



# Absence of G-protein activation by $\mu$ -opioid receptor agonists in the spinal cord of $\mu$ -opioid receptor knockout mice

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**1** The ability of  $\mu$ -opioid receptor agonists to activate G-proteins in the spinal cord of  $\mu$ -opioid receptor knockout mice was examined by monitoring the binding to membranes of the non-hydrolyzable analogue of GTP, guanosine-5'-O-(3-[<sup>35</sup>S]thio)triphosphate ([<sup>35</sup>S]GTP $\gamma$ S).

**2** In the receptor binding study, Scatchard analysis of [<sup>3</sup>H][D-Ala<sup>2</sup>,NHPhe<sup>4</sup>,Gly-ol]enkephalin ([<sup>3</sup>H]DAMGO;  $\mu$ -opioid receptor ligand) binding revealed that the heterozygous  $\mu$ -knockout mice displayed approximately 40% reduction in the number of  $\mu$ -receptors as compared to the wild-type mice. The homozygous  $\mu$ -knockout mice showed no detectable  $\mu$ -binding sites.

**3** The newly isolated  $\mu$ -opioid peptides endomorphin-1 and -2, the synthetic selective  $\mu$ -opioid receptor agonist DAMGO and the prototype of  $\mu$ -opioid receptor agonist morphine each produced concentration-dependent increases in [<sup>35</sup>S]GTP $\gamma$ S binding in wild-type mice. This stimulation was reduced by 55–70% of the wild-type level in heterozygous, and virtually eliminated in homozygous knockout mice.

**4** No differences in the [<sup>35</sup>S]GTP $\gamma$ S binding stimulated by specific  $\delta_1$ - ([D-Pen<sup>2,5</sup>]enkephalin),  $\delta_2$ - ([D-Ala<sup>2</sup>]deltorphin II) or  $\kappa_1$ - (U50,488H) opioid receptor agonists were noted in mice of any of the three genotypes.

**5** The data clearly indicate that  $\mu$ -opioid receptor gene products play a key role in G-protein activation by endomorphins, DAMGO and morphine in the mouse spinal cord. They support the idea that  $\mu$ -opioid receptor densities could be rate-limiting steps in the G-protein activation by  $\mu$ -opioid receptor agonists in the spinal cord. These thus indicate a limited physiological  $\mu$ -receptor reserve. Furthermore, little change in  $\delta_1$ -,  $\delta_2$ - or  $\kappa_1$ -opioid receptor-G-protein complex appears to accompany  $\mu$ -opioid receptor gene deletions in this region.

**Keywords:** knockout mice; endomorphins; G-proteins; homologous recombination; opioid peptides;  $\mu$ -opioid receptors; signal transduction; spinal cord

**Abbreviations:** DAMGO, [D-Ala<sup>2</sup>,NHPhe<sup>4</sup>,Gly-ol]enkephalin; DPDPE, [D-Pen<sup>2,5</sup>]enkephalin; GDP, guanosine-5'-O-(2-thio)-diphosphate; GTP $\gamma$ S, guanylyl-5'-O-( $\gamma$ -thio)-triphosphate; U50,488H, trans( $\pm$ )-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]; benzeneacetamide

## Introduction

The  $\mu$ -opioid receptor is expressed by neurons in several central nervous system regions, including the spinal cord dorsal horn. Its occupancy with agonist drugs such as morphine and agonist peptides produces many of the effects of opiate drugs, including much of the profound analgesic response attributed to the receptors localized in the spinal cord. Many synthetic opioids and derivatives of opium have reasonably-good affinities and selectivities for this receptor. Morphine and [D-Ala<sup>2</sup>,NHPhe<sup>4</sup>,Gly-ol]enkephalin (DAMGO) are useful ligands for probing  $\mu$ -opioid receptor function. However, selective endogenous ligands for this receptor had not been clearly defined until recent successes of Zadina and colleagues (1997) who isolated two high-affinity  $\mu$ -opioid receptor selective peptides ligands, endomorphin-1 (Tyr-Pro-Trp-Phe-NH<sub>2</sub>) and -2 (Tyr-Pro-Phe-Phe-NH<sub>2</sub>), from mammalian brain.

