports on the relevancy and modulation of these interactions (e.g. the role of the α_{2A}-adrenoceptor subtype in the mediation of morphine effects). Stone *et al.*[6] demonstrated a decreasing analgesic potency of spinally administered morphine in mice lacking functional α_{2A}-adrenoceptors compared with wild-type mice. In contrast with these findings, the antinociceptive action of morphine and, to a larger extent, of tramadol and buprenorphine was increased in α_{2A}-AR knockout mice.[38] These results suggested possible potentiation of the agonistic action of morphine with α_{2B}- and/or α_{2C}-adrenoceptors and may be further explained by a higher concentration of opioids binding to α_{2B}- and α_{2C}-adrenoceptors in the absence of α_{2A}-adrenoceptors. In agreement with our results, the antinociceptive responses to fentanyl were unaltered in the α_{2A}-AR knockout mice.[38]

Another study of Lähdesmäki *et al.*[39] found that the analgesic activity of morphine was not altered in α_{2A}-adrenoceptor deficient mice in normal or inflamed conditions. Therefore, the authors concluded that acute morphine antinociception was not dependent on α_{2A}-adrenoceptor activation and assumed that other α_2-adrenoceptor subtypes, especially the α_{2C}-adrenoceptor, were more likely to play a role in the modulation of inflammatory hyperalgesia.

In addition to the functional interactions described, also physical associations between both receptor families were demonstrated (e.g. coexpression of μ-opioid/α_{2A}-adrenergic receptor complexes in heterologous cells or primary neurons).[12] In these cells, the activation of either μ- or α_{2A}-receptors resulted in an increase of μ-/α_{2A}-receptor heterodimers with a corresponding increase of subsequent receptor signalling as measured by G-protein activation and mitogen-activated protein kinase phosphorylation. In contrast, activation of both receptors simultaneously was followed by a decreased expression of complexes.[12] Recently, Vilardaga *et al*.[13] demonstrated that in these μ-/α_{2A}-receptor heterodimers both receptors interacted with each other by a conformational switch (cross-talk) leading to direct inhibition of one receptor by the other. Morphine binding to the μ-receptor induced a conformational change of the ligand-occupied α_{2A}-adrenoreceptor, which was followed by an inhibition of further signalling to G_i-proteins and the mitogen-activated protein kinase cascade. Interestingly, morphine decreased the level of α_{2A}-adrenoceptor activation only in cells which expressed both μ- and α_{2A}-receptors and the cross-conformational switch was only observed when both agonists were present.[13] Conformational changes in the α_{2A}-adrenoceptor were also found in response to different ligands (e.g. noradrenaline, clonidine or dopamine).[40]

Therefore, these novel mechanisms of reciprocal interaction may contribute to explain the partly conflicting reports of μ-opioid/α_{2A}-adrenergic receptor interaction. It seems conceivable that both functional as well as direct physical interactions between μ- and α_2-adrenoceptors including conformational cross-talk of heterodimers may be responsible for the different effects observed *in vivo*.

Moreover, the lacking interaction of the μ-receptor agonist fentanyl with α_2-adrenoceptors in our study may have been related to an inhibited or decreased agonistic effect at μ-/α_2-receptor heterodimers. Zurn *et al*.[40] demonstrated that different ligands induced conformational changes in the α_2-receptor that were sensed differently in varying positions of the receptor molecule. Up to now, conformational changes of μ-/α_{2A}-receptor dimers were only demonstrated in the presence of morphine but no other μ-agonists and only for the α_{2A}-subtype.[13] In contrast to the selective μ-agonist fentanyl, morphine also shows affinity for the δ-opioid receptor, which may have affected our results. However, the existence of μ-/α_{2B}- and μ-/α_{2C}-heterodimers seems conceivable and may help to illuminate conflicting reports on the involvement of α_{2B}- and α_{2C}-adrenoceptors.

As a limitation of our study, our experimental methodology did not allow us to differentiate whether the interaction of morphine with α_2-adrenoceptors was affected by functional and/or physical receptor interaction, including receptor heterodimerisation or conformational cross-talk. Moreover, the calculations of the equilibrium dissociation constant K_d and maximum binding capacity B_{max} for saturation analysis were affected because in contrast to transfected cell lines, expressing one α_2-adrenoceptor subtype, knockout mice brain slices contained two different α_2-adrenoceptor subtypes. Therefore, the data have to be related to the cerebral receptor subtype density and distribution. Moreover, this may be responsible also for the regional differences of the calculated binding affinities (K_d values) for the α_2-adrenoceptor subtypes between different brain regions.

Conclusions

Our study demonstrated a concentration-dependent specific interaction of morphine with α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors in cortex, cerebellum, medulla oblongata, thalamus, hippocampus and pons of α_2-adrenoceptor knockout mice brain slices. In contrast, fentanyl (another μ-receptor agonist of the 4-anilopiperidine group) as well as the μ-receptor antagonist naloxone did not interact with α_2-adrenoceptors. The reasons for this discrepancy are not yet fully understood. Morphine binding affinity to α_{2B}- and α_{2C}-adrenoceptors seemed to be higher than to α_{2A}-adrenoceptors. These observations may help to improve the understanding of the complex pharmacological effects of morphine and may contribute to elucidation of its mechanisms of action with particular respect to functional receptor interactions with the α_2-adrenoceptor family.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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