The Journal of Neuroscience, November 1, 2002, *22*(21):9210–9220

Changes in Spinal δ and κ Opioid Systems in Mice Deficient in the A_{2A} Receptor Gene

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A large body of evidence indicates important interactions between the adenosine and opioid systems in regulating pain at both the spinal and supraspinal level. Mice lacking the A_{2A} receptor gene have been developed successfully, and these animals were shown to be hypoalgesic. To investigate whether there are any compensatory alterations in opioid systems in mutant animals, we have performed quantitative autoradiographic mapping of μ, δ, κ, and opioid receptor-like (ORL1) opioid receptors in the brains and spinal cords of wild-type and homozygous A_{2A} receptor knock-out mice. In addition, μ-, δ-, and κ−mediated antinociception using the tail immersion test was tested in wild-type and homozygous A_{2A} receptor knock-out mice. A significant reduction in [^{3}H]deltorphin-I binding to δ receptors and a significant increase in [^{3}H]CI-977 binding to κ receptors was detected in the spinal cords but not in the brains of the knock-out mice. μ and ORL1 receptor expression were not altered significantly. Moreover, a significant reduction in δ-mediated antinociception and a significant increase in κ-mediated antinociception were detected in mutant mice, whereas μ-mediated antinociception was unaffected. Comparison of basal nociceptive latencies showed a significant hypoalgesia in knock-out mice when tested at 55°C but not at 52°C. The results suggest a functional interaction between the spinal δ and κ opioid and the peripheral adenosine system in the control of pain pathways.

Key words: A_{2A} knock-out; μ opioid receptor; δ opioid receptor; κ opioid receptor; ORL1 receptor; autoradiography; opioid-mediated antinociception

There is evidence indicating that both acute and chronic effects of opioids are partly mediated by adenosine (Sawynok, 1998). Morphine enhances adenosine release from the spinal cord *in vitro* and *in vivo* (Sweeney et al., 1987, 1989; Cahill et al., 1995, 1996). Although neither δ- nor κ-selective opioid agonists enhance adenosine release from spinal cord synaptosomes (Cahill et al., 1995), a synergistic interaction between μ and δ receptors in mediating adenosine release has been suggested (Cahill et al., 1996). Adenosine in turn activates A_1 receptors in the spinal cord, which suppresses the transmission of nociceptive information (Sawynok et al., 1989). A_{2A} receptors (A_{2A}Rs) have been suggested to be involved in the expression of opioid-mediated antinociception. An additive interaction between μ-selective opioid receptor agonists and the A_{2A}-selective adenosine agonist CGS21680 has been observed at the spinal level (DeLander and Keil, 1994). However, synergistic or multiplicative interactions between δ- or κ-selective opioid receptor agonists and CGS21680 suggest a more complex functional interaction between those receptors (DeLander and Keil, 1994).

In addition to the spinal cord, opioid–adenosine interactions have been reported to be present in the brain. Morphine enhances purine release *in vivo* and *in vitro* from the cortex of rodents (Fredholm and Vernet, 1978; Phillis et al., 1980; Stone, 1981). A_{2A} receptors play a role in the development of opioid dependence, and chronic exposure to morphine has been demonstrated to downregulate adenosine A_{2A} receptors in the striatum of rats (De Montis, 1992). A specific interaction has been suggested between A_{2A} and δ receptors, because a selective δ receptor agonist has been shown to inhibit the A_{2A} receptor-mediated increase in DARPP-32 (dopamine- and cAMP-regulated phosphoprotein) occurring in striatopallidal neurons (Lindskog et al., 1999). Finally, the involvement of the opioid-related peptide nociceptin in stress and anxiety behaviors (Mogil et al., 1996; Jenck et al., 1997), combined with the modulatory role of A_{2A} adenosine receptors in these behaviors (Svenningsson et al., 1999), has led to the suggestion of a possible interaction between opioid receptor-like (ORL1) and A_{2A} receptors (Dassesse et al., 2000).

The generation of mice deficient in the A_{2A}R gene has been reported (Ledent et al., 1997; Chen et al., 1999). The knock-out mice generated by Ledent et al. (1997) show increased aggression, are more anxious, and are hypoalgesic. Moreover, there are decreases in transcripts for proenkephalin and protachykinin in the striatum (Ledent et al., 1997). These results suggest the likelihood of alterations in pain processing in these mice and in modulatory effects of the opioid systems. To test the hypothesis that opioid systems are altered in the absence of the A_{2A} receptor gene, we examined by quantitative autoradiography whether there are any changes in μ, δ, κ, and ORL1 opioid receptors in brains and spinal cords of A_{2A} adenosine receptor knock-out mice. To investigate further the involvement of A_{2A} adenosine receptors in modulating opioid effects, μ, δ, and κ receptor-mediated antinociception was also investigated in these animals.

MATERIALS AND METHODS

Generation of knock-out mice and experimental conditions. The experimental methodology for the generation of A_{2A} adenosine receptor-

Received July 12, 2002; revised July 12, 2002; accepted July 23, 2002.

This study was supported by a University of Surrey Research Scholarship and the Wellcome Trust (057088).

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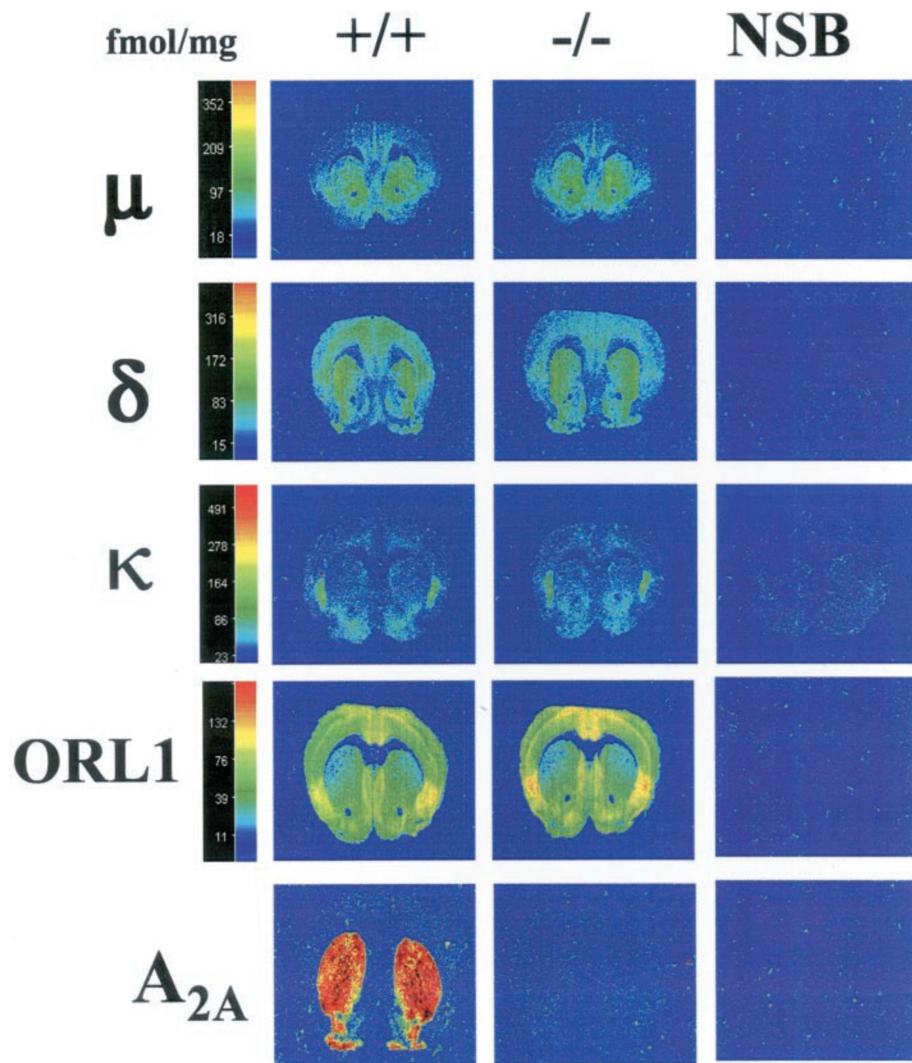