Recent cloning and expression studies have revealed that the  $\mu$ -opioid receptor, and the related  $\delta$ - and  $\kappa$ -opioid receptors

are seven-transmembrane domain receptors whose actions are mediated through activation of heterotrimeric guanine nucleotide binding proteins (G-proteins) of several classes, including Gi- and Go-proteins (Chen *et al.*, 1993; Law, 1995). The activation of G-proteins by the  $\mu$ -opioid receptor can be measured by assessing agonist stimulation of membrane binding of the non-hydrolyzable analogue of GTP, guanosine-5'-O-(3-[<sup>35</sup>S]thio)triphosphate ([<sup>35</sup>S]GTP $\gamma$ S) (Traynor & Nahorski, 1995; Sim *et al.*, 1995, 1996; Selley *et al.*, 1997; Shimohira *et al.*, 1997; Narita & Tseng, 1998). [<sup>35</sup>S]GTP $\gamma$ S addition results in accumulation of a stable G $\alpha$ -[<sup>35</sup>S]GTP $\gamma$ S complex in brain membranes. It has been recently reported that endomorphins increased [<sup>35</sup>S]GTP $\gamma$ S binding in rat brain (Sim *et al.*, 1998), mouse spinal cord (Narita *et al.*, 1998), SH-SY5Y human neuroblastoma cells (Harrison *et al.*, 1998) and C<sub>6</sub> rat glioma cells stably expressing a cloned rat  $\mu$  receptor (Alt *et al.*, 1998).  $\mu$ -Opioid agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in isolated membranes thus provide a functional measurement of agonist occupation of  $\mu$ -opioid receptors and its efficacy in leading to activation of G-proteins.

Knockout mice with  $\mu$ -opioid receptor gene deletions have been successfully developed by homologous recombination

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(Matthes *et al.*, 1996; Sora *et al.*, 1997b). These mice display profound gene-dose-dependent reductions in morphine analgesia in tests including tests of 'spinal' analgesia. The availability of transgenic  $\mu$ -opioid receptor knockout mice allows us to determine the extent to which the  $\mu$ -opioid receptor gene products are necessary for the expression of physiological actions by these newly discovered opioid peptides and alkaloids, including G-protein activation. The present study was thus designed to investigate the effects of  $\mu$ -opioid receptor agonists including newly isolated opioid peptides endomorphin-1 and -2 on [ $^{35}$ S]GTP $\gamma$ S binding to spinal cord membranes obtained from mice expressing normal (wild-type), half-normal (heterozygous), and absent (homozygous)  $\mu$ -opioid receptor complements. We discuss the data in light of the concepts of receptor reserve for  $\mu$ -opioid receptors and the possible compensatory changes in the functions of other opioid receptor types in the presence of  $\mu$ -opioid receptor gene deletions in this region.

## Methods

### Animals

$\mu$ -Opioid receptor knockout transgenic mice were maintained on C57BL/6 and 129Sv mixed genetic backgrounds as described (Sora *et al.*, 1997a,b). Animals were housed five per cage in a room maintained at  $22 \pm 0.5^\circ\text{C}$  with an alternating 12 h light-dark cycle. Food and water were available *ad libitum*.

### Membrane preparation

The spinal cord was removed from wild-type, and heterozygous and homozygous  $\mu$ -opioid receptor knockout mice after decapitation. The spinal cord was homogenized in 20 volumes of ice-cold Tris buffer containing Tris-HCl (pH 7.4) (50 mM) for the  $\mu$ -opioid receptor binding assay or ice-cold Tris-Mg $^{2+}$  buffer containing Tris-HCl (pH 7.4) 50 mM, MgCl $_2$

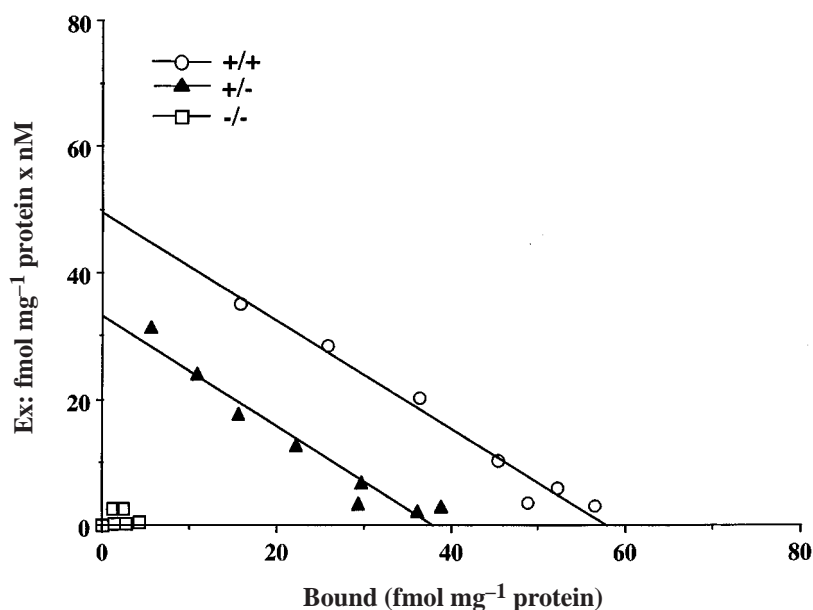
5 mM, and EGTA 1 mM for the [ $^{35}$ S]GTP $\gamma$ S binding assay. The homogenate was centrifuged at  $48,000 \times g$  at  $4^\circ\text{C}$  for 10 min. The pellets were resuspended in Tris buffer or [ $^{35}$ S]GTP $\gamma$ S binding assay buffer containing Tris-HCl (pH 7.4) 50 mM, MgCl $_2$  5 mM, EGTA 1 mM, and NaCl 100 mM and recentrifuged at  $48,000 \times g$  at  $4^\circ\text{C}$  for 10 min. The final pellets were resuspended in each assay buffer and stored at  $-70^\circ\text{C}$  until experiments.

