0026-895X/00/070159-08$3.00/0
MOLECULAR PHARMACOLOGY

MOL 58:159–166, 2000 /13141/828853

Overexpression of Dynamin Is Induced by Chronic Stimulation of μ- but Not δ-Opioid Receptors: Relationships with μ-Related Morphine Dependence

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Received December 3, 1999; accepted March 13, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Several studies using selective opioid agonists or mice with a deletion of the μ-opioid receptor, have shown that morphine dependence is essentially due to chronic stimulation of μ- but not δ-opioid receptors. Because dependence is assumed to be related to persistent intracellular modifications, we have investigated modifications putatively induced by chronic activation of μ receptors with morphine or selective agonists in vitro in SH-SY5Y cells and in vivo in different strains of mice, including mice lacking the μ-opioid receptor gene. The results show a similar down-regulation and desensitization of μ and δ binding sites, whereas an overexpression of dynamin occurred only with μ agonists, strongly suggesting the relevance of this up-regulation with the opiate dependence. Moreover, translocation of overexpressed dynamin from intracellular pools to plasma membranes was observed in chronic morphine-treated rats. This recruitment could be critically involved in long-lasting changes such as alterations of axonal transport observed in opioid dependence.

Drug addiction is assumed to result from intracellular adaptations occurring in specific brain neurons following repeated exposure to a drug. These modifications are believed to produce the complex behaviors that define an addicted state (review in Nestler and Aghajanian, 1997). Understanding the neurobiological mechanisms of addiction remains a challenging problem that could generate major changes in the way addiction is viewed and ultimately treated. Nevertheless, despite a great number of studies devoted to morphine dependence, the molecular and cellular mechanisms by which chronic opioid exposure elicits intracellular changes remains poorly understood (review in Cox, 1993; Nestler and Aghajanian, 1997). Since the initial reports of the negative coupling of opioid receptors to adenylyl cyclase in brain homogenates and in neuronal cell lines (review in Childers, 1991; Dhawan et al., 1996), biochemical studies have shown that chronic opioid treatment induces a feedback increase in the expression of adenylyl cyclase activity (review in Cox, 1993; Matsuoka et al., 1994; Nestler and Aghajanian, 1997). This leads to an up-regulated cAMP pathway, increasing the concentration of several phosphoproteins, such as the transcription factor cAMP response element-binding protein (review in Nestler and Aghajanian, 1997). This increase of phosphoprotein synthesis could overstep the physiological capacity of phosphatase regulation, resulting in long-lasting effects of these proteins. These changes may contribute to the very long-lived aspects of heroin addiction, leading to frequent relapse.

It is well known that repetitive stimulation by opiates of dopamine neurons located in the ventral tegmental area play a crucial role in opiate addiction (review in Koob and Le Moal, 1997). Interestingly, chronic activation of this reinforcing mesolimbic pathway was shown to result in selective reduction in the size of ventral tegmental area dopamine neurons (Beitner-Johnson et al., 1992; Sklair-Tavron et al., 1996). Consistent with this result, cytoskeletal or cytoskeletal-associated elements of dopamine neurons have been shown to be altered by chronic morphine treatment (Beitner-Johnson et al., 1992). However, no study has yet been per-

This work was supported by institutional grants from Centre National de la Recherche Scientifique and INSERM (France), grants from the European Community (BMH4 CT98 2267), and research grants OTKA T-16084, TÉT JFNo. 564. A short travel fund was provided by the French Embassy (Service Culturel, Scientifique et de Coopération) to M. Szücs.

ABBREVIATIONS: DAMGO, H-Tyr-D-Ala-Gly-*N*-Me-Phe-glycinol; SNC 80, (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide; BUBU, Tyr-D-Ser-(*O*-tertiobutyl)Gly-Phe-Leu-Thr(*O*-tertiobutyl); DPDPE, Tyr-D-Pen-Gly-Phe-D-Pen; PAGE, polyacrylamide gel electrophoresis; SPM, synaptic plasma membranes; MI, microsomes; MAP, mitogen-activated protein.

formed to investigate the possible modifications in the expression of proteins playing a role in synaptic plasma membrane regulation and cell morphology, although such cellular components may play a key role in the neuroplasticity reported to follow chronic administration of several drugs of abuse (Nakahara et al., 1998). Recent studies using mice with a deletion of the μ-opioid receptors have shown that morphine dependence is essentially caused by chronic stimulation of μ- but not δ-opioid receptors (Matthes et al., 1996), in agreement with previous pharmacological studies (Cowan et al., 1988; Maldonado et al., 1990).

