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Quantitative autoradiography of adenosine receptors in brains of chronic naltrexone-treated mice

[1]Alexis Bailey, [1]Rachel M. Hawkins, [1]Susanna M.O. Hourani & *,[1]Ian Kitchen

[1]Pharmacology Group, School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey GU2 7XH

1 Manipulation of μ opioid receptor expression either by chronic morphine treatment or by deletion of the gene encoding μ opioid receptors leads to changes in adenosine receptor expression. Chronic administration of the opioid receptor antagonist naltrexone leads to upregulation of μ receptor binding in the brain.

2 To investigate if there are any compensatory alterations in adenosine systems in the brains of chronic naltrexone-treated mice, we carried out quantitative autoradiographic mapping of A_1 and A_{2A} adenosine receptors in the brains of mice treated for 1 week with naltrexone ($8\,mg^{-1}\,kg^{-1}\,day^{-1}$), administered subcutaneously *via* osmotic minipump.

3 Adjacent coronal brain sections were cut from chronic saline- and naltrexone-treated mice for the determination of binding of [^{3}H] D-Ala^2-MePhe^4-Gly-ol^5 enkephalin ([^{3}H] DAMGO), [^{3}H]1,3-dipropyl-8-cyclopentylxanthine ([^{3}H] DPCPX) or [^{3}H] 2-[*p*-(2-carbonylethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H] CGS21680) to μ, A_1 and A_{2A} receptors, respectively.

4 A significant increase in μ and A_1 receptor binding was detected in chronic naltrexone-treated brains. The changes in μ receptors were significant in several regions, but changes in A_1 were relatively smaller but showed significant upregulation collectively. No significant change in A_{2A} receptor binding was detected in chronic naltrexone-treated brains.

5 The results show that blockade of opioid receptors causes upregulation of A_1 receptors, but not A_{2A} receptors, by as yet undefined mechanisms.
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Abbreviations: ANOVA, analysis of variance; CGS21680, 2-[*p*-(2-carbonylethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; CNS, central nervous system; CPA, N^6-cyclopentyladenosine; DAMGO, D-Ala^2-MePhe^4-Gly-ol^5 enkephalin; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; NECA, 5′-*N*-ethylcarboxamidoadenosine

Introduction

There is a large body of evidence indicating that both acute and chronic effects of opioids are partly mediated by adenosine in the central nervous system (CNS) (Sawynok, 1998). For example morphine and other μ receptor agonists were shown to enhance adenosine release from spinal cord and cortex *in vitro* and *in vivo* (Fredholm & Vernet, 1978; Phillis *et al*., 1980; Stone, 1981; Sweeney *et al*., 1987; 1989; Halimi *et al*., 2000). In addition to its involvement in the expression of opioid-mediated analgesia (Sawynok *et al*., 1989; Keil & DeLander, 1994; Keil & Delander, 1995; Sawynok, 1998; Lavand'homme & Eisenach, 1999), a role for adenosine in the development of opioid tolerance, dependence and withdrawal has also been suggested (Kaplan & Sears, 1996; Salem & Hope, 1997; 1999; Zarrindast *et al*., 1999). Further, cross-tolerance and cross-dependence between μ opioid, A_1 adenosine and α_2 adrenergic receptor-mediated antinociception in the periphery has been shown, suggesting a physical receptor interaction in the membrane or an interaction at the level of second messengers (Aley & Levine, 1997).

Some studies have demonstrated changes in adenosine receptor expression after chronic administration of morphine. Chronic exposure to morphine has been demonstrated to upregulate A_1 receptors in cortex (Kaplan *et al*., 1994) and brain homogenates (Ahlijanian & Takemori, 1986), and to downregulate adenosine A_{2A} receptors in striatum (De Montis, 1992) and A_1 receptors in the spinal cord of rats (Tao & Liu, 1992; Tao *et al*., 1995). In contrast, other groups have shown no change in A_1 and A_{2A} receptor numbers in cortex and striatum of chronic morphine-treated rats and mice, respectively (Tao *et al*., 1992; Kaplan *et al*., 1994).

Gene knockout technology has been recently used by us in order to study opioid–adenosine interactions in the CNS. A small but significant reduction in A_1 receptor binding was detected in the brains but not in the spinal cords of μ opioid receptor knockout mice, suggesting a functional interaction between μ receptors and A_1 receptors in the brain (Bailey *et al*., 2002b). No significant changes in A_{2A} adenosine receptors were detected in μ opioid receptor knockout mice brains (Bailey *et al*., 2002b).