### $\mu$ -Opioid receptor binding assay

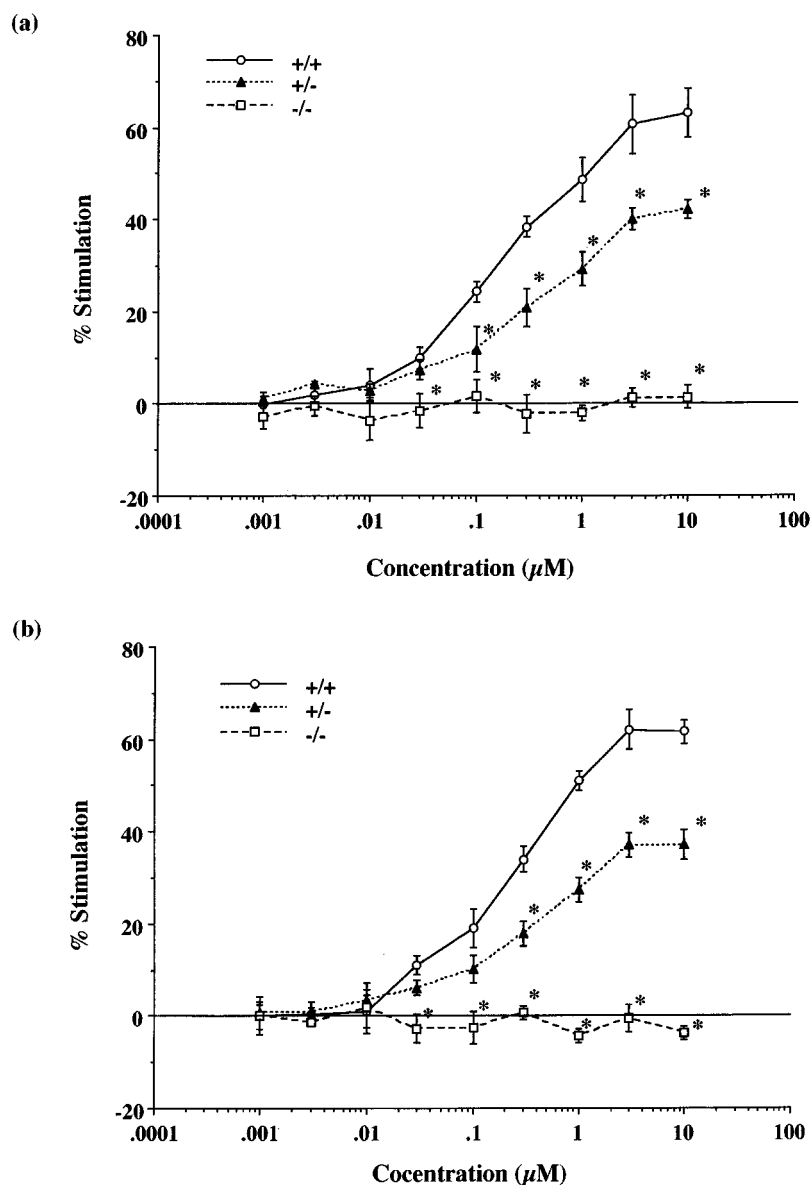
The  $\mu$ -opioid receptor binding assays were carried out in duplicate with [tyrosyl-3,5- $^3\text{H}$ (N)]-DAMGO ([ $^3\text{H}$ ]DAMGO; 55.3 Ci mmol $^{-1}$ ; NEN, Boston, MA, U.S.A.) at 0.2–20 nM in a final volume of 1.0 ml which contained Tris-HCl (pH 7.4) buffer (50 mM) and 0.1 ml of the homogenated membrane fraction. The amount of membrane protein used in each assay was in the range of 120–170  $\mu\text{g}$ , as determined by the method of Lowry *et al.* (1951). The test tubes were incubated for 120 min at  $25^\circ\text{C}$ . The specific binding was defined as the difference in binding observed in the absence and presence of  $10^{-5}$  M unlabelled naloxone. The incubations were terminated by collecting the membranes on Whatman GF/B filters using a Brandel cell harvester (Model M-24, Brandel, MD, U.S.A.). The filters were then washed three times with 5 ml Tris-HCl buffer (pH 7.4) at  $4^\circ\text{C}$  and transferred to scintillation vials. Then, 0.5 ml of Soluene-350 (Packard Instrument Company, Inc., Meriden, CT, U.S.A.) and 4 ml of Hionic Fluor Cocktail (Packard Instrument Company) were added to the vials. After a 12 h equilibration period, the radioactivity in the samples was determined in liquid scintillation analyzer (Model 1600 CA, Packard Instrument Company). Values for Scatchard analysis represent the means  $\pm$  s.e.mean of three independent determinations.