Because the phosphoprotein dynamin has a broad role in cellular signaling, including receptor endocytosis and binding to microtubules (review in McClure and Robinson, 1996), the aim of the present study was to assess whether chronic morphine treatment could alter intracellular regulation of this protein and to evaluate the respective effects induced by μ- or δ-opioid receptors stimulation. For this purpose we have combined in vitro and in vivo experiments, using SH-SY5Y cell lines, and different strains of mice and rats, including mice in which the μ-opioid receptor gene has been deleted resulting in lack of morphine dependence (Matthes et al., 1996).

Materials and Methods

Chemicals. Morphine hydrochloride was purchased from Sanofi (France), DAMGO (H-Tyr-D-Ala-Gly-*N*-Me-Phe-glycinol) from Bachem Biochimie SARL (France), SNC 80 [(+)-4-[(αR)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide) from Tocris (Bioblock-Scientific, France), and BUBU [Tyr-D-Ser-(*O*-tertiobutyl)Gly-Phe-Leu-Thr(*O*-tertiobutyl)] was synthesized in the laboratory (Gacel et al., 1988). SNC 80 is a systemically active, highly selective, and potent nonpeptide δ agonist (with 2000-fold δ/μ selectivity) (Calderon et al., 1994), and BUBU was a selective peptide agonist that displays a high affinity and a good in vitro selectivity ($K_i\mu/K_i\delta$ = 580) for δ-opioid receptors (Delay-Goyet et al., 1988). DAMGO is described as a highly selective μ agonist, with 600-fold δ/μ selectivity, and morphine as a preferential μ-opioid agonist (with 45-fold δ/μ selectivity). [^{3}H]cAMP (specific activity, 28.1 Ci/mmol) and [^{3}H]DAMGO (specific activity, 65 Ci/mmol) were purchased from Amersham Pharmacia Biotech (France), and [^{3}H]DPDPE (specific activity, 58 Ci/mmol) from NEN Life Science Products (France). The other reagents were obtained from Sigma (France).

Cell Culture and Treatments. The human neuroblastoma cell line SH-SY5Y were grown at 37°C in RPMI medium containing 10% fetal calf serum, in a humidified atmosphere containing 5% CO_2. Medium was changed every day, and the relevant concentration of drugs was replaced. SH-SY5Y were treated with morphine (0.1, 1, and 10 μM), DAMGO (10 μM) or BUBU (10 μM) for 1, 3, 5, or 6 days at 37°C. Cells were homogenized in Tris-HCl buffer (50 mM, pH 7.4) with phenylmethylsulfonyl fluoride (1 mM), and homogenates resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

Mice and Chronic Treatments. Male CD_1 mice (Charles River, France), and +/+ and −/− 129/Sv mice (obtained from B. Kieffer) were used. Mice were housed and used strictly in accordance with European Community guidelines for the care and use of laboratory animals and after approval of the proposed experiments by the ethical committee of the Université René Descartes. Animals were chronically treated with saline, naloxone (1 mg/kg, s.c.), SNC 80 (i.p.), morphine (i.p.), or morphine plus naloxone by repeated injection at an interval of 12 h during a 5-day period. The morphine doses were progressively increased as follows: day 1, 20 mg · kg^{-1}; day 2, 40 mg · kg^{-1}; day 3, 60 mg · kg^{-1}; day 4, 80 mg · kg^{-1}; day 5, 100 mg · kg^{-1}, and the SNC 80 doses were as follows: day 1, 50 mg · kg^{-1}; day 2, 70 mg · kg^{-1}; day 3, 80 mg · kg^{-1}; day 4, 100 mg · kg^{-1}; day 5, 120 mg · kg^{-1}. The brains were rapidly removed 12 h after the last injection, and the caudate putamen was dissected on ice. The caudate putamen was homogenized in Tris-HCl buffer (50 mM, pH 7.4) with phenylmethylsulfonyl fluoride (1 mM), and homogenates were resolved using SDS-PAGE.