As manipulation of the opioid system either by morphine treatment or by deletion of the gene encoding μ opioid receptors leads to changes in the adenosine system, we hypothesized that chronic blockade of opioid receptors with an opioid antagonist would also lead to alterations in adenosine receptor expression. Naltrexone is a well-charac-

*Author for correspondence; E-mail: I.Kitchen@surrey.ac.uk
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terised opioid receptor antagonist with highest affinity for μ opioid receptors (Corbett *et al*., 1993). It is clinically used for the treatment of alcohol dependence (Volpicelli *et al*., 1992). There are a large number of studies demonstrating that chronic naltrexone treatment produces an increase in the density of opioid receptors, particularly the μ but also the δ subtype in brain homogenates and slices (Tempel *et al*., 1984; 1985; Morris *et al*., 1988; Yoburn *et al*., 1989; Cote *et al*., 1993; Unterwald *et al*., 1995; 1998; Yoburn *et al*., 1995; Castelli *et al*., 1997). To investigate further the involvement of the adenosine receptors in mediating opioid effects, we examined by quantitative autoradiography if there are any changes in the binding and/or distribution of A_1 and A_{2A} receptors in the brains of chronic naltrexone-treated mice compared to saline-treated mice.

Materials and methods

Minipump implantation

Adult mice (25–30 g) of the strain C57BL/6 were purchased from Charles River (Margate, U.K.) and housed in a temperature-controlled room with freely accessible food and water. Osmotic minipumps (Alzet, Model 100D) were implanted subcutaneously in the dorsal midline of the animals under ether anaesthesia. The minipumps were filled either with sterile 0.9% saline or with approximately 20 mg ml^{-1} naltrexone (Sigma-Aldrich, Dorset, U.K.) dissolved in sterile 0.9% saline. Their contents were delivered at a constant rate of 0.5 μl h^{-1} resulting in a dose of naltrexone of 8 mg^{-1} kg^{-1} day^{-1} 7 days after the pumps were implanted, mice were killed by decapitation and the intact brains were removed immediately and rapidly frozen in isopentane at -20°C.

Autoradiographic procedure

General procedures for quantitative autoradiography were performed as detailed previously (Kitchen *et al*., 1997; Bailey *et al*., 2002b). Adjacent frozen coronal sections (20 μm thick) were cut at 300 μm intervals throughout the brains of chronic saline- and naltrexone-treated mice for the determination of total and nonspecific binding of [^{3}H]D-Ala^2-MePhe^4-Gly-ol^5 enkephalin (DAMGO), [^{3}H]1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and [^{3}H]2-[*p*-(2-carbonylethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680) to μ opioid, A_1 adenosine and A_{2A} adenosine receptors, respectively. Sections from chronic saline- and naltrexone-treated animals were processed together. Ligand concentrations were approximately three to four times K_d with [^{3}H]DAMGO used at a concentration of 4 nM, [^{3}H]DPCPX at 3 nM and [^{3}H]CGS21680 at 10 nM. Nonspecific binding was determined in the presence of 1 μM naloxone for [^{3}H]DAMGO, 1 μM N^6-cyclopentyladenosine (CPA) for [^{3}H]DPCPX and 20 μM 5′-*N*-ethylcarboxamidoadenosine (NECA) for [^{3}H]CGS21680 binding. The incubation periods were 1 h for [^{3}H]DAMGO binding and 2 h for [^{3}H]DPCPX and [^{3}H]CGS21680 binding, and following washing the slides were apposed to [^{3}H]Hyperfilm (Amersham) for a period of 3 weeks (Bailey *et al*., 2002b). Films were developed using 50% Kodak D19 developer.

Quantitative analysis and statistical procedures

Quantitative analysis of brain sections was carried out as detailed previously (Kitchen *et al*., 1997) using an MCID image analyser (Imaging Research, Canada) and [^{3}H]microscale standards. Brain structures were identified by reference to the mouse atlas of Franklin & Paxinos (1997). Comparison of quantitative measurements of autoradiographic binding for each ligand in brains from chronic saline to chronic naltrexone-treated animals was carried out using two-way analysis of variance (ANOVA) for factors treatment and region. Where significant effects for the factor treatment were observed, Scheffe's *post hoc* test was carried out on individual regions. To determine if there was an association between regions where changes in A_1 receptor binding in chronic naltrexone-treated mice were observed, and regions of high μ receptor expression in saline-treated animals, a correlation analysis was carried out for all regions where μ receptors are coexpressed with A_1 receptors. This correlation was also carried out between changes in A_1 receptor binding in chronic naltrexone-treated mice with the changes in μ receptor binding in the same animals.

