Overexpression of Type 7 Adenylyl Cyclase in the Mouse Brain Enhances Acute and Chronic Actions of Morphine

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ABSTRACT

The mechanisms by which morphine-induced analgesia and tolerance and physical dependence on morphine arise have been the subject of intense study, and much work has pointed to the involvement of cAMP-mediated events in the neuroadaptive phenomena leading to morphine tolerance and/or dependence. We overexpressed an opioid receptor-stimulatable form of adenylyl cyclase (type 7) in the central nervous system of mice and demonstrated significant effects of this manipulation on the animals' acute response to morphine, the development of morphine tolerance, and development of sensitization to morphine. Measurements of the acute analgesic response to morphine demonstrated that the ED_{50} values for the transgenic mice were significantly lower than the ED_{50} values determined for the "wild-type" animals. During chronic treatment with morphine, the transgenic mice developed tolerance more rapidly than the wild-type mice, and transgenic animals of the C57BL/6xSJL background showed a larger sensitization to morphine's effects on locomotor activity than did wild-type mice of the same background. These results indicated that cAMP-generating systems may simultaneously modulate the development of tolerance and sensitization. Interestingly, the signs of physical dependence on morphine in the transgenic mice did not differ from those in their wild-type litter mates, indicating that separate mechanisms may modulate opiate tolerance and opiate dependence.

Adenylyl cyclases (ACs) constitute a family of enzymes that convert ATP to the intracellular second messenger cAMP. Nine ACs have been cloned and characterized to date, and each isoform has a particular regulatory characteristic that distinguishes it from the others. Particularly relevant to this report is the fact that various members of the AC family respond quite differently to receptor-mediated activation of the G_i/G_o-proteins, such as that produced through opioid receptors. Whereas the activity of type 1, 5, and 6 ACs is inhibited by the $G_i\alpha$-subunit, the activity of type 2 and 7 ACs has been shown to be insensitive to $G_i\alpha$. However, type 2 and 7 ACs are stimulated by the $\beta\gamma$-subunits of the G_i/G_o-proteins when these enzymes are coordinately activated by G_S (Lustig et al., 1993; Yoshimura et al., 1996).

Much evidence has been presented during the last 25 years to indicate that morphine-induced analgesia is related to opiate-induced lowering of cellular cAMP levels (Collier and Roy, 1974; Duman et al., 1988; Harrison et al., 1998), although opiate effects on the function of voltage-gated calcium and potassium channels have also been considered as medi-

ators of the analgesic response to opiates (Christie, 1991). The development of tolerance to and dependence on the opiates has been proposed to involve an adaptive response (upregulation) of the AC signal transduction system, including quantitative changes in AC (Collier and Tucker, 1984; Matsuoka et al., 1994; Avidor-Reiss et al., 1995, 1997; Wang and Gintzler, 1995; Chakrabarti et al., 1998) and changes in downstream signaling elements such as protein kinase A and cAMP response element-binding protein (CREB; Terwilliger et al., 1991; Guitart et al., 1992; Nestler et al., 1994). A recent variant of this hypothesis is that chronic exposure of a tissue to morphine produces a substitution of the opiate-inhibitable forms of ACs with the opiate-stimulatable forms of the ACs (Wang and Gintzler, 1995). To further assess the role of the AC system in the actions of opiates, we generated transgenic (TG) mice that overexpress type 7 AC in brain and determined their responses to acute and chronic administration of morphine.

Materials and Methods

Mice. The transgene was constructed from a 3.6-kb *Bam*HI/*Xba*I fragment of the human AC7 cDNA (Hellevuo et al., 1993; Nomura et al., 1994; the *Bam*HI site was created 5′ upstream of the coding sequence by in vitro mutagenesis), a 650-bp *Kpn*I/*Rsa*I fragment of

ABBREVIATIONS: AC, adenylyl cyclase; AC7, type VII adenylyl cyclase; TG, transgenic; WT, wild type; DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin; CREB, cAMP response element-binding protein; VIP, vasoactive intestinal peptide; CNS, central nervous system.