Figure 1. Computer-enhanced autoradiograms of coronal brain sections from wild-type (+/+) and homozygous (−/−) A_{2A} adenosine receptor knock-out mice. All the sections shown are from the level of the caudate (bregma 1.34 mm, apart from A_{2A} receptor-labeled sections, which were bregma 1.10 mm). μ receptors were labeled with [^{3}H]DAMGO (4 nM), δ receptors with [^{3}H]deltorphin-I (7 nM), κ receptors with [^{3}H]CI-977 (2.5 nM), ORL1 receptors with [^{3}H] nociceptin (0.4 nM), and A_{2A} receptors with [^{3}H]CGS216800 (10 nM). Nonspecific binding (*NSB*), shown in the *far right column*, was determined in the presence of naloxone (1 μM for μ and κ and 10 μM for δ), unlabeled nociceptin (100 μM for ORL1), or 5′-*N*-ethylcarboxamidoadenosine (20 μM for A_{2A}). The *color bar* shows a pseudocolor interpretation of the relative density of the black and white film image calibrated in femtomoles per milligram of tissue. Sections from +/+ and −/− brains were processed in parallel.

deficient mice has been described in detail elsewhere (Ledent et al., 1997). Knock-out and wild-type mice from the same litters were bred from heterozygous mice and genotyped by PCR at weaning. Male mice aged between 10 and 12 weeks were used in all studies. Mice were housed in groups in a temperature-controlled room with a 12 hr light/dark schedule. Food and water were available *ad libitum*. For antinociception experiments, animals were tested in the same building in which they were housed between 2 and 5 P.M. and were allowed to acclimatize to the testing room for at least 1 hr before each experiment. All studies were performed in accordance with protocols approved by the Home Office (Animals Act 1986) UK.

Autoradiographic procedures and quantitative analysis. Autoradiography was performed as detailed previously (Kitchen et al., 1997; Clarke et al., 2001). Adjacent 20 μm coronal sections were cut at intervals of 300 μm from wild-type (+/+) and homozygous (−/−) brains and transverse sections from spinal cords for the determination of total and nonspecific binding of [^{3}H] D-Ala^2-MePhe^4-Gly-ol^5 enkephalin (DAMGO), [^{3}H] D-Ala^{2}deltorphin-I (deltorphin-I), [^{3}H] ((-)-*N*-methyl-*N*-[7-(1-pyrrodinyl)-1-oxospirol[4,5]dec-8-yl]-4-benzofuranacetamide (CI-977), and [^{3}H] nociceptin at μ, δ, κ, and ORL1 opioid receptors, respectively. Ligand concentrations were ~3–4× K_D with [^{3}H]DAMGO used at a concentration of 4 nM, [^{3}H]deltorphin-I at 7 nM, [^{3}H]CI-977 at 2.5 nM, and [^{3}H]nociceptin at 0.4 nM. Nonspecific binding was defined in the presence of naloxone (1 μM for [^{3}H]DAMGO and [^{3}H]CI-977 and 10 μM for [^{3}H]deltorphin-I) or unlabeled nociceptin (100 μM for [^{3}H]nociceptin). An incubation time of 60 min was used for μ, δ, and κ ligands, whereas 180 min was used for ORL1. Sections from +/+ and −/− animals were processed together in a paired protocol. After binding, brain sections were apposed to [^{3}H] Hyperfilm (Amersham) for a period of 3 weeks (μ, δ, and ORL1 receptors) or 6 weeks (κ receptors), whereas spinal cord sections were apposed for 11 weeks (μ receptors), 14 weeks (δ receptors), 18 weeks (k receptors), or 8 weeks (ORL1 receptors). Films were developed using 50% Kodak D19 developer. Quantitative analysis of brain receptors was performed as detailed previously (Kitchen et al., 1997; Clarke et al., 2001) using an MCID image analyzer (Imaging Research). Measurements for quantitative analysis of spinal cords were taken from both right and left sides for each region, therefore representing a duplicate determination apart from lamina X, where only one measurement was taken. All anatomical areas of the spinal cord were analyzed by freehand drawing. Brain structures were identified using the mouse brain atlas of Franklin and Paxinos (1997), and spinal cord structures were referenced to the rat atlas of Paxinos and Watson (1986). [^{3}H] CGS21680 (10 nM) binding to spinal cord and brain sections taken at the level of the striatum of each mouse used in this study was determined as described by Bailey et al. (2002) to confirm the genotype.

Drug administration and assay for antinociception. A time course for antinociceptive effects was established. All drugs were administered intraperitoneally in a volume of 0.1 ml. Antinociceptive responses to the μ-selective agonist morphine, the δ-selective agonist [D-Ala^2]deltorphin (deltorphin-I), and the κ-selective agonist CI-977 were studied using the tail immersion test as described by Janssen et al. (1963). The thermal stimulus for the tail immersion assay was warm water at 55°C in the studies with morphine and 52°C for deltorphin-I and CI-977. Control latencies were determined by measuring the time required for the mouse to flick or withdraw its tail from the water 20 min before drug treatment. A 10 sec cutoff time was used to prevent tissue damage. Each naive +/+ and −/− mouse was administered vehicle or a dose of an opioid agonist intraperitoneally. Each mouse was tested for antinociceptive responses 5,