Materials

[^{3}H]DPCPX (111.6 Ci mmol^{-1}) and [^{3}H]CGS21680 (42.5 Ci mmol^{-1}) were purchased from NEN Life Science Products (Hounslow, U.K.). [^{3}H]DAMGO (56.0 Ci mmol^{-1}) was purchased from Amersham International Plc (Buckinghamshire, U.K.). CPA, NECA, naloxone and adenosine deaminase type VIII were purchased from Sigma-Aldrich (Dorset, U.K.).

Results

μ opioid receptor autoradiography

The qualitative and quantitative distribution of μ opioid receptors labelled with [^{3}H]DAMGO (4 nM) in coronal sections of brain of chronic saline-treated mice (Figure 1) was similar to previous studies reported by our group (Kitchen *et al*., 1997; Slowe *et al*., 1999; Bailey *et al*., 2002a). The pattern of distribution of μ receptors was identical in chronic naltrexone- and saline-treated mice. However, large quantitative changes in specific binding were detected between these treatment groups. All regions analysed showed an upregulation in μ receptor expression in naltrexone-treated mice ($P<0.001$, Table 1). Increases in [^{3}H]DAMGO binding ranged from only 10% in the nucleus accumbens shell up to 129% in the preoptic area. Other regions of the naltrexone-treated brains that demonstrated very large increases in μ receptor binding were the vertical limb of the diagonal band (126%), the basomedial amygdala (105%), the zona incerta (94%) and the hypothalamus (91%). The mean and median percentage increase in [^{3}H]DAMGO binding was 49 and 37%, respectively (Table 1).

A_1 adenosine receptor autoradiography

The qualitative and quantitative distribution of A_1 adenosine receptors labelled with [^{3}H]DPCPX (3 nM) in coronal sections of brains of chronic saline-treated mice was similar to our