Rats and Treatments. Male Wistar rats (Charles River) ranging in weight from 200 to 220 g at the beginning of the experiment were used. Saline (control animals) and morphine were injected s.c. twice daily at 9:00 AM and 6:00 PM in a volume of 1 ml/kg. The morphine dose was progressively increased from 10 to 40 mg/kg over a period of 2 days, and this dose was maintained during 3 more days. The first and second number inside parentheses represent the dose of morphine (mg/kg) injected at 09:00 AM and 6:00 PM, respectively, on consecutive days: 1st day (10, 20), 2nd day (20, 40), 3rd through 5th days (40, 40). The rats were sacrificed on the morning of the 6th day, and the brains were rapidly dissected. For acute experiments, 10 mg/kg s.c. morphine was given, and the rats were sacrificed 2 h later.

Assessment of Physical Dependence. Mice or rats were chronically treated with morphine as described above. On the 6th day, animals received a final injection of morphine, and 2 h later the withdrawal was precipitated by injection of naloxone hydrochloride (1 mg/kg, s.c.). Somatic signs of withdrawal (jumps, paw shakes, wet dog shakes, tremor) were evaluated immediately after naloxone injection during a period of 30 min.

Subcellular Fractionation of Rat Brains. Membrane fractions highly enriched in synaptic plasma membranes (SPM) or endoplasmic reticulum and Golgi complexes [microsomes (MI)] were prepared from rat brains minus cerebellum by sucrose density gradient centrifugation as described elsewhere (Szücs and Coscia, 1992). Briefly, all sucrose solutions contained 5 mM Tris-HCl (pH 7.4), 50 μM $CaCl_2$, 0.5 mM dithiothreitol. Rat brains were homogenized in 10 volumes of 10% sucrose by 5 up and down passes of a loosely fitting, slowly rotating pestle. The resulting homogenate was centrifuged at 1000*g* for 10 min to remove cellular debris and nuclei. The supernatant obtained (S_1) was centrifuged at 12,000*g* for 20 min to yield the crude synaptosomal pellet (P_2) and the supernatant (S_2). To remove adhering MIs, P_2 was washed three times by gentle resuspension and recentrifugation. The pellet obtained after the third washing step was lysed at pH 8.1 and allowed to incubate for 30 min at 4°C. The lysate was then adjusted to 34% sucrose. This formed the bottom of a three-step gradient of 10%/28.5%/34% sucrose. Following centrifugation at 100,000*g* (SW 28 rotor) for 120 min, the 28.5%/34% interface was collected (SPM). For preparation of MIs, S_2 was centrifuged at 20,000*g* for 25 min. This 20,000*g* supernatant was centrifuged at 100,000*g* for 60 min to yield the crude microsomal pellet (P_3). MI were resuspended in 10% sucrose, and this formed the top of a two-step gradient of 10%/28.5%. Following centrifugation at 100,000*g* (SW 28 rotor) for 120 min, the 10%/28.5% interface was collected (MI).

Western Blot. Protein extracts (homogenates, SPM or MI) were fractionated by SDS-PAGE on a 7.5% acrylamide gel. Proteins were transferred onto nitrocellulose filters, and blots were hybridized with the mouse anti-dynamin antibody (Transduction Laboratories, France). Specificity of the anti-dynamin antibody has been previously demonstrated in rat brain homogenates in which only one band was revealed by Western blot, with a molecular weight corresponding to that of purified dynamin (Montiel et al., 1997). Immunocomplexes were revealed by a peroxidase-labeled antimouse IgG conjugate associated with the enhanced chemiluminescence detection system (Amersham, France). Autoradiograms were quantified by scanning laser densitometry (Imager, Bio1d software; Vilber Lourmat, Marne La Vallée, France). To determine the amount of dynamin in the SPM and MI fractions, a standard curve was performed with purified dynamin (Montiel et al., 1997).