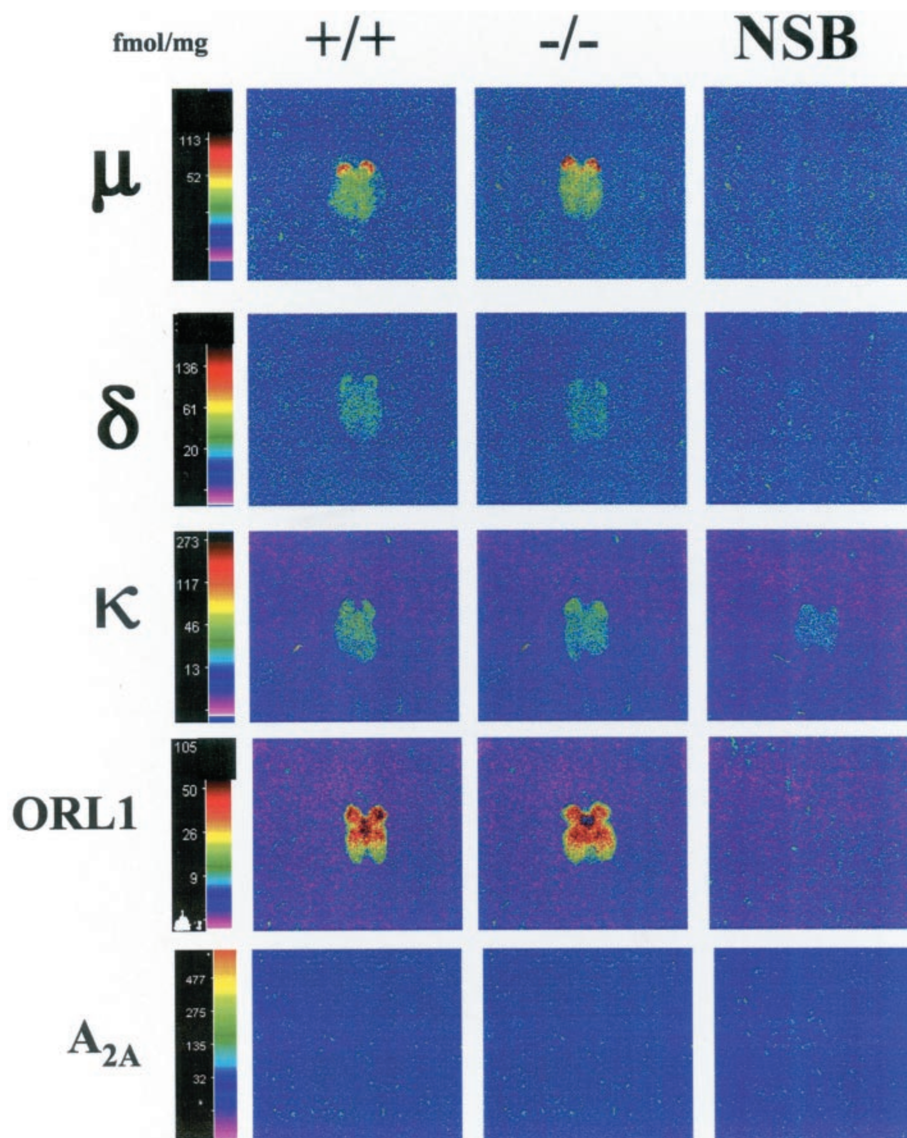

Figure 2. Computer-enhanced autoradiograms of coronal sacral spinal cord sections from wild-type (+/+) and homozygous (−/−) A_{2A} knock-out mice. The sections were taken were the S4 segment according to the rat atlas of Paxinos and Watson (1986). μ receptors were labeled with [^{3}H]DAMGO (4 nM), δ receptors with [^{3}H]deltorphin-I (7 nM), κ receptors with [^{3}H]CI-977 (2.5 nM), ORL1 receptors with [^{3}H]nociceptin (0.4 nM), and A_{2A} receptors with [^{3}H]CGS21680 (10 nM). Nonspecific binding (*NSB*), shown in the *far right column*, was determined in the presence of naloxone (1 μM for μ and κ and 10 μM for δ), unlabeled nociceptin (100 μM for ORL1), or 5′-*N*-ethylcarboxamidoadenosine (20 μM for A_{2A}). The *color bar* shows a pseudocolor interpretation of the relative density of the black and white film image calibrated in femtomoles per milligram of tissue. Sections from +/+ and −/− spinal cords were processed in parallel.

15, 30, 45, and 60 min after the administration of morphine, 5, 10, 15, 30 and 45 min after the administration of deltorphin-I, and 5, 10, 20, 30, 45, 60, and 120 min after the administration of CI-977. Dose–response curves for each agonist were plotted from data at times when the analgesic response to the opioid agonists was maximum (45 min for morphine, 15 min for deltorphin-I, 10 min for CI-977). Basal nociceptive responses of wild-type and A_{2A} receptor knock-out mice to mechanical stimuli were determined by the use of the tail pressure test as described by Kitchen (1984).

Statistics. Comparison of quantitative measures of autoradiographic binding for each ligand in brains and spinal cords from +/+ and −/− animals was performed using two-way ANOVA (for factors region or lamina and genotype). Comparison of antinociceptive dose–response curves of +/+ and −/− animals for each agonist was performed using repeated measures two-way ANOVA (for factors dose and genotype) followed by Scheffe's *post hoc* analysis. Comparison of basal control latencies from +/+ and −/− animals was performed using Student's *t* test. Comparison of tail immersion latencies of +/+ and −/− vehicle-treated animals was performed using repeated measures two-way ANOVA (for factors time and genotype).

Materials and drug preparation. [^{3}H]DAMGO, 56.0 Ci/mmol, [^{3}H]deltorphin-I, 50.0 Ci/mmol, [^{3}H]CI-977, 41 Ci/mmol, and [^{3}H]nociceptin, 164 Ci/mmol, were purchased from Amersham International (Buckinghamshire, UK). [^{3}H]CGS21689, 42.5 Ci/mmol, was purchased from NEN Life Science Products (Hounslow, UK). Naloxone and morphine sulfate were purchased from Sigma-Aldrich (Dorset, UK), unlabeled nociceptin and deltorphin-I were from Bachem (Essex, UK), and unlabeled CI-977 was a gift from Parke-Davis (Cambridge, UK). Morphine sulfate and CI-977 were dissolved in saline, and deltorphin-I was dissolved in saline that was acidified with glacial acetic acid (1% v/v) and buffered with NaOH (75 mM) to pH 7.

RESULTS

Quantitative autoradiography of μ, δ, κ, and ORL1 opioid receptors in brains and spinal cords of A_{2A} adenosine receptor knock-out mice

The qualitative and quantitative distribution of μ, δ, κ, and ORL1 opioid receptors labeled with [^{3}H]DAMGO (4 nM), [^{3}H]deltorphin-I (7 nM), [^{3}H]CI-977 (2.5 nM), and [^{3}H]nociceptin (0.4 nM) in coronal sections of brain of wild-type mice were similar to previous studies reported by our group (Kitchen et al., 1997; Slowe et al., 1999; Clarke et al., 2001) (Fig. 1). In spinal cord, the highest levels of [^{3}H]DAMGO, [^{3}H]deltorphin-I, and [^{3}H]CI-977 binding were detected in the superficial layers. High levels of [^{3}H]nociceptin binding were detected in the superficial layers and in lamina X of the spinal cord (Fig. 2). The pattern of distribution of all receptor types studied in homozygous A_{2A} adenosine receptor knock-out mice brains and spinal cords was identical to wild

Table 1. Representative noncortical regions of quantitative autoradiography of μ, δ, κ, and ORL1 receptor binding in wild-type (+/+) and homozygous (−/−) A_{2A} mutant mice brains

Region	Bregma coordinates (mm)	[³H]ligand-specific binding (fmol/mg)		% Change in binding
		Wild-type (+/+)	Homozygous (−/−)	(−/−)
[³H]DAMGO binding				
Nucleus accumbens core	1.34	103 ± 14.5	106 ± 12.7	2.9
Endopiriform nucleus	1.10	93.4 ± 6.1	92.4 ± 8.9	−1.1
Medial habenula nucleus	−1.70	132 ± 16.2	126 ± 16.7	−4.5
Thalamus	−1.70	50.1 ± 7.4	49.6 ± 7.2	−1.0
Basolateral amygdala	−1.70	108 ± 18.3	109 ± 13.0	0.9
Periaqueductal gray	−3.40	65.1 ± 14.1	69.7 ± 11.5	7.1
Superficial gray layer of superior colliculus	−3.40	103 ± 9.0	93.3 ± 16.2	−9.4
Intermediate gray layer of superior colliculus	−3.40	80.0 ± 9.0	74.6 ± 14.8	−6.8
[³H]deltorphin-1 binding				
Olfactory bulb	4.28			
Exterior plexiform layer		64.2 ± 4.6	68.3 ± 6.3	6.4
Interior granular layer		148 ± 20.3	160.0 ± 20.1	8.1
Nucleus accumbens core	1.34	56.3 ± 4.8	60.4 ± 4.5	7.3
Caudate-putamen	1.10	82.2 ± 6.2	93.5 ± 2.9	13.7
Olfactory tubercle	1.10	80.0 ± 6.4	68.8 ± 7.5	−14.0
Thalamus	−1.70	25.5 ± 1.8	24 ± 1.1	−5.9
Basolateral amygdala	−1.70	74.1 ± 5.0	75.0 ± 8.6	1.2
Presubiculum	−4.04	61.6 ± 6.3	50.8 ± 3.1	−17.5
[³H]CI-977 binding				
Claustrum	1.94	114 ± 10.2	119 ± 14.5	4.4
Nucleus accumbens core	1.34	35.0 ± 4.7	28.9 ± 2.5	−17.4
Olfactory tubercle	1.10	29.2 ± 3.5	35.2 ± 2.8	20.5
Dorsal endopiriform nucleus	1.10	125.5 ± 7.6	136.2 ± 15.4	8.5
Ventral pallidum	0.14	47.7 ± 2.4	46.0 ± 6.2	−3.6
Basolateral amygdala	−1.70	37.8 ± 5.6	38.6 ± 3.7	2.1
Hypothalamus	−1.70	35.9 ± 4.4	32.6 ± 1.1	−9.1
Substantia nigra	−3.40	37.7 ± 2.3	31.8 ± 2.5	−15.6
Superficial gray of the superior colliculus	−3.40	7.3 ± 1.8	8.8 ± 3.2	20.5
[³H]nociceptin binding				
Anterior olfactory bulb	3.08	88.2 ± 6.4	83.9 ± 5.6	−4.9
Nucleus accumbens	1.34	55.4 ± 2.2	55.1 ± 3.1	−0.5
Thalamus	−1.70	39.5 ± 1.4	37.1 ± 1.4	−6.1
Amygdala	−1.70	111 ± 8.0	105 ± 7.9	−5.4
Hippocampus	−2.46	60.0 ± 3.6	52.8 ± 2.0	−12.0
Periaqueductal gray	−3.40	48.6 ± 3.5	48.6 ± 5.8	0
Superficial gray layer of superior colliculus	−3.40	78.2 ± 6.1	78.4 ± 1.9	0.3
Intermediate gray layer of superior colliculus	−3.40	46.4 ± 2.0	47.2 ± 2.0	1.7
Presubiculum	−3.40	75.8 ± 1.6	69.4 ± 3.1	−8.4