the human growth hormone gene (Andersson et al., 1989; the *Kpn*I and *Rsa*I sites were converted to *Xba*I and *Not*I sites, respectively, with specific linkers), and a 4.5-kb *Sal*I/*Xho*I fragment of the rat synapsin I gene (Hoesche et al., 1993; the *Xho*I site was converted to a *Bam*HI site; Fig. 1a). FVB/N mice and hybrids (F$_2$) of C57BL/6 and SJL mice were used as hosts for the transgene. The FVB/N TGs were bred with FVB/N mice, and the C57BL/6xSJL TGs were backcrossed to C57BL/6 mice. For Southern blotting, *Bam*HI-digested genomic DNA was probed with an 876-bp *Sac*I fragment from the 3′-region of the human AC7 cDNA. For the RNase protection assays, a 269-bp fragment from the coding region of human AC7 cDNA (position 3050 to 3318) (Nomura et al., 1994) and a 200-bp fragment from the 3′-noncoding region of mouse AC7 cDNA (position 4301 to 4500) (Watson et al., 1994) were used as probes.

Measurement of AC Activity. Membrane preparation from mouse cerebral cortex was obtained, and assays were carried out as described by Olianas and Onali (1994) with the following modifications. Membrane preparation was carried out in the presence of proteinase inhibitors: 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml antipain, 20 μg/ml soybean trypsin inhibitor, 2 μg/ml pepstatin A, and 0.5 mM benzamidine. The enzyme activity was assayed by measuring the conversion of [α-^{32}P]ATP to [^{32}P]cAMP as previously described (Olianas and Onali, 1994; Tabakoff et al., 1995). The 100-μl reaction mixture contained 50 mM HEPES/NaOH (pH 7.4), 2.3 mM MgCl$_2$, 1.3 mM dithiothreitol, 0.3 mM EGTA, 1 mM GTP, 1 mM 3-isobutyl-1-methylxanthine, 0.25 mM Ro20-1724, 5 mM phosphocreatine, 50 U/ml creatine phosphate, and 0.5 mg/ml bovine serum albumin. Twenty-five microliters of the membrane preparations (35–50 μg of protein) were used for the reaction. The assay was carried out at 30°C for 10 min and terminated by the addition of 150 μl of 2% SDS. cAMP was isolated as described by Salomon et al. (1974).

Behavioral Testing. All experiments were performed in compliance with the *NRC Guide for the Care and Use of Laboratory Animals* (1996) and were approved by the Institutional Animal Care and Use Committee of the University of Colorado Health Sciences Center. Successive doses of morphine were injected s.c. into mice to generate cumulative dose-response curves (Duttaroy et al., 1997). The morphine injections were spaced 32 min apart, and the doses reported in Fig. 3 represent the total amount of morphine administered before each testing period. Immediately after each morphine injection, locomotor activity was measured for 30 min (Gwynn and Domino, 1984; Moskowitz et al., 1985). Analgesia was then assessed using the hot-plate test (Marubio et al., 1999) with a cut-off of 60 s, and another dose of morphine was then administered. Data from dose-response curves for each mouse were fitted to a logistic equation, using the NFIT curve-fitting program (University of Texas, Galveston, TX). ED$_{50}$ values were calculated from these equations. When mice were treated chronically with morphine, they received s.c. injections of 5 or 10 mg/kg morphine, once daily for 4 days, starting on the day after the initial behavioral testing. On the 5th day, they were again tested for morphine-induced stimulation of locomotor activity and analgesia. On the 6th day, withdrawal was assessed by measuring jumping, rearing, and/or nociception (hyperalgesia) after naloxone injection (5 mg/kg, s.c.; Koob et al., 1992; Maldonado et al., 1996). Hyperalgesia is expressed as the difference in paw-lick latency when animals were tested before and 30 min after naloxone injection. A chronic saline group, which received four daily saline injections, but no morphine treatment, was included in the withdrawal experiments.