### [ $^{35}$ S]GTP $\gamma$ S binding assay

The reaction was initiated by the addition of membrane suspension (30–80  $\mu\text{g ml}^{-1}$  of membrane proteins) into the



**Figure 1** Scatchard analysis of [ $^3\text{H}$ ]DAMGO binding to spinal cord membranes obtained from wild-type (+/+), heterozygous (+/-) and homozygous (-/-)  $\mu$ -opioid receptor knockout mice. Membranes were incubated with [ $^3\text{H}$ ]DAMGO at 0.2–20 nM in a final volume of 1.0 ml which contained Tris-HCl (pH 7.4) 50 mM buffer and 0.1 ml of the homogenated membrane fraction (120–170  $\mu\text{g}$ ) for 120 min at  $25^\circ\text{C}$ . The specific binding was defined as the difference in binding observed in the absence and presence of  $10^{-5}$  M unlabelled naloxone. A representative experiment that was replicated three times is shown.



**Figure 2** Comparison of the stimulation of  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  binding to spinal cord membranes of wild-type (+/+), and heterozygous (+/-) and homozygous (-/-)  $\mu$ -opioid receptor knockout mice by endomorphin-1 (a) and -2 (b). Membranes were incubated with  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  (50 pM) and GDP (30  $\mu\text{M}$ ) with and without various concentrations of endomorphin-1 or -2 for 120 min at 25°C. The data are expressed as the percentage of basal  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  (50 pM) binding measured in the presence of GDP and absence of agonist, and represent the means  $\pm$  s.e.mean from at least three separate experiments. \* $P < 0.05$  vs wild-type mice.

assay buffer with the opioid receptor agonists, GDP 30  $\mu\text{M}$  and  $[^{35}\text{S}]\text{GTP}\gamma\text{S}$  (50 pM) (1000 Ci  $\text{mmol}^{-1}$ ; Amersham, Arlington Heights, IL, U.S.A.). The suspensions were incubated at 25°C for 120 min and the reaction was terminated by filtering through Whatman GF/B glass filters using a Brandel cell harvester. The filters were then washed three times with 5 ml of Tris-HCl buffer (pH 7.4) and transferred to scintillation counting vials containing scintillation cocktail (0.5 ml of Soluene-350 and 4 ml of Hionic Fluor Cocktail). The radioactivity in the samples was determined with a liquid scintillation analyzer. Non-specific binding was measured in the presence of 10  $\mu\text{M}$  unlabelled  $\text{GTP}\gamma\text{S}$ .

#### Drugs

The drugs used in the present studies were: Tyr-Pro-Trp-Phe-NH<sub>2</sub> (endomorphin-1, Tocris Cookson Inc., Ballwin, MO, U.S.A.); Tyr-Pro-Phe-Phe-NH<sub>2</sub> (endomorphin-2, Tocris Cookson Inc.); [D-Ala<sup>2</sup>,NHPh<sup>4</sup>,Gly-ol]enkephalin (DAMGO,

Bachem California, Torrance, CA, U.S.A.); morphine (Mallinckrodt Chemical Works, St. Louis, MO, U.S.A.), [D-Pen<sup>2,5</sup>]enkephalin (Bachem California), Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH<sub>2</sub> ([D-Ala<sup>2</sup>] deltorphin II, Molecular Research Laboratories, Durham, NC); trans( $\pm$ )-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidiny)-cyclohexyl] benzeneacetamide (U50, 488H, Research Biochemicals International, Natick, MA, U.S.A.); naloxone (Research Biochemicals International); guanylyl-5'-*O*-( $\gamma$ -thio)-triphosphate ( $\text{GTP}\gamma\text{S}$ , Research Biochemicals International); and guanosine-5'-*O*-(2-thio)-diphosphate (GDP, Sigma Chemical Company, St. Louis, MO, U.S.A.).

#### Statistical analysis

The data were expressed as means  $\pm$  s.e.mean. The binding data for the determination of the density ( $B_{\text{max}}$ ) and affinity ( $K_d$ ) of binding sites were evaluated by a computer-assisted analysis, ED<sub>01</sub> and LIGAND (Biosoft, Cambridge, U.K.).

Student's *t*-test was used for the statistical analysis of  $B_{\max}$  and  $K_d$  values. Comparisons of all other data were performed *via* ANOVA followed by Newman-Keuls' test (Tallarida & Murray, 1987).