The mean specific binding ($n = 4$) of [³H]DAMGO, [³H]deltorphin-1, [³H]CI-977, or [³H]nociceptin (femtomoles per milligram) ± SEM in brain regions of wild-type (+/+) and homozygous (−/−) A_{2A} receptor knock-out mice. Measurements in the regions were performed at the bregma coordinates taken from the mouse atlas of Franklin and Paxinos (1997). Regional determinates were made from both left and right sides of the sections, which were cut 300 μm apart. The labeling was performed on sections from +/+ and −/− mice in a completely paired protocol. Specific binding of [³H]DAMGO, [³H]deltorphin-1, and [³H]nociceptin was >85% in all regions. Specific binding of [³H]CI-977 was >50% in all regions. The data show only regions expressing high receptor levels and regions associated with pain processing. Analysis of all 40–50 regions in which receptor measurements were made showed that comparison of genotypes was not statistically significant for any ligand ($p > 0.05$; ANOVA). The percentage (%) change in binding represents the change in binding levels in −/− brains compared with +/+. A minus sign indicates a percentage decrease in binding levels.

type. Binding of A_{2A} adenosine receptors with [³H]CGS21680 showed a complete absence of A_{2A} sites in homozygous mice, which confirmed the genotype of the A_{2A} adenosine receptor knock-out mice used in this study (Fig. 1). Finally no [³H]CGS21680 binding was detected in wild-type or knock-out spinal cords (Fig. 2).

In the brain, there were no significant differences in the levels of μ, δ, κ, and ORL1 opioid expression between genotypes for between 40 and 50 brain regions analyzed for each receptor ($p > 0.05$). For illustration, Table 1 shows only noncortical regions that either exhibit high level of receptor expression or are known to play an important role in pain. Binding in all cortical structures was very similar across genotypes for each receptor (data not shown). In contrast, in the spinal cord a significant quantitative difference in the levels of [³H]deltorphin-I binding between genotypes was observed ($p < 0.01$), and three-quarters of the regions analyzed in A_{2A} knock-out mice showed a downregulation of δ receptor expression. The largest loss of δ sites (up to 19%) was found in the superficial layers of the spinal cord (Table 2). There was also a significant quantitative increase in the levels

Table 2. Quantitative autoradiography of δ receptor binding in wild-type (+/+) and homozygous (−/−) A_{2A} mutant mice

Region	Segments from rat atlas	[³H]deltorphin-1-specific binding (fmol/mg)		% Change in binding
		Wild-type (+/+)	Homozygous (−/−)	(−/−)
Cervical	C1 and C6			
Superficial layers (laminas I and II)		30.0 ± 2.6	25.4 ± 2.6	−15.3
Laminas III–VI		15.5 ± 1.0	13.7 ± 0.8	−11.6
Lamina X		18.3 ± 2.0	18.0 ± 0.9	−1.6
Ventral horn (laminas VII–IX)		13.7 ± 0.9	12.4 ± 0.7	−9.5
Dorsal horn (laminas I–VI)		18.7 ± 1.3	15.4 ± 0.9	−17.6
Thoracic	T3 and T6			
Superficial layers (laminas I and II)		27.7 ± 1.8	24.9 ± 2.2	−10.1
Laminas III–VI		15.7 ± 1.0	15.4 ± 1.6	−1.9
Lamina X		16.7 ± 1.2	15.8 ± 1.5	−5.4
Ventral horn (laminas VII–IX)		14.9 ± 0.9	14.4 ± 1.1	−3.4
Dorsal horn (laminas I–VI)		19.3 ± 0.8	18.8 ± 2.0	−2.6
Sacral	S4			
Superficial layers (laminas I and II)		21.2 ± 1.3	17.2 ± 1.5	−18.9
Laminas III–VI		11.2 ± 0.9	12.0 ± 0.9	7.1
Lamina X		11.2 ± 0.8	11.6 ± 0.5	3.6
Ventral horn (laminas VII–IX)		10.9 ± 1.0	11.7 ± 0.4	7.3
Dorsal horn (laminas I–VI)		14.2 ± 1.2	13.5 ± 0.7	−4.9

The mean specific binding ($n = 4$) of [³H]deltorphin-I (femtomoles per milligram) ± SEM in spinal cord regions of wild-type (+/+) and homozygous (−/−) A_{2A} receptor knock-out mice. Measurements in the regions were performed at the bregma coordinates taken from the rat atlas of Paxinos and Watson (1986). Regional determinates were made from both left and right sides of the sections, which were cut 300 μm apart. The labeling was performed on sections from +/+ and −/− mice in a completely paired protocol. Specific binding was >80% in all regions. Comparison of genotypes was statistically significant ($p < 0.01$; ANOVA). The percentage (%) change in binding represents the change in binding levels in −/− spinal cords compared with +/+. A minus sign indicates a percentage decrease in binding levels. The overall mean and median percentage changes across regions were −5.8 and −5.4%, respectively.

Table 3. Quantitative autoradiography of κ receptor binding in wild-type (+/+) and homozygous (−/−) A_{2A} mutant mice

Region	Segments from rat atlas	[³H]CI-977-specific binding (fmol/mg)		% Change in binding
		Wild-type (+/+)	Homozygous (−/−)	(−/−)
Cervical	C1 and C6			
Superficial layers (laminas I and II)		15.9 ± 1.7	15.7 ± 1.3	−1.3
Laminas III–VI		4.8 ± 0.9	4.5 ± 1.0	−6.3
Lamina X		9.8 ± 1.2	11.4 ± 1.1	16.3
Ventral horn (laminas VII–IX)		2.5 ± 0.7	2.4 ± 0.6	−4.0
Dorsal horn (laminas I–VI)		7.4 ± 1.4	8.0 ± 1.1	8.1
Thoracic	T3 and T6			
Superficial layers (laminas I and II)		16.9 ± 2.1	20.1 ± 1.4	18.9
Laminas III–VI		7.0 ± 1.3	9.2 ± 1.0	31.4
Lamina X		11.7 ± 0.9	11.8 ± 1.0	0.9
Ventral horn (laminas VII–IX)		5.1 ± 1.1	6.1 ± 1.0	19.6
Dorsal horn (laminas I–VI)		9.9 ± 1.4	12.7 ± 1.2	28.3
Sacral	S4			
Superficial layers (laminas I and II)		17.2 ± 1.6	21.8 ± 2.8	26.7
Laminas III–VI		8.4 ± 1.0	7.0 ± 2.4	−16.7
Lamina X		11.2 ± 1.1	13.4 ± 3.4	19.6
Ventral horn (laminas VII–IX)		4.7 ± 0.8	2.4 ± 1.0	−48.9
Dorsal horn (laminas I–VI)		12.0 ± 1.1	13.7 ± 2.7	14.2

The mean specific binding ($n = 4$) of [³H]CI-977 (femtomoles per milligram) ± SEM in spinal cord regions of wild-type (+/+) and homozygous (−/−) A_{2A} receptor knock-out mice. Measurements in the regions were performed at the bregma coordinates taken from the rat atlas of Paxinos and Watson (1986). Regional determinates were made from both left and right sides of the sections, which were cut 300 μm apart. The labeling was performed on sections from +/+ and −/− mice in a completely paired protocol. Specific binding was >45% in all regions. Comparison of genotypes was statistically significant ($p < 0.05$; ANOVA). The percentage (%) change in binding represents the change in binding levels in −/− spinal cords compared with +/+. A minus sign indicates a percentage decrease in binding levels. The overall mean and median percentage changes across regions were 7.1 and 14.2%, respectively.