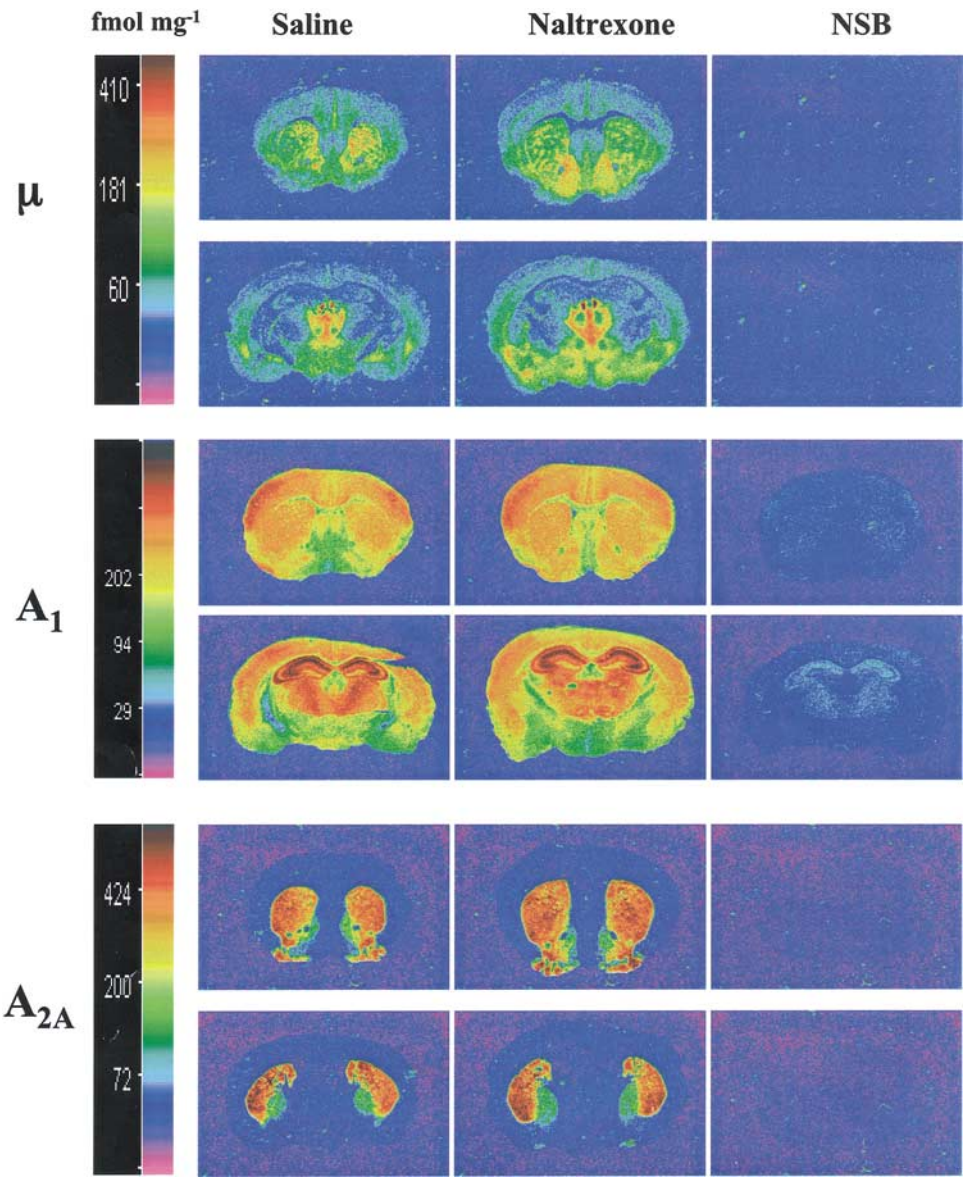

Figure 1 Colour autoradiograms of adjacent coronal brain sections showing μ receptor binding in the brains of chronic saline- and naltrexone-treated mice. μ opioid receptors were labelled with [^{3}H]DAMGO (4 nM), adenosine A_1 receptors were labelled with [^{3}H]DPCPX (3 nM) and adenosine A_{2A} receptors were labelled with [^{3}H]CGS-21680 (10 nM). Nonspecific binding images, shown in the far right column, were determined in the presence of unlabelled naloxone (1 μM) for μ opioid receptor binding, CPA (1 μM) for adenosine A_1receptor binding and NECA (20 μM) for adenosine A_{2A} receptor binding. The sections shown are from the level of the caudate (Bregma 1.10 mm) and the hippocampus (Bregma −1.70 mm) for μ- and A_1receptor binding and at the level of the caudate (Bregma 1.10 mm) and the globus pallidus (Bregma −0.22 mm) for A_{2A} receptor binding. The colour bar represents a pseudo-colour interpretation of relative black and white film images in fmol mg^{-1} tissue equivalent. Sections cut from chronic saline- and naltrexone-treated brains were processed in parallel.

previous study (Bailey *et al*., 2002b). The pattern of distribution of A_1 receptors was identical between chronic naltrexone- and saline-treated mice (Figure 1). However, small but significant quantitative changes in [^{3}H]DPCPX binding were detected in brain regions of chronic naltrexone-treated mice compared to saline-treated animals. ANOVA demonstrated a significant quantitative difference for factor treatment ($P<0.01$) in the levels of [^{3}H]DPCPX binding (Table 2). Although the median change for all regions was only 5.5%, 75% of the regions analysed in the naltrexone-treated mice showed an upregulation in A_1 expression. The greatest increase of A_1 sites (30–40%) in chronic naltrexone-treated mice brains was found in areas of high μ opioid receptor expression (accumbens and superficial grey layer of superior colliculus, where *post hoc* analysis of these individual changes approached significance, $P=0.056$ and 0.054, respectively) (Table 2). Regression analysis was carried out to determine if the degree of increase in A_1 receptor binding observed in naltrexone-treated brains correlated with regions of high μ receptor expression in saline-treated brains. A small but significant correlation was observed ($r=0.48$, $n=25$, $P<0.05$). It has been shown that regions of great A_1 receptor decrease in homozygous (−/−) μ opioid receptor knockout mice also correlate with regions of high μ receptor expression in wild-

Table 1 Quantitative autoradiography of μ opioid receptors in the brains of chronic saline- and naltrexone-treated mice