Results

The TG mice showed no overt, distinctive phenotype. All lines of the TG mice which we examined (nine lines) demonstrated the expression of the transgene in three areas of brain (Fig. 1c). Given the higher expression of the transgene in the brains of certain lines of mice (Fig. 1c), lines 11004, 11012, and 10115 were used for our behavioral studies of morphine's actions. Lines 11004 and 11012 were derived on the C57BL/6xSJL background, and line 10115 was derived on the FVB/N background. Mice of these three lines were also used for analysis of the expression of the transgene in other tissues. It is evident from Fig. 1d that the expression of the synapsin promoter-containing transgene was limited to the central nervous system (CNS; brain and spinal cord) of the TG mice. Figure 2 demonstrates the levels of AC activity in cortical tissue of mice of lines 11012 and 10115. When AC activity was measured in the presence of GTP, we noted approximately 20% greater activity in the cortical tissue of the TG mice. Activity in the presence of vasoactive intestinal peptide (VIP) and GTP was also higher in the TG mice, and this difference reached statistical significance in line 11012. The inhibition of the activity by the μ-receptor agonist [D-Ala^2,*N*-MePhe^4,Gly-o1^5]enkephalin (DAMGO) was less pronounced in the tissue from the TG mice. DAMGO (10 μM) produced 25% inhibition of GTP-stimulated AC activity in cor-
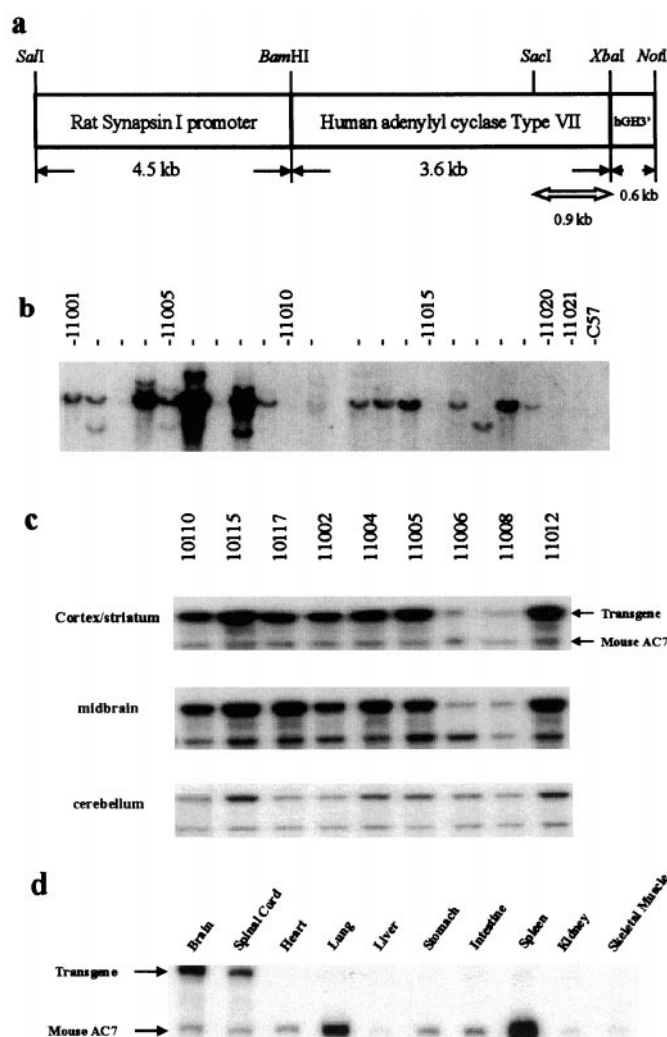

Fig. 1. Construction and characterization of AC7 TG mice. a, schematic illustration of the construct used for creation of the AC7 TG mice. b, Southern blots from various lines of TG mice. c, expression of AC7 mRNA in brains of TG mice measured using RNase protection assays. d, expression of AC7 mRNA in various organs of TG mice of line 10115 measured using RNase protection assays. Similar results were obtained for mice of lines 11004 and 11012.

tical tissue of the TG mice and 35% inhibition in the wild-type (WT) mice (line 10115). For line 11012, the inhibition by DAMGO was 14% for the TG animals and 21% for the WT mice.

The measure of nociception thresholds in the TG mice and their WT litter mates, using the hot-plate test (Marubio et al., 1999), demonstrated no differences between the TG and WT mice in basal responses of paw-lick latency. Cumulative dose-response curves (Duttaroy et al., 1997) like those shown in Fig. 3 were used to determine ED_{50} values for morphine to increase paw-lick latency on the hot-plate. For both backgrounds, the ED_{50} values for the TG mice were significantly lower ($P < .05$) than the ED_{50} values determined for the WT mice [C57BL/6xSJL (lines 11004 and 11012, combined), WT: $ED_{50} = 14.7 \pm 2.2$ mg/kg, mean $\pm$ S.E., $n = 10$, TG: $ED_{50} = 8.1 \pm 1.6$ mg/kg, $n = 11$; FVB/N (line 10115), WT: $ED_{50} = 11.3 \pm 1.3$ mg/kg, $n = 12$, TG: $ED_{50} = 6.5 \pm 1.8$ mg/kg, $n = 12$]. These data showed that the TG animals of either the FVB/N background or the C57BL/6xSJL background were more sensitive than the corresponding WT mice to the analgesic action of morphine.