Table 4. Quantitative autoradiography of μ receptor binding in wild-type (+/+) and homozygous (−/−) A_{2A} mutant mice

Region	Segments from rat atlas	[^{3}H]DAMGO-specific binding (fmol/mg)		% Change in binding
		Wild-type (+/+)	Homozygous (−/−)	(−/−)
Cervical	C1 and C6			
Superficial layers (laminas I and II)		96.2 ± 4.1	95.9 ± 3.0	−0.3
Laminas III–VI		31.2 ± 2.2	30.2 ± 2.2	−3.2
Lamina X		27.2 ± 1.7	28.9 ± 2.4	6.3
Ventral horn (laminas VII–IX)		18.9 ± 1.8	19.1 ± 2.1	1.1
Dorsal horn (laminas I–VI)		43.0 ± 1.7	41.3 ± 3.1	−4.0
Thoracic	T3 and T6			
Superficial layers (laminas I and II)		99.5 ± 4.6	91.5 ± 3.6	−8.0
Laminas III–VI		30.5 ± 2.7	34.5 ± 3.0	13.1
Lamina X		22.6 ± 2.4	18.8 ± 1.6	−16.8
Ventral horn (laminas VII–IX)		17.4 ± 3.0	20.5 ± 2.0	17.8
Dorsal horn (laminas I–VI)		48.9 ± 2.6	48.8 ± 2.4	−0.2
Sacral	S4			
Superficial layers (laminas I and II)		91.5 ± 8.6	92.4 ± 4.9	1.0
Laminas III–VI		31.6 ± 3.1	31.1 ± 2.6	−1.6
Lamina X		29.7 ± 4.5	29.4 ± 6.7	−1.0
Ventral horn (laminas VII–IX)		23.7 ± 1.7	19.7 ± 2.9	−16.9
Dorsal horn (laminas I–VI)		47.5 ± 4.2	44.6 ± 4.7	−6.1

The mean specific binding ($n = 4$) of [^{3}H]DAMGO (femtomoles per milligram) ± SEM in spinal cord regions of wild-type (+/+) and homozygous (−/−) A_{2A} receptor knock-out mice. Measurements in the regions were performed at the bregma coordinates taken from the rat atlas of Paxinos and Watson (1986). Regional determinates were made from both left and right sides of the sections, which were cut 300 μm apart. The labeling was performed on sections from +/+ and −/− mice in a completely paired protocol. Specific binding was >90% in all regions. Comparison of genotypes was not statistically significant ($p > 0.05$; ANOVA). The percentage (%) change in binding represents the change in binding levels in −/− spinal cords compared with +/+. A minus sign indicates a percentage decrease in binding levels. The overall mean and median percentage changes across regions were −1 and −0.9%, respectively.

Table 5. Quantitative autoradiography of ORL1 receptor binding in wild-type (+/+) and homozygous (−/−) A_{2A} mutant mice

Region	Segments from rat atlas	[^{3}H]nociceptin-specific binding (fmol/mg)		% Change in binding
		Wild-type (+/+)	Homozygous (−/−)	(−/−)
Cervical	C1 and C6			
Superficial layers (laminas I and II)		52.9 ± 3.6	55.8 ± 6.3	5.5
Laminas III–VII		36.9 ± 1.7	36.5 ± 1.9	−1.1
Lamina X		46.8 ± 3.7	50.5 ± 2.8	7.9
Laminas VIII and IX		20.4 ± 1.4	19.0 ± 2.1	−6.9
Dorsal horn (laminas I–VI)		40.8 ± 2.1	42.0 ± 2.7	2.9
Thoracic	T3 and T6			
Superficial layers (laminas I and II)		54.6 ± 3.6	67.8 ± 8.5	24.2
Laminas III–VII		45.9 ± 2.5	48.8 ± 2.6	6.3
Lamina X		54.0 ± 2.8	57.7 ± 2.6	6.9
Laminas VIII and IX		23.7 ± 1.0	23.1 ± 1	−2.5
Dorsal horn (laminas I–VI)		48.3 ± 3.3	54.4 ± 4.6	12.6
Sacral	S4			
Superficial layers (laminas I and II)		66.3 ± 3.6	75.6 ± 11.2	14.0
Laminas III–VII		50.5 ± 1.8	51.4 ± 2.6	1.8
Lamina X		73.6 ± 6.6	82.1 ± 7.2	11.5
Laminas VIII and IX		24.0 ± 1.7	22.4 ± 1.3	−6.7
Dorsal horn (laminas I–VI)		56.2 ± 2.3	59.2 ± 5.6	5.3

The mean specific binding ($n = 4$) of [^{3}H]nociceptin (femtomoles per milligram) ± SEM in spinal cord regions of wild-type (+/+) and homozygous (−/−) A_{2A} receptor knock-out mice. Measurements in the regions were performed at the bregma coordinates taken from the rat atlas of Paxinos and Watson (1986). Regional determinates were made from both left and right sides of the sections, which were cut 300 μm apart. The labeling was performed on sections from +/+ and −/− mice in a completely paired protocol. Specific binding was >90% in all regions. Comparison of genotypes was not statistically significant ($p > 0.05$; ANOVA). The percentage (%) change in binding represents the change in binding levels in −/− spinal cords compared with +/+. A minus sign indicates a percentage decrease in binding levels. The overall mean and median percentage changes across regions were 5.3 and 5.3%, respectively.