		[^{3}H]DAMGO-specific binding (fmol mg^{-1})		
Region	*Bregma co-ordinates (mm)*	*Saline*	*Naltrexone*	*% Change in binding*
Olfactory bulb	4.28			
Ext. plexiform layer		29.7±1.8	34.0±3.7	14.5
Granule layer		23.9±1.9	35.9±2.9	50.2*
Cortex				
Motor	2.46			
Superficial layers		22.1±3.2	29.4±2.8	32
Deep layers		38.1±3.0	49.1±3.1	28.9*
Orbital	2.46			
Superficial layers		44.6±5.7	63.7±4.3	42.8*
Deep layers		41.8±3.0	57.4±3.2	37.3*
Rostral somatosensory	1.10			
Superficial layers		19.4±3.7	26.0±2.5	34
Deep layers		34.2±3.9	44.4±2.3	29.8
Cingulate	1.10			
Superficial layers		29.8±4.1	32.2±4.3	8
Deep layers		40.3±5.6	51.5±4.7	27.8
Auditory	−2.80			
Superficial layers		24.5±3.2	32.5±4.6	32.7
Deep layers		39.0±4.6	55.5±4.0	42.3*
Visual	−2.80			
Superficial layers		17.6±3.9	22.5±2.1	27.8
Deep layers		25.2±4.6	38.7±1.2	53.6*
Retrosplenial	−2.80			
Superficial layers		22.5±2.9	34.1±2.0	51.6*
Deep layers		26.3±1.4	37.1±3.3	41.1*
Nucleus accumbens	1.34			
Shell		111±5.7	122±13.1	10
Core		114±8.6	150±13.4	31.6
Caudate-putamen	1.10	79.3±16.2	93.9±9.8	18.4
Endopiriform nucleus	1.10	81.6±6.2	102±8.7	25
Septum	0.86			
Medial		58.0±6.6	94.6±8.1	63.1*
Lateral		43.1±6.9	54.5±7.0	26.5
Vertical limb of diagonal band	0.86	44.3±7.3	100±5.6	125.7***
Bed nucleus of stria terminalis	−0.22	84.9±4.7	135±19.8	59*
Preoptic area	−0.22	55.0±4.2	126±15.3	129**
Medial habenula nucleus	−1.70	203±43.8	265±41.8	30.5
Thalamus	−1.70	63.0±5.3	97.2±15.2	54.3
Zona incerta	−1.70	54.1±10.7	105±19.0	94
Amygdala	−1.70			
Basolateral		109±7.7	148±14.0	35.8
Basomedial		49.8±3.9	102±16.6	104.8*
Medial		86.7±6.9	148±21.9	70.7*
Hypothalamus	−1.70	59.6±4.9	114±12.1	91.3**
Hippocampus	−2.46	20.8±2.0	28.5±3.5	37
Periaqueductal grey	−3.40	72.5±7.2	130±11.8	79.3**
Substantia nigra	−3.40	63.5±11.9	114±16.5	79.5*
Superficial grey layer of superior colliculus	−3.40	105±8.9	144±9.6	37.1*
Intermediate grey layer of superior colliculus	−3.40	91.6±8.3	143±10.8	56.1**

The mean specific binding ($n=4$)±s.e.m. of [^{3}H]DAMGO (fmol mg^{-1}) in the brain regions of chronic saline- and naltrexone-treated mice. Quantitative measurements were carried out in the regions of the brains at the Bregma co-ordinates taken from the mouse atlas of Franklin & Paxinos (1997). Regional determinations were made from both left and right sides of the sections that were 300 μm apart. The labelling was carried out on sections from saline- and naltrexone-treated mice in a completely paired protocol. Specific binding was >95% in regions of high binding. The percentage change in binding represents the increase in binding levels in the brains of chronic naltrexone-treated mice compared to those of saline-treated mice. There was a significant difference between treatment groups ($P<0.001$, ANOVA). Scheffe's *post hoc* test for individual brain regions; *$P<0.05$, **$P<0.01$, ***$P<0.001$. The overall mean and median percentage changes across all regions were 49 and 37.3%, respectively.

Table 2 Quantitative autoradiography of adenosine A_1 receptors in the brains of chronic saline- and naltrexone treated mice