## Results

The spinal cord membranes obtained from wild-type, heterozygous and homozygous  $\mu$ -opioid receptor knockout mice were incubated with a range of [ $^3$ H]DAMGO concentrations (0.2–20 nM) for 2 h at 25°C. Figure 1 illustrates Scatchard plots for three genotypes. A mean  $B_{\max}$  value of  $61.6 \pm 4.9$  fmol  $\text{mg}^{-1}$  protein with a  $K_d$  of  $1.1 \pm 0.1$  nM was found in the wild-type mice. The membranes obtained from heterozygous  $\mu$ -opioid receptor knockout mice displayed a 41.3% reduction in the number of [ $^3$ H]DAMGO binding sites ( $36.1 \pm 3.6$  fmol  $\text{mg}^{-1}$  protein;  $P < 0.01$ , as compared to the wild-type mice) with no change in affinity ( $1.1 \pm 0.1$  nM). The membranes obtained from homozygous  $\mu$ -opioid receptor knockout mice showed no detectable [ $^3$ H]DAMGO binding sites.

Both endomorphins-1 and -2 (0.001–10  $\mu\text{M}$ ) produced concentration-dependent increases in [ $^{35}$ S]GTP $\gamma$ S binding to spinal cord membranes obtained from wild-type mice (Figure 2a and b). Maximal stimulation was  $63.1 \pm 5.3$  and  $61.6 \pm 2.5\%$ , respectively, using 10  $\mu\text{M}$  peptide concentrations. As shown in Figure 3, the synthetic selective  $\mu$ -opioid receptor agonist DAMGO and the prototype of  $\mu$ -opioid receptor agonist morphine also stimulated [ $^{35}$ S]GTP $\gamma$ S binding in a concentration-dependent manner and produced a maximal stimulation of  $94.4 \pm 9.6$  and  $65.1 \pm 1.3\%$ , respectively, at 10  $\mu\text{M}$ .

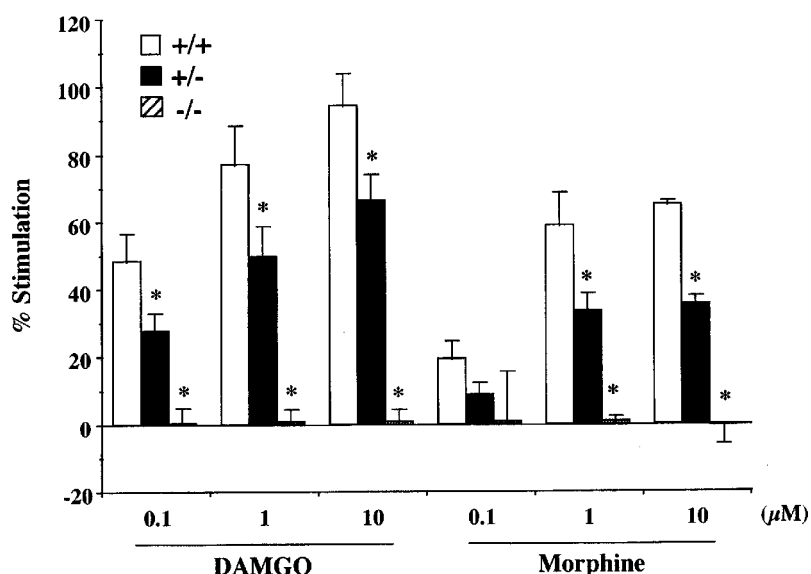
In heterozygous  $\mu$ -opioid receptor knockout mice, [ $^{35}$ S]GTP $\gamma$ S binding stimulated by either endomorphin-1, -2, DAMGO or morphine was markedly decreased to 67, 60, 70 or 55% of the stimulation observed in wild-type mice, respectively. In homozygous mice, no stimulation of [ $^{35}$ S]GTP $\gamma$ S binding stimulated by either endomorphin-1, -2, DAMGO or morphine could be detected (Figures 2A, B and 3).

No significant changes in  $\delta_1$ -,  $\delta_2$ - or  $\kappa_1$ -opioid receptor functions were noted in  $\mu$ -opioid receptor knockout mice. The  $\delta_1$ -opioid receptor agonist [D-Pen $^{2,5}$ ]enkephalin (DPDPE), the  $\delta_2$ -opioid receptor agonist [D-Ala $^2$ ]deltorphin II and the  $\kappa_1$ -opioid receptor agonist U-50,488H both produced robust stimulation of [ $^{35}$ S]GTP $\gamma$ S binding. As shown in Figure 4, DPDPE, [D-Ala $^2$ ]deltorphin II and U50,488H (10  $\mu\text{M}$ ) produced  $17.6 \pm 2.5$ ,  $31.6 \pm 8.6$  and  $7.3 \pm 5.9\%$  stimulation, respectively, in wild-type mice. The levels of [ $^{35}$ S]GTP $\gamma$ S binding stimulated by DPDPE, [D-Ala $^2$ ]deltorphin II or U50,488H in both heterozygous and homozygous  $\mu$ -opioid receptor knockout mice were similar to those found in wild-type mice.