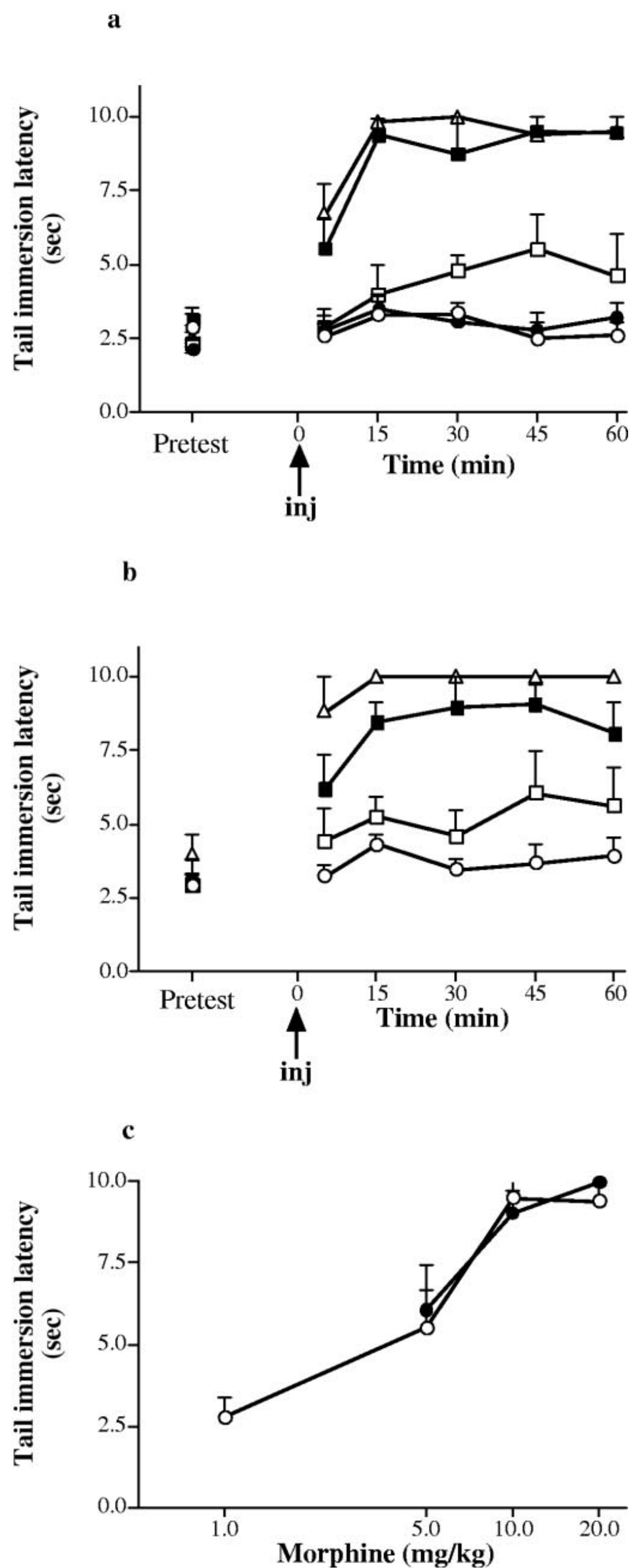

Figure 3. Dose-related antinociception induced by morphine sulfate in 12-week-old wild-type (+/+) and A_{2A} receptor knock-out (−/−) mice. *a*, Time course of morphine-induced antinociception in the +/+ mice; *b*, time course of morphine-induced antinociception in the −/− mice measured as tail immersion latency at 55°C. ○, Saline control values; ●, 1 mg/kg morphine sulfate; □, 5 mg/kg morphine sulfate; ■, 10 mg/kg morphine sulfate; △, 20 mg/kg morphine sulfate. Doses (0.1 ml volume, i.p) were administered at 0 min. *pretest*, Pretest responses 20 min before injection. *c*, Dose–response relationship for morphine sulfate at peak antinociception (45 min after injection) in wild-type (○) and A_{2A} receptor knock-out (●) mice. Values are means ± SEM of five determinations ($n = 5$). Differences in dose–response curves between genotypes were not statistically significant ($p > 0.05$; ANOVA). The difference in tail immersion latencies between +/+ and −/− saline-treated animals was statistically significant ($p < 0.05$; ANOVA).

of [^{3}H]CI-977 binding between genotypes ($p < 0.05$), and the greatest overall upregulation was detected in the superficial layers of the spinal cord (up to 27%) (Table 3). In contrast to δ and κ binding, there were no significant differences in the levels of μ or ORL1 receptor expression between genotypes in the spinal cord ($p > 0.05$) (Tables 4, 5).

μ, δ, and κ opioid receptor-mediated antinociception in wild-type (+/+) and A_{2A} receptor knock-out (−/−) mice

An antinociceptive time course and dose–response relationships were established for the μ agonist morphine (Fig. 3), the δ agonist deltorphin-I (Fig. 4), and the κ agonist CI-977 (Fig. 5). The antinociceptive effect was at a maximum at 45, 15, and 10 min after injection for morphine, deltorphin-I, and CI-977, respectively, in both wild-type and knock-out mice. Comparison of antinociceptive dose–response relationships between +/+ and −/− mice showed that there was a significant decrease in δ-mediated antinociception ($p < 0.05$) and a significant increase in κ-mediated antinociception ($p < 0.01$) but no change in μ-mediated antinociception ($p > 0.05$).

Comparison of basal reactivity with thermal stimuli of 55°C between knock-out and wild-type animals showed that there was a significant increase in latency in knock-out animals ($p < 0.05$) (Fig. 6*a*). However, when the animals were exposed to a stimulus of 52°C, no significant difference in basal antinociception was found between genotypes ($p > 0.05$) (Fig. 6*b*). Similarly, a comparison of tail immersion latencies in vehicle-treated animals also demonstrated a significant increase in knock-out mice when they were exposed to a thermal stimuli of 55°C ($p < 0.05$) but not when they were exposed to 52°C (p > 0.05) (Figs. 3–5). Finally, no significant change in basal nociceptive threshold (135.7 ± 12.6 gm for +/+ vs 157.9 ± 10.2 gm for −/−; $n = 43$) was found when +/+ and −/− mice were tested with the tail pressure test.

DISCUSSION

Although quantitative mapping of all four opioid receptors has been performed in the mouse brain (Clarke et al., 2001), the current study now reports the first complete quantitative autoradiography in spinal cord using highly selective ligands in this species. The qualitative pattern of μ, δ, κ, and ORL1 receptors in the spinal cord was similar to the studies that have been reported by others in the rat (Besse et al., 1990, 1991; Neal et al., 1999) and mouse (Narita et al., 1999; Mogil et al., 2000).

The lack of change of μ, δ, κ, or ORL1 binding in the brains of −/− mice clearly suggests that a direct A_{2A} receptor interaction with any opioid receptor subtype is not relevant in the mouse brain. This is in agreement with a study that failed to find changes in A_{2A} receptor binding in the striatum of chronically morphine-treated mice (Kaplan et al., 1994) in contrast with the rat, where A_{2A} receptors are downregulated (De Montis, 1992). Moreover, the decrease in proenkephalin mRNA, observed in A_{2A} receptor