Region	*Bregma co-ordinates (mm)*	*[^{3}H]DPCPX-specific binding (fmol mg^{-1})* *Saline*	*Naltrexone*	*% Change in binding*
Cortex				
Motor	2.46			
Superficial layers		174±4.8	167±7.1	−4.0
Deep layers		202±8.3	196±6.7	−3.0
Rostral somatosensory	1.10			
Superficial layers		271±24.3	264±13.5	−2.6
Deep layers		260±9.6	263±11.4	1.1
Cingulate	1.10			
Superficial layers		162±7.3	174±12.6	7.4
Deep layers		205±6.4	221±9.6	7.8
Caudal somatosensory	−1.7			
Superficial layers		226±12.4	237±12.2	4.9
Deep layers		244±10.7	261±13.6	7
Auditory	−2.80			
Superficial layers		214±17.3	224±15.8	4.7
Deep layers		256±16.3	257±14.8	0.4
Visual	−2.80			
Superficial layers		195±7.9	211±13.6	8.2
Deep layers		233±13.1	256±11.4	9.9
Reterosplenial	−2.80			
Superficial layers		166±12.8	180±15.2	8.5
Deep layers		225±7.9	239±9.2	6.2
Nucleus accumbens	1.34			
Shell		158±17.3	226±23.0	43.0
Core		176±5.5	200±14.0	13.6
Caudate-putamen	1.10	209±10.7	210±13.4	0.5
Septum	0.86			
Medial		88.3±3.1	116±17.4	31.3
Lateral dorsal		206±10.7	240±22.8	16.5
Lateral intermediate		117±8.7	150±14.8	28.2
Vertical limb of diagonal band	0.86	71.9±5.8	96.8±13.9	34.6
Corpus callosum	0.86	114±9.6	128±14.4	12.3
Ventral pallidum	0.14	172±6.3	185±17.4	7.6
Globus pallidus	−0.10	179±10.3	192±21.9	7.3
Thalamus	−1.70	268±12.7	270±15.7	0.7
Lateroposterior nucleus		304±10.9	302±19.3	−0.7
Ventroposterior nucleus		226±16.6	213±15.1	−5.8
Amygdala	−1.70			
Basolateral		219±8.7	234±9.2	6.8
Hypothalamus	−1.70	94.9±9.9	87.2±8.0	−8.1
Hippocampus	−2.46			
Stratum oriens		391±17.7	409±28.7	4.6
Stratum radiatum		435±14.5	472±30.0	8.5
Stratum moleculare		308±8.7	323±22.9	4.9
Dentate gyrus	−2.46	260±2.8	264±11.0	1.5
Periaqueductal grey	−3.40	108±8.8	111±0.5	2.8
Substantia nigra	−3.40	193±12.5	178±14.3	−7.8
Subiculum	−3.40	261±19.0	262±5.4	0.4
Superficial grey layer of superior colliculus	−3.40	138±18.5	183±3.5	32.6
Presubiculum	−4.04	215±20.1	22S±12.6	4.7

The mean specific binding ($n=4$)±s.e.m. of [^{3}H]DPCPX (fmol mg^{-1}) in brain regions of chronic saline- and naltrexone-treated mice. Quantitative measurements were carried out in the regions of the brains at the Bregma co-ordinates taken from the mouse atlas of Franklin & Paxinos (1997). Regional determinations were made from both left and right sides of the sections which were cut 300 μm apart. The labelling was carried out on sections from saline- and naltrexone-treated mice in a completely paired protocol. Specific binding was >80% in regions of high binding. The percentage change in binding represents the change in binding levels in the brains of chronic naltrexone-treated mice compared to the brains of saline-treated ones. A minus sign indicates a percentage decrease in binding. There was a significant difference between the treatment groups ($P<0.01$). Scheffe's *post hoc* test for individual regions showed no significant differences. The overall mean and median percentage changes across all regions were 7.8 and 5.6%, respectively.

Table 3 Quantitative autoradiography of adenosine A_{2A} receptors in the brains of chronic saline- and naltrexone-treated mice

Region	*Bregma co-ordinates (mm)*	*[^{3}H]CGS-21680-specific binding fmol mg^{-1})* *Saline*	*Naltrexone*	*% Change in binding*
Nucleus accumbens	1.34			
Shell		118±12.5	134±13.1	13.6
Core		235±17.1	218±14.8	−7.2
Caudate-putamen	1.10	328±20.3	319±19.8	−2.7
Olfactory tubercle	1.10	243±23.9	256±31.5	5.3
Globus pallidus	−0.10	97.4±5.5	92.9±9.0	−4.6

The mean specific binding ($n=4$)±s.e.m. of [^{3}H]CGS-21680 (fmol mg^{-1}) in the brain regions of chronic saline- and naltrexone-treated mice. Quantitative measurements were carried out in the regions of the brains at the Bregma co-ordinates taken from the mouse atlas of Franklin & Paxinos (1997). Regional determinations were made from both left and right sides of the sections, 300 μm apart. The labelling was carried out on sections from saline- and naltrexone-treated mice in a completely paired protocol. Specific binding was >95% in regions of high binding. The percentage change in binding represents the change in binding levels in the brains of chronic naltrexone-treated mice compared to the brains of saline-treated mice. A minus sign indicates a percentage decrease in binding. There was no significant difference between the treatment groups ($P>0.05$). The overall mean and median percent changes across all regions were 0.9 and −2.7%, respectively.