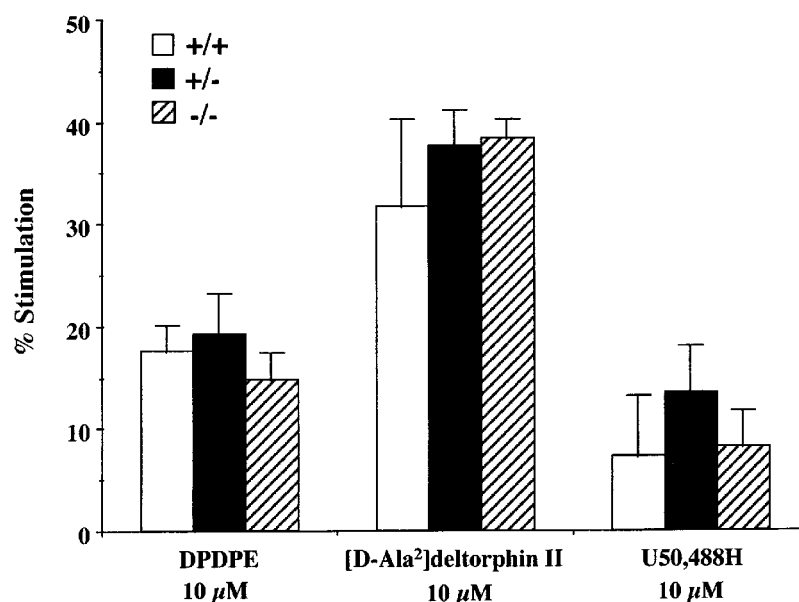
## Discussion

The knockout mice used in the present study display gene dose-dependent reductions in the levels of  $\mu$ -opioid receptor expression in the spinal cord. Heterozygous knockout animals manifest 40–50% reductions in  $\mu$ -opioid receptor densities, whereas homozygous knockout mice display no detectable  $\mu$ -opioid receptor binding or immunoreactivity (this study; Sora *et al.*, 1997b). Analyses of these animals provides direct evidence for  $\mu$ -opioid receptor involvement in the expression of physiological actions by  $\mu$ -opioid receptor agonists. The data from these studies documents that disruption of the mouse  $\mu$ -opioid receptor gene ablates the G-protein activation in the mouse spinal cord by endomorphin-1 and -2 found in wild-type mice. Elimination of  $\mu$ -opioid receptor expression in mutant mice virtually abolished both DAMGO- and morphine-stimulated [ $^{35}$ S]GTP $\gamma$ S binding. This agrees with our previous findings that  $\mu$ -opioid receptor agonist-stimulated [ $^{35}$ S]GTP $\gamma$ S binding was completely blocked by  $\mu$ -opioid receptor antagonists (Narita & Tseng, 1998). These findings clearly indicate that  $\mu$ -opioid receptor gene products are the molecular target of endomorphins, DAMGO and morphine relevant to activation of G-proteins in the mouse spinal cord.

Regional differences in the relationships between  $\mu$ -opioid receptor densities and pharmacological efficacies produced by



**Figure 3** Effect of DAMGO and morphine on [ $^{35}$ S]GTP $\gamma$ S binding to spinal cord membranes of wild-type (+/+), and heterozygous (+/-) and homozygous (-/-)  $\mu$ -opioid receptor knockout mice. Membranes were incubated with [ $^{35}$ S]GTP $\gamma$ S (50 pM) and GDP (30  $\mu\text{M}$ ) with and without different concentrations of DAMGO or morphine. The data are expressed as the percentage of basal [ $^{35}$ S]GTP $\gamma$ S (50 pM) binding measured in the presence of GDP and absence of agonist, and represent the means  $\pm$  s.e. mean from at least three separate experiments. \* $P < 0.05$  vs wild-type mice.



**Figure 4** No differences in the [<sup>35</sup>S]GTP $\gamma$ S binding stimulated by specific  $\delta_1$ - (DPDPE),  $\delta_2$ - ([D-Ala<sup>2</sup>]deltorphin II) or  $\kappa_1$ - (U50,488H) opioid receptor agonists in the  $\mu$ -opioid receptor knockout mice. The spinal cord membranes obtained from wild-type (+/+), and heterozygous (+/-) and homozygous (-/-)  $\mu$ -opioid receptor knockout mice were incubated with [<sup>35</sup>S]GTP $\gamma$ S (50 pM) and GDP (30  $\mu$ M) with and without opioid agonists. The data are expressed as the percentage of basal [<sup>35</sup>S]GTP $\gamma$ S (50 pM) binding measured in the presence of GDP and absence of agonist, and represent the means  $\pm$  s.e.mean from at least three separate experiments.