To examine the development of morphine tolerance in TG and WT mice, we determined the ED_{50} values for morphine analgesia for individual mice in the various experimental groups before and after chronic morphine treatment. The differences between ED_{50} values for each mouse, before and after the 4-day treatment with morphine, were used as a measure of the amount of tolerance development. Figures 3 and 4a indicate that chronic daily treatment with 5 or 10 mg/kg morphine produced substantial increases in ED_{50} values (indicating the development of tolerance) in the TG mice (Fig. 4a). The WT mice of the FVB/N strain (line 10115), however, developed no tolerance when given 5 mg/kg of morphine for 4 days, and the WT mice of the C57BL/6xSJL strain (lines 11004 and 11012, combined) developed only a fraction of the tolerance of the TG mice in this paradigm (Fig. 4a). When the daily dose of morphine was increased to 10 mg/kg, the WT mice of the FVB/N strain did develop measurable levels of tolerance (Fig. 4a). Interestingly, increasing the chronic dose (10 mg/kg/day) did not further increase the

magnitude of tolerance in the TG FVB/N mice (line 10115, compare center and right panels in Fig. 4a). Such results provided an initial indication that the AC7 transgene may increase the rate of morphine tolerance development rather than changing the final magnitude of morphine tolerance. This possibility was supported by experiments in which WT and TG FVB/N mice (line 10115) were treated with 10 mg/kg of morphine for 8 days (Fig. 4b). Given this 8-day period of morphine administration, the WT mice did finally develop tolerance of the magnitude seen in the TG mice after only 4 days of morphine treatment. However, the 8-day treatment did not further increase the magnitude of tolerance in the TG mice (line 10115).

We also assessed naloxone (5 mg/kg)-precipitated morphine withdrawal (Maldonado et al., 1996) in animals that had received morphine chronically. It has to be clear that the WT and TG animals treated with naloxone had previously received graded increments in morphine dosing, which resulted in a total dose of 50 mg/kg morphine over a 3-h period on the first day, during the session in which the initial
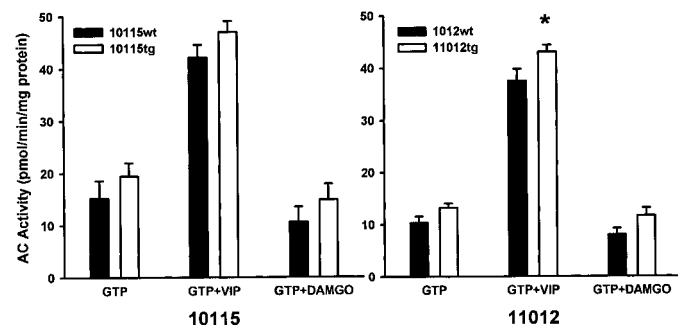

Fig. 2. AC activity in mouse cortex. AC activity in the cortex of TG and WT littermates was measured in the presence of 1 mM GTP, 1 mM GTP plus 1 μM VIP (GTP + VIP), or 1 mM GTP plus 10 μM DAMGO (GTP + DAMGO). Data are the means $\pm$ S.E. For line 10115, eight mice (four TG and four WT) were used. For line 11012, 12 mice (six TG and six WT) were used. Two-way repeated measures ANOVA revealed genotype (TG versus WT) effects ($P = .05$ for line 11012). The results were similar for line 10115 but did not reach statistical significance. Post hoc analysis revealed that VIP significantly increased the enzyme activity over GTP alone ($P < .001$) in all genotypes, but the effect was significantly greater (*$P < .05$) in the TG versus WT mice of line 11012. DAMGO inhibited the AC enzyme activity compared with enzyme activity in the presence of GTP alone, but the inhibition was less pronounced in the TG mice (see text).
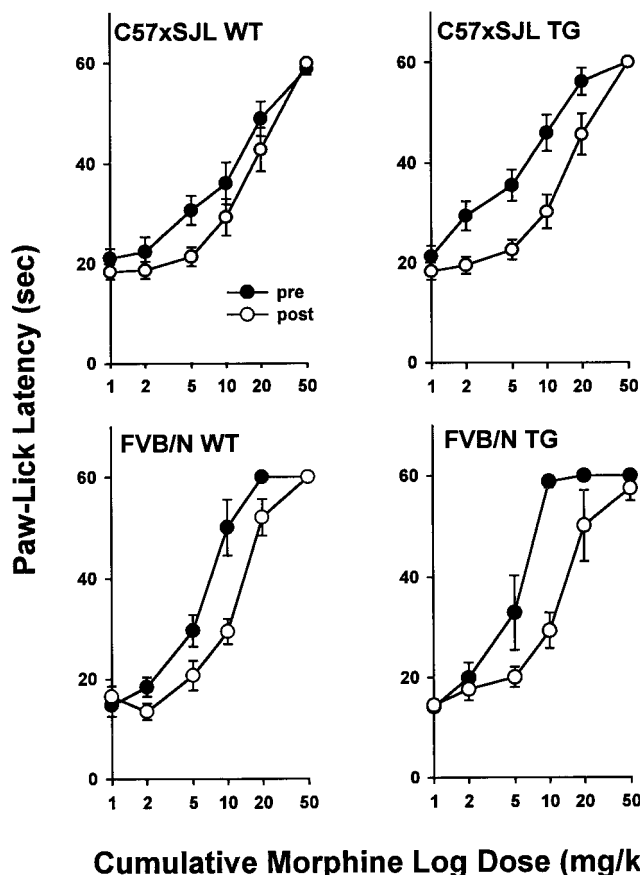