type animals (Bailey *et al*., 2002b). Accordingly, we investigated whether regions showing A_1 receptor downregulation in homozygous (−/−) μ opioid receptor knockout mice correlated with regions of upregulation of A_1 receptors in chronic naltrexone-treated brains. Again, the regression analysis revealed a small but significant correlation ($r=0.43$, $n=25$, $P<0.05$).

A_{2A} adenosine receptor autoradiography

The qualitative and quantitative distribution of A_{2A} adenosine receptors labelled with [^{3}H]CGS21680 (10 nM) in coronal sections of brains of chronic saline-treated mice (Figure 1) was similar to our previous study (Bailey *et al*., 2002b). The qualitative distribution of A_{2A} receptors in chronic naltrexone-treated mice was identical with that observed in chronic saline-treated animals (Table 3). ANOVA demonstrated that there were no significant differences in the levels of A_{2A} receptor expression between treatment groups ($P>0.05$).

Discussion

[^{3}H]DAMGO, [^{3}H]DPCPX and [^{3}H]CGS21680 were chosen to label selectively μ, A_1 and A_{2A} receptors, respectively, not only because of their selectivity (Lohse *et al*., 1987; Yeadon & Kitchen, 1988; Jarvis *et al*., 1989), but also because all the three ligands have extremely low non-specific binding. There was a clear upregulation of μ opioid receptors in the brains of chronic naltrexone-treated mice in all regions analysed. This is in agreement with a large body of studies which have shown an increase in the μ receptor binding in mice and rats after chronic exposure to naltrexone (Zukin *et al*., 1982; Tempel *et al*., 1984; Yoburn *et al*., 1986; 1988; 1989; 1995; Danks *et al*., 1988; Rothman *et al*., 1989; Cote *et al*., 1993; Unterwald *et al*., 1995; Castelli *et al*., 1997; Duttaroy *et al*., 1999). The data suggest that chronic naltrexone exposure induces a general upregulation, although there were differences in the level of increase in μ binding throughout regions. The preoptic area, the vertical limb of the diagonal band, the basolateral amygdala, the zona incerta, the hypothalamus, the periaqueductal grey and the substantia nigra showed a large increase (80–125%) in μ binding, whereas the cingulate cortex, the nucleus accumbens and the caudate putamen showed relatively less upregulation (10–30%). This is not in agreement with Diaz *et al*. (2002) who described large increases in [^{3}H]DAMGO binding in the caudate putamen and the nucleus accumbens of rats. There is indeed some disagreement in the literature about which areas are most sensitive to chronic naltrexone-induced μ opioid receptor upregulation. Zukin *et al*. (1982) observed high increases in opioid receptors in the limbic system in contrast with other groups that found only small changes (Tempel *et al*., 1984; Morris *et al*., 1988). The reasons for these discrepancies could be differences in treatment protocol (pellets, minipump, injections, dose and exposure time) and differences in species. Indeed, a greater increase of μ receptor density has been observed in animals which have been treated with a higher dose or longer exposure of opioid receptor antagonist, suggesting that the upregulation is dependent on the proportion of occupancy of opioid receptors by the antagonist (Morris *et al*., 1988). Moreover, a great range of ligands, with different selectivity to opioid receptors, have been used in order to label the μ receptors. The difference in selectivity of the ligands to the μ receptors and the lack of good differentiation of some ligands between subtypes of opioid receptors could account for the discrepancies observed. In this study, however, we used [^{3}H]DAMGO which is highly selective for μ receptors and allows good differentiation from the other subtypes of opioid receptors.

The mechanism of μ opioid receptor upregulation induced by naltrexone has yet to be elucidated. Regulatory mechanisms other than control of transcription or mRNA stability are likely, since no change was observed in μ opioid receptor mRNA levels following chronic naltrexone treatment (Unterwald *et al*., 1995; Castelli *et al*., 1997; Duttaroy *et al*., 1999). A possible mechanism by which naltrexone could produce μ receptor upregulation is by inhibiting normal downregulation of μ receptors, presumably by preventing the binding of endogenous opioids (Unterwald *et al*., 1995). However, other mechanisms by which naltrexone could produce μ receptor upregulation have been suggested. As it has been shown that naltrexone decreases lysosomal enzyme activity in neuroblas-

toma hybrid cells (Belcheva *et al*., 1991), naltrexone-induced upregulation might be due to a decrease in receptor degradation. Finally, naltrexone may also enhance the coupling of μ receptors to G proteins (Belcheva *et al*., 1991).