$\mu$ -opioid receptor agonists could result from different patterns of the  $\mu$ -opioid receptor reserve (Chavkin & Goldstein, 1984). Studies using heterozygous mice with half of the normal, wild-type  $\mu$ -opioid receptor densities can provide information about physiologic  $\mu$ -opioid receptor reserves. Previous analgesic tests in knockout mice suggest that even 50% reductions in spinal  $\mu$ -opioid receptor densities alter baseline nociception and agonist-induced changes in nociceptive responses in the tail-flick assay, which largely reflects spinal antinociceptive influences (Sora *et al.*, 1997b). In the present study,  $\mu$ -opioid receptor agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding was reduced by 55–70% in heterozygous mice, and virtually eliminated in homozygous knockout mice. Both this behavioural result and the current biochemical data thus support the idea that  $\mu$ -opioid receptors are rate-limiting steps in the biochemical and physiological spinal cord pathways stimulated by endogenous and exogenous  $\mu$ -opioid receptor agonists. Both lines of evidence indicate a limited physiological ' $\mu$ -opioid receptor reserve' in these spinal cord mechanisms.

It is possible that gene deletion strategies would produce compensatory changes in other receptor types associated with the deleted receptor. Quantitative autoradiography has revealed trends toward changes (10–15%) for  $\delta$ - and  $\kappa$ -opioid receptor binding in some brain regions of  $\mu$ -opioid receptor knockout mice (Kitchen *et al.*, 1997). In the present study, however, we did not find any significant changes in  $\delta_1$ -,  $\delta_2$ - or  $\kappa_1$ -opioid receptor agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding to

spinal cord membranes in  $\mu$ -opioid receptor knockout mice compared to wild-type mice. Matthes *et al.* (1998) have recently reported that mice lacking  $\mu$ -opioid receptors do not display significant changes in either  $\delta_1$ -,  $\delta_2$ - or  $\kappa_1$ -opioid receptor agonist-activated G-proteins in the brain. These findings suggest that functional coupling of  $\delta_1$ -,  $\delta_2$ - and  $\kappa_1$ -opioid receptors to G-protein appears to be preserved in the process of  $\mu$ -opioid receptor deletions. However, it should be also noted that some of antinociceptive actions of  $\delta_1$ - and  $\delta_2$ - agonists when given intracerebroventricularly are less effective in  $\mu$ -opioid receptor knockout mice than in wild-type mice (Sora *et al.*, 1997a; Matthes *et al.*, 1998). It is therefore worthwhile to examine more closely whether there could be physiological  $\mu$ -receptor dependence of  $\delta$ -receptor actions at the spinal cord level.

In conclusion, we have demonstrated profound gene-dose-dependent reductions in G-protein activation by  $\mu$ -opioid receptor agonists including newly isolated peptides endomorphins in the mouse spinal cord. Furthermore, the preservation of  $\delta_1$ -,  $\delta_2$ - and  $\kappa_1$ -opioid receptor signalling properties in the process of  $\mu$ -opioid receptor deletions provides no evidence for cross-talk among opioid receptors at the cellular level.

This work was supported by U.S. Public Health Service Grant DA 03811 from the National Institute on Drug Abuse (NIDA), National Institutes of Health and the Intramural Research Program, NIDA.

## References

- ALT, A., MANSOUR, A., AKIL, H., MEDZIHRADESKY, F., TRAYNOR, J.R. & WOODS, J.H. (1998). Stimulation of guanosine-5'-O-(3-[<sup>35</sup>S]thio)triphosphate binding by endogenous opioids acting at a cloned mu receptor. *J. Pharmacol. Exp. Ther.*, **286**, 282–288.
- CHAVKIN, C. & GOLDSTEIN, A. (1984). Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus. *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 7253–7257.
- CHEN, Y., MESTEK, A., LIU, J., HURLEY, J.A. & YU, L. (1993). Molecular cloning and functional expression of a  $\mu$ -opioid receptor from the rat brain. *Mol. Pharmacol.*, **44**, 8–12.
- HARRISON, L.M., KASTIN, A.J. & ZADINA, J.E. (1998). Differential effects of endomorphin-1, endomorphin-2, and Tyr-W-MIF-1 on activation of G-proteins in SH-SY5Y human neuroblastoma membranes. *Peptides*, **19**, 749–753.