Fig. 3. Acute analgesic effect of morphine and tolerance to the analgesic effect of morphine in WT and AC7 TG mice. Cumulative dose-response curves for morphine analgesia before (pre, closed circles) and after (post, open circles) chronic 4-day morphine treatment [5 mg/kg/day for C57BL/6xSJL (lines 11004 and 11012) mice; 10 mg/kg/day for FVB/N (line 10115) mice; data for FVB/N mice treated with 5 mg/kg of morphine/day are not shown]. Data from the two lines of C57BL/6xSJL mice did not differ significantly and have been combined. Two-way ANOVA with repeated measures revealed a significant effect of dose (C57BL/6xSJL WT and TG: $P < .001$; FVB/N WT and TG: $P < .001$); a significant effect of treatment (C57BL/6xSJL WT: $P < .008$; TG: $P < .001$; FVB/N WT: $P < .041$; TG: $P < .014$); and significant dose $\times$ treatment interactions (C57BL/6xSJL WT: $P < .032$; TG: $P < .001$; FVB/N WT and TG: $P < .001$).

morphine ED_{50} was determined (see *Materials and Methods*). They then received 5 or 10 mg morphine/kg/day for 4 days; a day later they again received 50 mg/kg morphine over a 3-h period during tolerance testing. Naloxone was administered 21 h after the last dose of morphine, and naloxone-precipitated jumping and rearing were measured (Koob et al., 1992; Maldonado et al., 1996). Rearing is considered to be a mild withdrawal sign, whereas jumping is an indicator of more severe withdrawal. The TG and WT mice of either C57BL/6xSJL (lines 11004 and 11012) or FVB/N (line 10115) background exhibited few withdrawal signs upon naloxone administration after the chronic treatment with 5 mg/kg morphine for 4 days (Fig. 5a). Although a significant increase in naloxone-induced rearing was evident in the morphine-treated TG and WT mice of both genetic backgrounds, compared with chronically saline-treated mice (data not shown), the magnitude of naloxone-precipitated rearing did not differ between the TG and WT mice (Fig. 5a). When the four daily doses of morphine were increased to 10 mg/kg for the FVB/N (line 10115) mice, a more severe morphine withdrawal syn-

drome was precipitated with naloxone. This syndrome included persistent jumping (Koob et al., 1992), but again, neither the number of jumps nor the amount of rearing differed significantly between the WT and TG FVB/N mice of line 10115 (Fig. 5a). We also examined withdrawal-induced hyperalgesia in the WT and TG FVB/N (line 10115) mice using the hot-plate test (Suaudeau et al., 1998). Twenty-one hours after the last dose of morphine, or after chronic treatment with saline, a baseline paw-lick latency score was established, which did not differ between the WT and TG FVB/N mice. Thirty minutes later, the mice received 5 mg/kg naloxone and again were tested on the hot-plate. The change in latency for paw lick between the initial hot-plate test and the test after naloxone treatment is plotted in Fig. 5b. Naloxone treatment did not produce a significant change in paw-lick latency in chronically saline-treated mice. In chronically morphine-treated animals, naloxone shortened the paw-lick latency (i.e., produced hyperalgesia) identically in the FVB/N TG and WT mice of line 10115 (Fig. 5b).