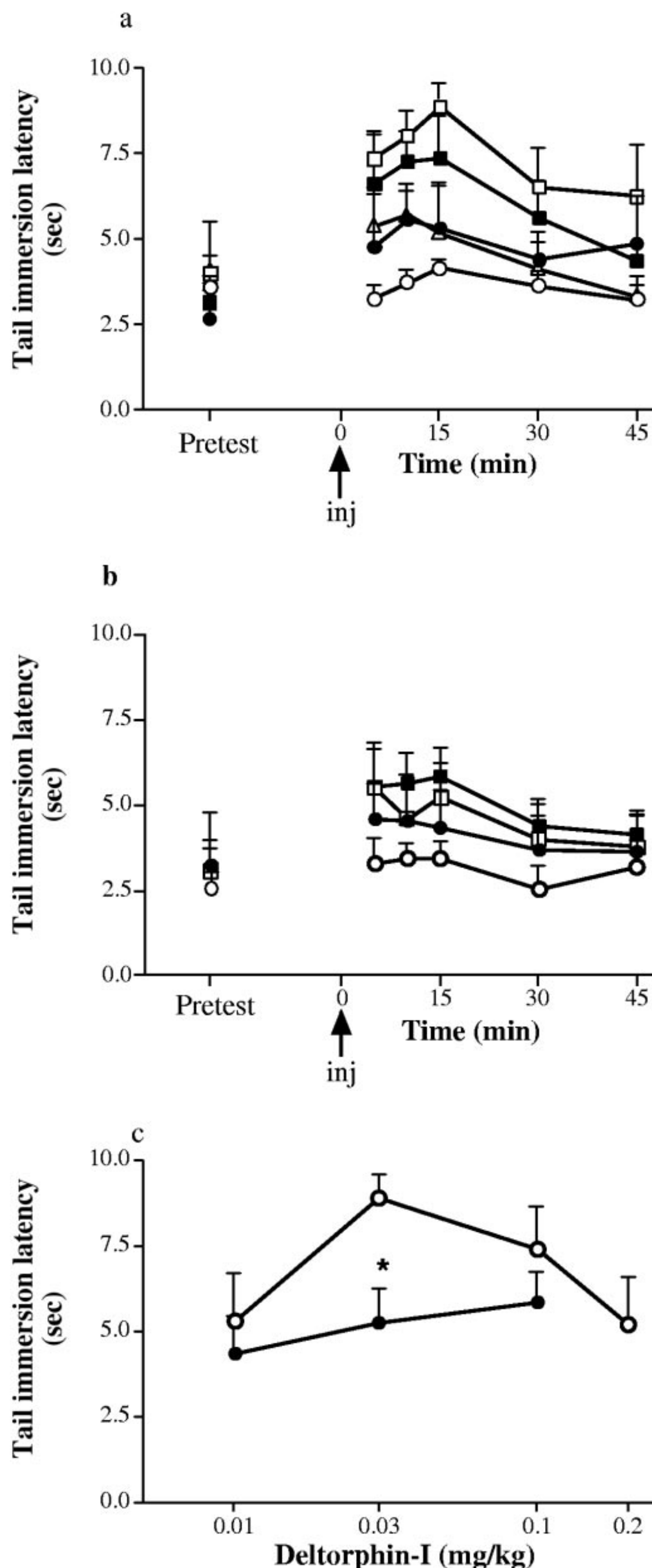

Figure 4. Dose-related antinociception induced by deltorphin-I in 12-week-old wild-type (+/+) and A_{2A} receptor knock-out (−/−) mice. *a*, Time course of deltorphin-I-induced antinociception in the +/+ mice; *b*, time course of deltorphin-I induced antinociception in the −/− mice measured as tail immersion latency at 52°C. ○ represents vehicle control values; ●, 0.01 mg/kg deltorphin-I; □, 0.03 mg/kg deltorphin-I; ■, 0.1 mg/kg deltorphin-I; △, 0.2 mg/kg deltorphin-I. Doses (0.1 ml volume, i.p) are administered at 0 min. *pretest*, Pretest responses 20 min before injection. *c*, Dose–response relationship for deltorphin-I at peak antinociception (15 min after injection) in wild-type (○) and A_{2A} receptor knock-out (●) mice. Values are means ± SEM of five determinations ($n = 5$). Differences in dose–response curves between genotypes were statistically significant ($p < 0.05$; ANOVA); *$p < 0.05$ (Scheffe's *post hoc* analysis). The difference in tail immersion latency between +/+ and −/− vehicle-treated animals was not statistically significant ($p > 0.05$; ANOVA).

knock-out mice striatum (Ledent et al., 1997), does not appear to trigger any changes in opioid receptor numbers. It is possible, however, that the decrease in proenkephalin mRNA in −/− mice does not result in a decrease in the levels of the transmitter itself. Indeed, enkephalin immunoreactivity is unaltered in another strain of A_{2A} knock-out mice (Chen et al., 1999). The lack of change in nociceptin mRNA (Dassesse et al., 2000) in the striatum of −/− mice supports the lack of changes in ORL1 binding observed here in the brains of −/− mice of the same strain.

In contrast to the brain, significant changes in δ and κ receptors were observed in the spinal cord of −/− mice, with no change in μ and ORL1 binding. To investigate whether the small changes in binding had any behavioral relevance, μ-, δ-, and κ-mediated antinociception assays were performed. Deltorphin-I and CI-977 were used to enable direct correlation with the results from the binding studies. Morphine was used as a prototypic μ agonist and because the μ receptor is its primary target (Matthes et al., 1996). Tail immersion was used as an analgesic test to investigate changes in spinal rather than supraspinal antinociception, because this assay is classically considered to reflect the actions of drugs on pain transmission at spinal sites (DeLander et al., 1992). A temperature of 55°C was used for testing morphine antinociception because it has been shown that no supraspinal circuits are involved at that temperature (Ossipov et al., 1995); 52°C was also used to test deltorphin-I- and CI-977-mediated antinociception in common with previous studies (Millan, 1989; Crook et al., 1993; Kitchen et al., 1994, 1995; Matthes et al., 1998) and because these drugs have limited or no antinociceptive activity at higher intensity thermal stimuli.

In accordance with the autoradiography in the spinal cord, we showed a decrease and increase in δ- and κ-mediated antinociception, respectively, and no change in μ-mediated antinociception in A_{2A} receptor knock-out mice. This strongly suggests that even relatively small changes in the receptor density in −/− mice result in significant changes in the antinociceptive responses. In wild-type mice, the dose–response curve to deltorphin-I was bell shaped. It is not clear why this occurs, but we have observed a similar profile with this peptide in neonatal rats (our unpublished observations).

The possibility that the changes in δ and κ receptors are caused by lack of spinal A_{2A} receptors in −/− mice can be ruled out, because we have been unable to detect any A_{2A} receptors in the spinal cord of wild-type mice. We have also shown the absence of A_{2A} receptors in spinal cord of another strain of mice (Bailey et al., 2002). This is at odds with the observation of DeLander and Keil (1994) that antinociception induced by intrathecal coadministration of the selective A_{2A} agonist CGS 21680 and μ-, δ-, or κ-selective opioid agonists showed an additive, synergistic, or multiplicative interaction between them. However, the issue as to whether CGS21680 actually induces antinociception by acting on A_{2A} receptors is debatable because significantly greater doses of CGS21680, as compared with a selective A_1 receptor agonist,

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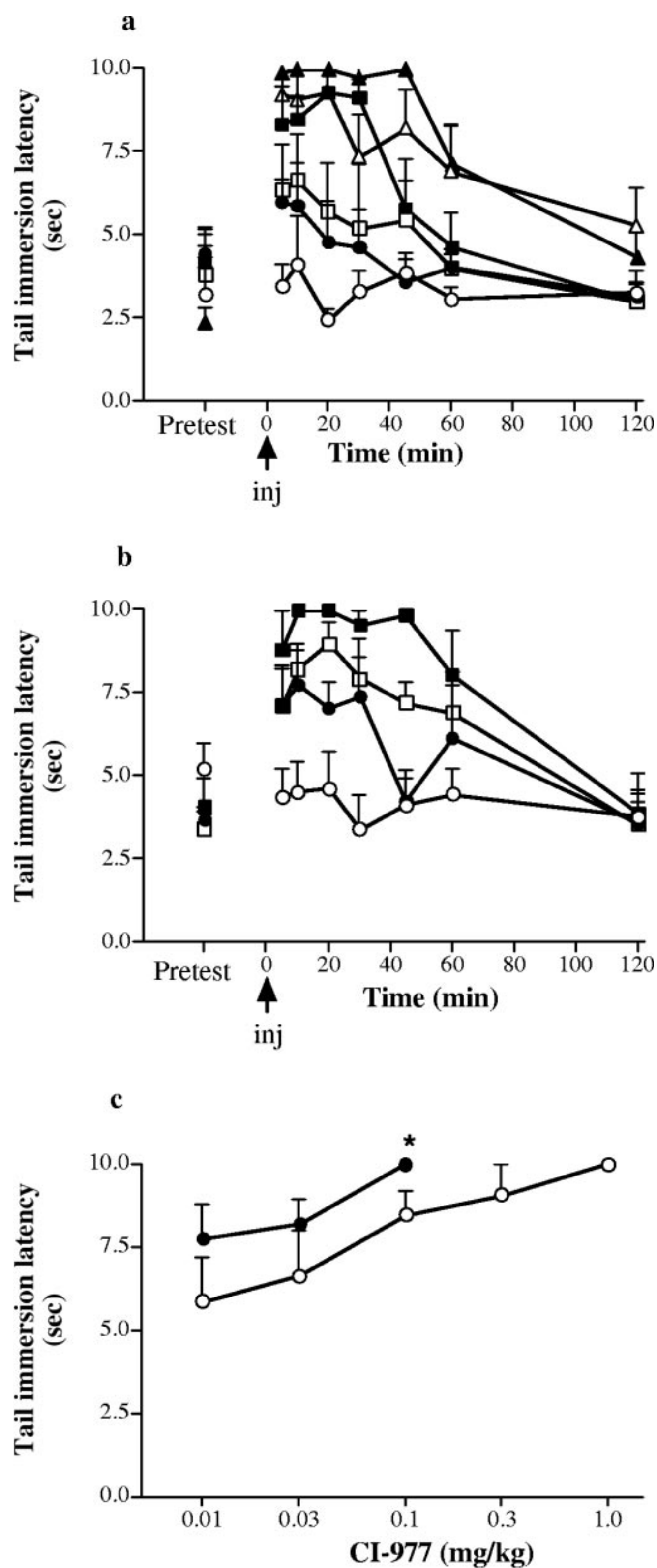