Opiate Receptor Assays. For opiate receptor binding assays, cell membranes (~200 μg of protein) were incubated in 50 mM

Tris-HCl (pH 7.4) in the presence of varying concentrations (0.1–4.0 nM) of [^{3}H]DAMGO or [^{3}H]DPDPE for 90 or 120 min, respectively, at 25°C. Nonspecific binding was determined in the presence of 10 μM levorphanol. B_{max} and K_D values were estimated from linear regression methods (EBDA-LIGAND program; Biosoft, Cambridge, UK).

Adenylyl Cyclase Activity. Cell membranes (15–30 μg of protein in 10 μl) was added on ice to assay tubes (final volume, 60 μl) containing 80 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM $MgSO_4$, 0.8 mM EGTA, 30 mM NaCl, 0.25 mM ATP, 0.01 mM GTP, and either the drug being tested or water. Triplicate samples for each treatment were incubated at 30°C for 5 min. Adenylyl cyclase activity was terminated by placing the tubes into boiling water for 2 min. The amount of cAMP formed was determined using a [^{3}H]cAMP protein binding assay. [^{3}H]cAMP (final concentration, 4 nM) in citrate-phosphate buffer (pH 5.0), followed by binding protein prepared from bovine adrenal glands, was added to each sample. Additional samples were prepared, without tissue, containing known amounts of cAMP and served as standards for quantification. The binding reaction was allowed to reach equilibrium by incubation for 90 min at 4°C, and the assay was terminated by the addition of charcoal and by centrifugation (1000*g* for 10 min, at 4°C) to separate the free tritiated cAMP from that bound to the binding protein. Aliquots from the supernatant containing bound cAMP were placed into scintillation vials to which scintillation mixture (Wallac, Evry, France) was added, and radioactivity was determined with liquid scintillation spectrometry. Results are expressed as percentage of basal activity measured in the absence of opioid.

Results

Regulation of Dynamin Immunoreactivity by Chronic Morphine Treatment in SH-SY5Y Cells. Chronic morphine treatment of SH-SY5Y cells, which express μ- and δ-opioid receptors, results in a significant 1.8-fold increase in dynamin immunoreactivity. This increase was dependent on the concentration of morphine used (Fig. 1A), on the duration of the chronic treatment, and the saturation (Fig. 2). Moreover, the morphine-stimulated dynamin overexpression is mediated by opioid receptors, because cotreatment with the opioid antagonist, naloxone, abrogates the alkaloid-mediated increase of the protein immunoreactivity (Fig. 1B). The results reported in Fig. 2 show that the increase of dynamin immunoreactivity induced by morphine is significant after 3 days of treatment and reaches a plateau after 5 days.

***μ*- but Not *δ*-Opioid Receptors Are Involved in Up-Regulation of Dynamin Immunoreactivity Induced by Chronic Morphine Treatment of SH-SY5Y Cells.** Morphine has only a factor 45 of selectivity for the μ- versus δ-opioid binding sites. Therefore, with the aim to determine whether μ- and/or δ-opioid receptor activation results in dynamin overexpression, SH-SY5Y cells were treated with the highly selective μ agonist DAMGO (10 μM) or the δ-selective agonist BUBU (10 μM), which have about the same nanomolar affinity for their own receptor and a selectivity factor higher than 600 for their specific target. After 1, 3, 5, or 6 days of cells treatment, modifications of dynamin immunoreactivity were detected by Western blot. As shown in Fig. 2, the μ-selective agonist DAMGO induced a 1.9-fold increase in amounts of dynamin immunoreactivity and this increase was significant after 1 day, reaching a plateau after 5 days of treatment. In contrast, dynamin immunoreactivity in SH-SY5Y cells remained unchanged following chronic δ-opioid receptor activation with BUBU.

Binding Parameters and Functionality of Opioid Receptors following Chronic Morphine Treatment. In the same experimental conditions, we have also evaluated the binding parameters and functional responses coupled to μ- and δ-opioid receptors chronically treated with DAMGO or BUBU. As shown in Tables 1 and 2, DAMGO altered both the binding properties and the functional responses associated with the μ-opioid receptors. Thus, when SH-SY5Y cells were treated for 1 and 5 days with 10 μM DAMGO, a desensitization of μ-opioid receptors, and a reduction of the B_{max} value for [^{3}H]DAMGO binding were observed in comparison with control cells.