A number of studies have demonstrated that chronic treatment with morphine can also produce a sensitization to certain actions of morphine, including morphine-induced increases in locomotor activity in mice (Gwynn and Domino,
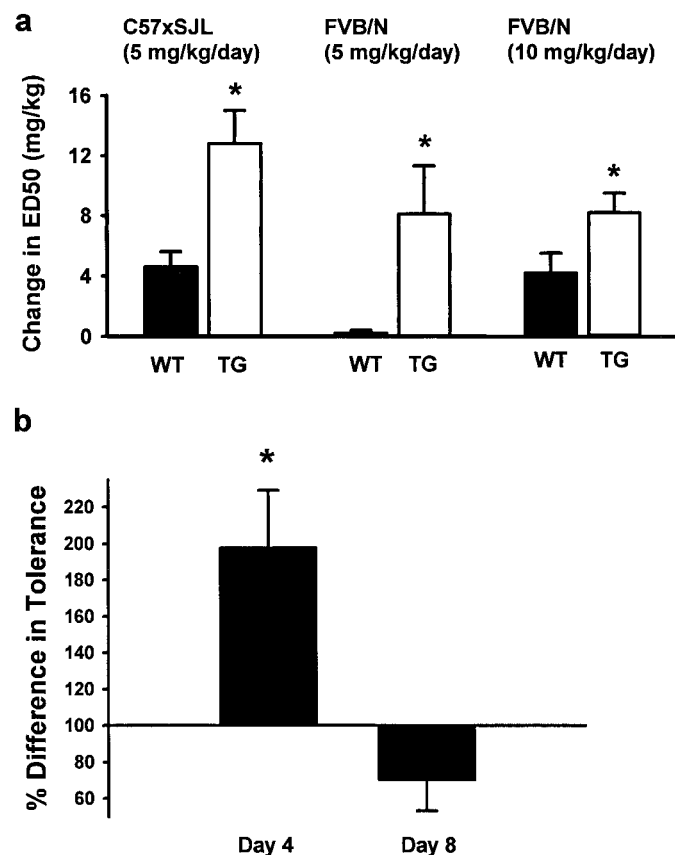

Fig. 4. Changes in the analgesic effect of morphine after chronic treatment. a, change in morphine ED_{50} when measured before and after 4-day morphine treatment. Left: 5 mg/kg/day, combined data for the two lines of C57BL/6xSJL (lines 11004 and 11012) mice, $n = 12$ mice per group; center: 5 mg/kg/day for FVB/N (line 10115) mice, $n = 6$ mice per group; right: 10 mg/kg/day for FVB/N mice, $n = 7$ (WT) or $n = 5$ (TG) mice per group. *$P < .05$, compared with WT (Student's t test). b, percentage differences in the magnitude of tolerance development in TG and WT FVB/N (line 10115) mice. Morphine, 10 mg/kg, were injected into mice for 4 or for 8 days. The change in morphine ED_{50} was ascertained as in a, and the values for the TG mice are expressed as a percentage of the tolerance measures for the WT mice (*$P < .05$). The numerical changes in the ED_{50} (mg/kg) values after 8 days of treatment with morphine were 8.0 ± 2.2 mg/kg for WT mice and 6.5 ± 1.4 mg/kg for the TG mice. Values in all panels represent means $\pm$ S.E.
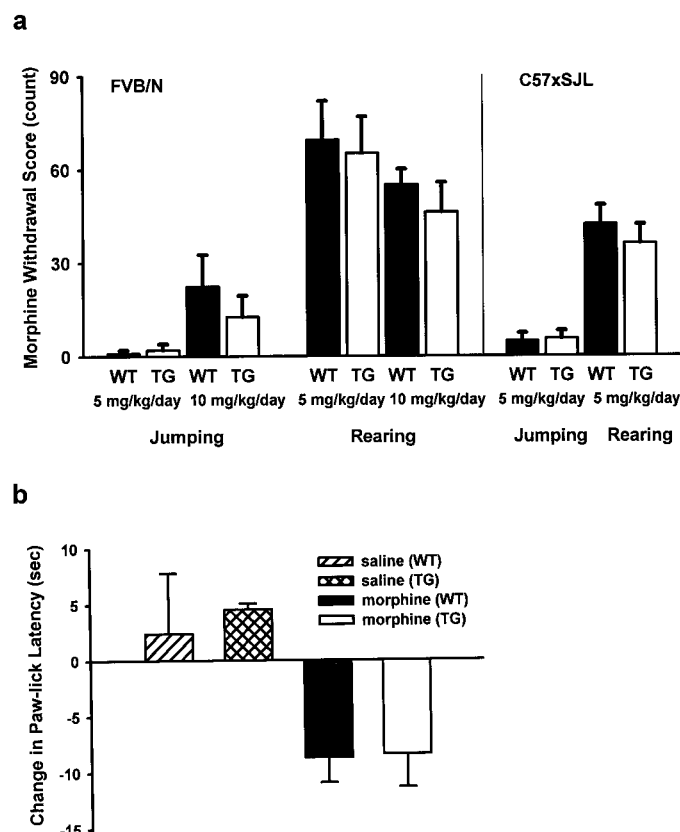