Figure 5. Dose-related antinociception induced by CI-977 in 12-week-old wild-type (+/+) and A_{2A} receptor knock-out (−/−) mice. *a*, Time course of CI-977-induced antinociception in the +/+ mice; *b*, time course of CI-977-induced antinociception in the −/− mice measured as tail immersion latency at 52°C. ○ represents saline control values; ●, 0.01 mg/kg CI-977; □, 0.03 mg/kg CI-977; ■, 0.1 mg/kg CI-977; △, 0.3 mg/kg CI-977; ▲ 1 mg/kg CI-977. Doses (0.1 ml volume, i.p) are administered at 0 min. *pretest*, Pretest responses 20 min before injection. *c*, Dose–response relationship for CI-977 at peak antinociception (10 min after injection) in wild-type (○) and A_{2A} receptor knock-out (●) mice. Values are means ± SEM of five determinations ($n = 5$). Differences in dose–response curves between genotypes were statistically significant ($p < 0.05$; ANOVA); $^*p < 0.05$ (Scheffe's *post hoc* analysis). Differences in tail immersion latency between +/+ and −/− saline-treated animals were not statistically significant ($p > 0.05$; ANOVA).

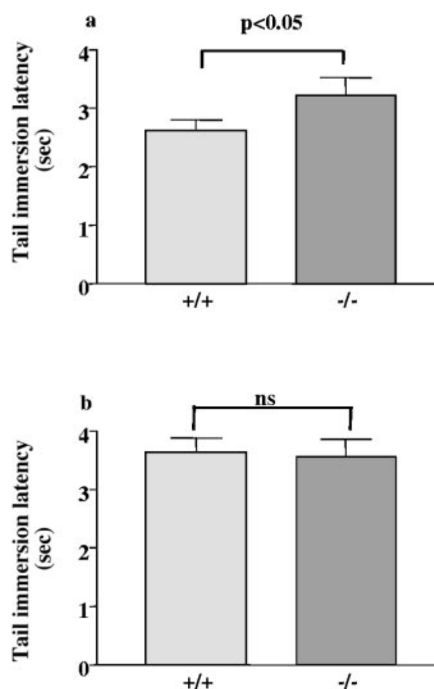

Figure 6. Tail immersion responses to acute thermal stimulus in wild-type (+/+) and A_{2A} receptor knock-out (−/−) mice. *a*, Basal reactivity to a thermal stimulus of 55°C for wild-type (+/+) mice ($n = 25$) and A_{2A} receptor knock-out (−/−) mice ($n = 20$); *b*, basal reactivity to a thermal stimulus of 52°C for wild-type (+/+) mice ($n = 55$) and A_{2A} receptor knock-out (−/−) mice ($n = 40$). Error bars represent SEM. *ns*, Not significant. $p < 0.05$; significant difference between genotypes (Student's *t* test).

were required to induce antinociception. Thus it is likely that the opioid–A_{2A} receptor agonist interactions observed in that study were caused by interactions at an A_1 receptor at which CGS21680 has lesser potency (DeLander and Keil, 1994). However, expression of the A_{2A} receptor gene has been reported in the dorsal root ganglia in mouse (Kaelin-Lang et al., 1998), and A_{2A} receptors are present on sensory nerve terminals in the periphery. It has been shown that A_{2A} receptors play a role in peripheral regulation of pain. Local administration of a selective A_{2A} receptor agonist, CGS21680, has been reported to produce mechanical hyperalgesia (Khasar et al., 1995) and enhance pain responses in the low-concentration formalin model in rat (Doak and Sawynok, 1995). That action has been proposed to result from the stimulation of adenylate cyclase resulting in an increase in cAMP levels in the sensory nerve terminal (Taiwo and Levine, 1991; Khasar et al., 1995). As a result, the higher nociceptive threshold observed in A_{2A} knock-out mice has been suggested to be attributable to the peripheral lack of A_{2A} receptors (Ledent et al., 1997). Accordingly, the changes in δ and κ opioid receptor-mediated antinociception and binding in the superficial layers of the spinal cord of −/− mice might be the result of the lack of A_{2A} receptors

in sensory nerve terminals. Activation of μ, δ, and κ opioid receptors located on the synaptic terminals of primary afferent fibers has been proposed as the basis for the depressive effects of opioid agonists on pronociceptive information coming from the peripheral nerve terminals (Besson and Chaouch, 1987). Thus it seems possible that the changes in δ and κ opioid-mediated antinociception and binding in A_{2A} knock-out mice are caused by changes occurring in peripheral pain transmission as a consequence of lack of A_{2A} receptors at sensory terminals. Because A_{2A} receptors are strictly localized solely in the striatum of mouse brain and are not expressed in any major pain circuitry, it is highly unlikely that the lack of supraspinal A_{2A} receptors could be the cause of the changes in the spinal opioid receptors and opioid-mediated antinociception in $-/-$ animals. Why the changes in δ and κ receptor-mediated antinociception and binding are in opposition and why no change in μ receptor-mediated antinociception and binding was observed in $-/-$ mice are not clear. However, it implies the presence of different spinal mechanisms of opioid receptor subtypes in modulating peripheral pain. Nonetheless, this study strongly suggests an interaction between peripheral A_{2A} receptors and spinal δ and κ receptors but not μ receptors in regulating pain.

To check whether the changes observed in the opioid-mediated antinociception experiments were actually caused by opioid-mediated antinociception and not changes in basal pain sensitivity in $-/-$ mice, a comparison of basal nociceptive latencies was performed between genotypes. The significant increase in tail withdrawal latency in $-/-$ mice when they are exposed to 55°C water, the temperature used for morphine-mediated antinociception, is in agreement with the findings of Ledent et al. (1997) and the suggestion that peripheral A_{2A} receptors enhance pain. However, it is known that blood flow influences nociceptive thresholds. A_{2A} receptor knock-out mice have been shown to have high blood pressure (Ledent et al., 1997), which could reduce the skin temperature of the tail (Coupar and Tran, 2002) and lead to apparent antinociception (Hole and Tjolsen, 1993). Indeed, no significant change in basal nociceptive threshold was observed when $+/+$ and $-/-$ mice were tested with the tail pressure test, a nociceptive response that would not be influenced by blood flow. In any case, the differences in δ-and κ-mediated antinociception shown in this paper could not be attributed to differences in blood flow or tail temperature because there was no significant difference in basal tail immersion latencies between genotypes when tested at 52°C. The lack of significant difference in nociceptive threshold between genotypes in the response to a 52°C stimulus could be caused by the reduced importance of peripheral A_{2A} receptors at the lower temperature or to the involvement of supraspinal circuits, which are absent when the stimulus is 55°C (Ossipov et al., 1995).

In conclusion, deletion of the A_{2A} receptor gene causes changes in δ and κ receptor-mediated antinociception and binding in the spinal cord but not in the brain of mutant mice. This supports a functional interaction between the spinal opioid and the peripheral adenosine system in control of pain pathways.

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