In addition to the upregulation of μ receptor binding, quantitative autoradiography revealed a small but significant increase in A_1 receptor binding following chronic naltrexone treatment, with 75% of the brain regions analysed showing an upregulation. The observed change in A_1 receptor binding is consistent with the large body of evidence indicating the involvement of adenosine in a number of functional opioid effects including antinociception and the expression of tolerance and dependence. Moreover, it is in agreement with studies that demonstrated changes in A_1 receptor binding following the manipulation of the opioid system by chronic morphine treatment (Ahlijanian & Takemori, 1985) or by removal of μ opioid receptors (Bailey *et al*., 2002b).

The nucleus accumbens shell and the superficial grey layers of the superior colliculus, which have very high levels of μ receptor expression, showed the greatest upregulation of A_1 binding (32–42%). Overall increases in the binding of the regions correlated significantly with the levels of μ receptor expression in saline-treated animals, suggesting that where μ receptors were in abundance, there were increments in A_1 receptor binding following chronic naltrexone treatment. This is in accordance with our previous study where the decreases in A_1 receptor binding in μ opioid receptor knockout mice correlated significantly with the regions showing high μ receptor expression in wildtype animals (Bailey *et al*., 2002b). Indeed, the regions showing increases in A_1 binding in naltrexone-treated mice brains also correlated significantly with the decreases in A_1 binding in μ receptor knockout mice brains. On the other hand, the regions where large increases in A_1 binding were observed in chronic naltrexone-treated mice did not correlate with the regions where large increases of μ binding were observed. These data imply the existence of a functional and/or structural interaction in the brain that is more prominent in areas where μ receptors are normally highly expressed. However, this μ–A_1 interaction seems to be independent from the upregulation of μ receptors in the brains of chronic naltrexone-treated mice.

In relation to the underlying molecular mechanism, it is possible that naltrexone inhibits normal downregulation of a μ–A_1 receptor complex by preventing the binding of endogenous opioids to μ receptors. The existence of a μ–A_1 physical complex in sensory nerves in the periphery has been proposed by Aley & Levine (1997). Another possibility is that the increase of A_1 binding is the consequence of a naltrexone-induced decrease of lysosomal enzyme activity (Belcheva *et al*., 1991), which would decrease receptor degradation. However, this is unlikely because no change in A_{2A} receptor binding occurred following chronic naltrexone treatment. The possibility of a regulatory effect exerted at the G-protein level is unlikely, as the ligand used for labelling A_1 receptors (DPCPX) is an antagonist that has been shown not to discriminate between coupled and uncoupled receptors (Lohse *et al*., 1987). Alternatively, a reduction of adenosine release which might result from the lack of or decrease in μ and δ opioid receptor stimulation in chronic naltrexone-treated mice brains, might be the mechanism by which A_1 receptor number is increased. A reduction in adenosine release would probably lead to an upregulation of A_1 receptors, as it has been shown that adenosine A_1 receptors were downregulated following prolonged incubation of adipocytes with the A_1 receptor agonist N^6-phenylisopropyladenosine (Green, 1987). In addition, an upregulation of brain A_1 receptors was observed in animals chronically treated with the nonselective adenosine receptor antagonist caffeine (Fredholm, 1982; Boulenger *et al*., 1983; Wu & Coffin, 1984; Green, 1987).

No significant overall changes were observed in A_{2A} receptor binding in chronic naltrexone-treated mice brains, which is in accordance with a study that failed to find changes in A_{2A} receptor binding in the brains of chronically morphine-treated mice (Kaplan *et al*., 1994). It is also in agreement with the results from our previous study where no change in A_{2A} receptor binding was observed in the brains of mice deficient in the μ opioid receptor gene (Bailey *et al*., 2002b). All these studies suggest that A_{2A}–μ receptor interactions are not relevant in the mouse brain.

In conclusion, chronic naltrexone treatment causes increases in μ and A_1 receptor binding in the brains of mice. The largest increase in A_1 receptors was observed in areas of high μ expression, supporting an interaction between these two receptors and therefore between opioid and adenosine systems, which may be of functional importance.

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