Fig. 5. Naloxone-precipitated morphine withdrawal in WT and AC7 TG mice. a, naloxone-precipitated jumping and rearing in mice treated chronically with the indicated doses of morphine is presented. Data from the two C57BL/6xSJL lines (lines11004 and 11012) did not differ significantly and have been combined. ANOVA revealed no significant differences between WT and TG mice of either line with respect to withdrawal scores. Values represent means $\pm$ S.E. from five to 12 mice in each group. b, naloxone-induced hyperalgesia in FVB/N (line 10115) mice treated chronically with morphine or saline. ANOVA revealed a significant effect of treatment (saline versus chronic morphine) on naloxone-induced hyperalgesia ($P < .015$) but no significant effect of genotype (WT versus TG). Values represent means $\pm$ S.E. from five to seven mice in each group.

1984; Moskowitz et al., 1985). It has been demonstrated that morphine increases locomotor activity in a strain-dependent manner (Gwynn and Domino, 1984; Moskowitz et al., 1985) and that this increase in activity is enhanced after chronic treatment with morphine [particularly in paradigms that include once-daily treatments with morphine (Gwynn and Domino, 1984)]. When we examined the acute effect of morphine (2, 5, and 10 mg/kg) in the FVB/N WT or TG (line 10115) mice, we noted no increase in locomotor activity, and there was no significant change in response to morphine after chronic morphine treatment. On the other hand, the WT and TG mice of the C57BL/6xSJL background (lines 11004 and 11012, combined) showed a dose-dependent increase in locomotor activity after the acute administration of morphine (Fig. 6). After chronic morphine treatment, the magnitude of the change in morphine's effect on locomotor activity was significantly greater in the TG animals than in the WT C57BL/6xSJL mice (Fig. 6).

Discussion

Nestler and Aghajanian (1997) recently posited that the cAMP-signaling system is of major importance in the development of tolerance to and physical dependence on morphine, whereas others have implicated morphine's ability to inhibit cAMP generation as a mechanism for explaining the acute analgesic effects of opiates (Collier and Roy, 1974; Duman et al., 1988; Harrison et al., 1998). The introduction into the CNS of a transgene that responds to activation of the μ-opioid receptor with an increase in cAMP production, rather than a decrease in cAMP production (Yoshimura et al., 1996), generated mice with an increased sensitivity to the analgesic effect of morphine (Fig. 3). We ascertained that the lines of TG animals that we tested behaviorally demonstrated the expression of mRNA for the human form of AC7 in several brain areas and demonstrated a measurable increase in receptor/$G_S\alpha$-stimulated AC activity in their cortical tissue. Although the magnitude of this increase in AC activity was relatively small, it has to be pointed out that the measurement of AC activity takes place on the activity background of

Fig. 6. Morphine-induced locomotor activity after acute (pre) and chronic morphine treatment (post; 5 mg/kg/day) of WT and AC7 TG mice (C57BL/6xSJL). Two-way ANOVA with repeated measures revealed a significant effect of acute morphine dose in WT and TG mice ($P < .001$). However, the effect of chronic morphine treatment was significant only in the TG mice ($P < .01$). Values represent means ± S.E. from 12 mice in each group. *$P < .05$, compared with before chronic morphine treatment (pre) (post hoc comparisons).

all endogenously expressed isoforms of the AC enzyme such that even a large increase in expression of a single isoform may be somewhat masked in an in vitro assay. In vivo, the cellular localization of various enzyme isoforms, and coupling to specific receptors, can, however, allow for an increased influence of particular forms of the enzyme. It was thus interesting to note that the μ-opioid agonist DAMGO had a lesser inhibitory effect on GTP-stimulated AC activity in the cortical tissue of the AC7 TG animals, as would be expected if an opiate-stimulatable form of AC had been overexpressed.