- KITCHEN, I., SLOWE, S.J., MATTHES, H.W.-D. & KIEFFER, B. (1997). Quantitative autoradiographic mapping of  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors in knockout mice lacking the  $\mu$ -opioid receptor gene. *Brain Res.*, **778**, 73–88.
- LAW, P.-Y. (1995). G-proteins and opioid receptors' function. In *The Pharmacology of Opioid Peptides*, ed. Tseng, L.F. Amsterdam: Harwood Academic Publishers, pp.109–130.
- LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.
- MATTHES, H.W.D., MALDONADO, R., SIMONIN, F., VALVERDE, O., SLOWE, S., KITCHEN, I., BEFORT, K., DIERICH, A., LE, M.M., DOLLE, P., TZAVARA, E., HANOUNE, J., ROQUES, B.P. & KIEFFER, B.L. (1996). Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the  $\mu$ -opioid-receptor gene. *Nature*, **383**, 819–823.
- MATTHES, H.W.D., SMADJA, C., VALVERDE, O., VONESCH, J.-L., FOUTZ, A.S., BOUDINOT, E., DENAVIT-SAUBIÉ, M., SEVERINI, C., NEGRI, L., ROQUES, B.P., MALDONADO, R. & KIEFFER, B.L. (1998). Activity of the  $\delta$ -opioid receptor is partially reduced, whereas activity of the  $\kappa$ -receptor is maintained in mice lacking the  $\mu$ -receptor. *J. Neuroscience*, **18**, 7285–7295.
- NARITA, M., MIZOGUCHI, H., OJI, G.S., TSENG, E.L., SUGANUMA, C., NAGASE, H. & TSENG, L.F. (1998). Characterization of endomorphin-1 and -2 on [<sup>35</sup>S]GTP $\gamma$ S binding in the mouse spinal cord. *Eur. J. Pharmacol.*, **351**, 383–387.
- NARITA, M. & TSENG, L.F. (1998). Evidence for the existence of the  $\beta$ -endorphin-sensitive ' $\epsilon$ -opioid receptor' in the brain: the mechanisms of  $\epsilon$ -mediated antinociception. *Jpn. J. Pharmacol.*, **76**, 233–253.
- SELLY, D.E., SIM, L.J., XIAO, R., LIU, QIXU & CHILDERS, S.R. (1997).  $\mu$ -Opioid receptor-stimulated guanosin-5'-O-( $\gamma$ -thio)-triphosphate binding in rat thalamia and cultured cell lines: Signal transduction mechanisms underlying agonist efficacy. *Mol. Pharmacol.*, **51**, 87–96.
- SIM, L.J., LIU, Q., CHILDERS, S.R. & SELLEY, D.E. (1998). Endomorphin-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding in rat brain: Evidence for partial agonist activity at  $\mu$ -opioid receptors. *J. Neurochem.*, **70**, 1567–1576.
- SIM, L.J., SELLY, D.E. & CHILDERS, S.R. (1995). In vitro autoradiography of receptor-activated G-proteins in rat brain by agonist-stimulated guanylyl 5'-[<sup>35</sup>S]thio]-triphosphate binding. *Proc. Natl. Acad. Sci. USA*, **92**, 7242–7246.
- SIM, J.S., SELLY, D.E., XIAO, R. & CHILDERS, S.R. (1996). Differences in G-protein activation by  $\mu$ - and  $\delta$ -opioid, and cannabinoid, receptors in rat striatum. *Eur. J. Pharmacol.*, **307**, 97–105.
- SHIMOHIRA, I., TOKUYAMA, S., HIMENO, A., NIWA, M. & UEDA, H. (1997). Characterization of nociceptin-stimulated in site [<sup>35</sup>S]GTP $\gamma$ S binding in comparison with opioid agonist-stimulated ones in brain regions of the mice. *Neurosci. Lett.*, **237**, 113–116.
- SORA, I., FUNADA, M. & UHL, G.R. (1997a). The  $\mu$ -opioid receptor is necessary for [D-Pen<sup>2</sup>,D-Pen<sup>5</sup>]enkephalin-induced analgesia. *Eur. J. Pharmacol.*, **324**, R1–R2.
- SORA, I., TAKAHASHI, N., FUNADA, M., UJIKE, H., REVAY, R.S., DONOVAN, D.M., MINER, L.L. & UHL, G.R. (1997b). Opiate receptor knockout mice define  $\mu$  receptor roles in endogenous nociceptive responses and morphine-induced analgesia. *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 1544–1549.
- TALLARIDA, R.J. & MURRAY, R.B. (1987). *Manual of Pharmacological Calculation with Computer Programs*. New York: Springer.
- TRAYNOR, J.R. & NAHORSKI, S.R. (1995). Modulation by  $\mu$ -opioid agonists of guanosine-5'-O-(3-[<sup>35</sup>S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol. Pharmacol.*, **47**, 848–854.
- ZADINA, J., HACKLER, L., GE, L.-J. & KASTIN, A.J. (1997). A potent and selective endogenous agonist for the  $\mu$ -opiate receptor. *Nature*, **386**, 499–502.

(Received September 28, 1998

Revised October 27, 1998

Accepted November 3, 1998)