Furthermore, a substantial change in the development of opiate tolerance was noted in our TG animals. Under conditions of chronic morphine administration (5 mg/kg/day, see Figs. 3 and 4a), we noted more than a 2-fold change in ED_{50} values in chronically morphine-treated TG mice, whereas the sensitivity of WT mice changed little (i.e., approximately 30% in C57BL/6xSJL WT mice) or not at all (i.e., FVB/N WT mice). One can conclude from these data and the data in Fig. 6 that the mice overexpressing the AC7 transgene adapt more quickly to chronic morphine treatment. The more pronounced adaptive response is evident in particular measures of either tolerance or sensitization. Although we have selectively expressed the transgene for AC7 in the CNS of our TG mice, one cannot, at this point, discount the possibility that pharmacokinetic factors may play a role in generating our results. Another important caveat is also evident from the studies of sensitization to the locomotor-activating effect of morphine. The introduction of the human AC7 transgene into the CNS can modify morphine-induced behavioral/physiological events but apparently does not introduce behavior not previously evident in animals of a particular genetic background. The FVB/N WT animals (and FVB/N mice, in general) did not respond to the administration of morphine with an increase in locomotor behavior, and the absence of this morphine-induced behavior was not altered by the introduction into the CNS of the AC7 transgene.

It was of major interest to note that the significant differences in tolerance development were not paralleled by major differences between WT and TG mice in the signs of physical dependence as measured after treatment with naloxone. Although claims for dissociation of morphine tolerance and dependence have to be, many times, tempered because of the use of different measures of ascertaining tolerance and dependence, the lack of difference in withdrawal signs between the TG and WT mice in the current study was found using measures of hyperalgesia, as well as hyperactivity.

Maldonado et al. (1996) examined the development of tolerance to and dependence on morphine in mice with disrupted forms of CREB-α and -δ. The inactivation of these forms of CREB generated a suppression of the signs of naloxone-precipitated withdrawal, as well as a reduction in development of morphine tolerance. The dissociation of changes in development of tolerance from changes in the development of physical dependence in our studies indicates that the cAMP-generating systems that were modified in our TG mice are more related to the development of tolerance than to physical dependence. This dissociation also reinforces the proposition (Schulz and Herz, 1984) that opiate tolerance and physical dependence are not simply mirror images of the same adaptive process. It would seem from our studies and those of Maldonado et al. (1996) that cAMP and other signaling cascades may use CREB as a final common path for
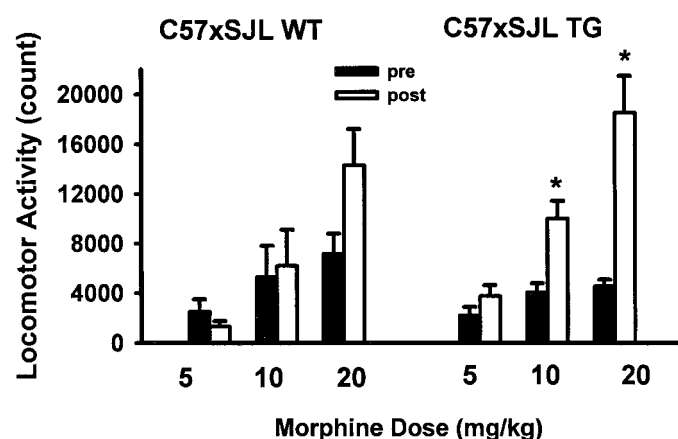
development of morphine tolerance and dependence, but upstream components may determine whether tolerance, dependence, or both are altered by genetic manipulations.

The usefulness of morphine as an analgesic agent is many times compromised by the development of tolerance. Sensitization to the locomotor-activating effects of morphine has been utilized as a surrogate for measuring compulsive drug-seeking behavior and drug craving (Robinson and Berridge, 1993). Novel approaches can be developed for increasing the therapeutic utility of the opiates without increasing their abuse or addictive potential by identifying the CNS-signaling systems that modulate these phenomena and producing animal models in which the development of tolerance and sensitization are altered.

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