Similar results were obtained after chronic treatment with

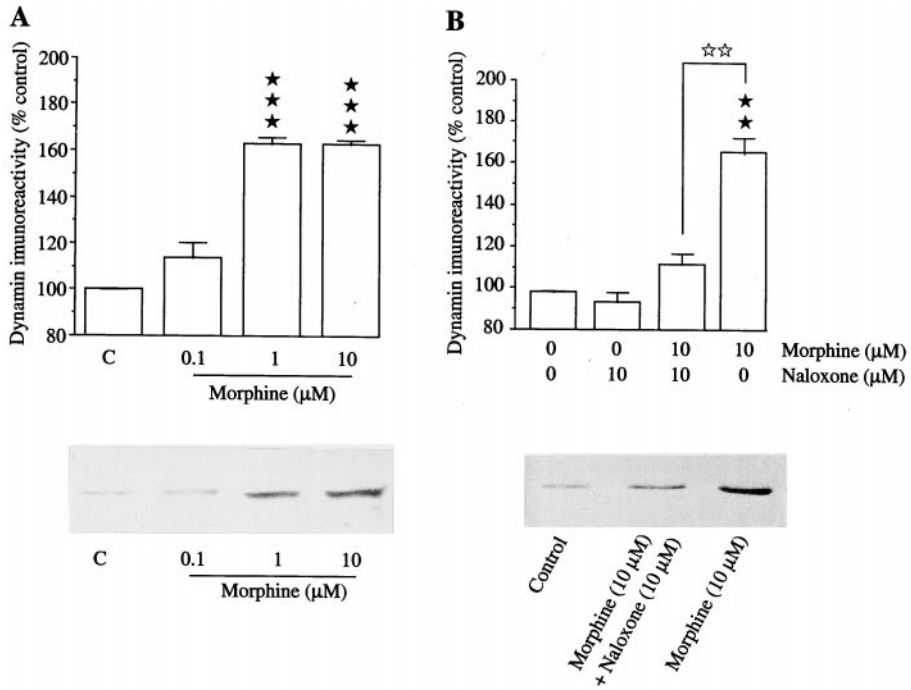

Fig. 1. Regulation of dynamin immunoreactivity in SH-SY5Y cells by chronic morphine treatment and blockade by naloxone. A, cells were treated with different concentrations of morphine for 5 days. B, cells were treated with morphine, naloxone, or morphine plus naloxone for 5 days. Quantification of dynamin immunoreactivity in whole cells (representative example under the histogram) was determined by Western blotting. $^{☆☆}P < .01$, $^{★★}P < .01$, and $^{★★★}P < .001$ compared with control cells (C) (Newman-Keuls test).

the δ-selective agonist BUBU, i.e., a desensitization and a down-regulation of the δ-opioid receptors (Tables 1 and 2).

Dynamin Up-Regulation Was Also Observed in Morphine-Dependent Mice after Activation of μ- but Not δ-Opioid Receptors. The biological relevance of the increase in dynamin immunoreactivity observed in SH-SY5Y cells has been confirmed in CD_1 mice chronically treated by i.p. morphine. In these in vivo conditions, morphine was also shown to produce an increase in dynamin immunoreactivity in the caudate putamen as compared with animals treated with saline and morphine plus naloxone. In contrast, no change in dynamin levels were observed following chronic treatment by i.p. route with the selective δ-opioid agonist SNC 80 (Fig. 3). Moreover, no change in dynamin immunoreactivity was observed in dissected cerebellum, which is devoid of opioid binding sites (data not shown). This suggests that the morphine-induced increase of dynamin immunoreactivity is restricted to some brain areas containing opioid receptors.

To firmly demonstrate that μ-opioid receptor activation selectively induces the increase in dynamin immunoreactivity, 129/Sv mice (−/−) lacking the μ-opioid receptor gene were used (Matthes et al., 1996). As shown in Fig. 4, in 129/Sv control (+/+) mice, chronic morphine induced up-regulation of dynamin immunoreactivity in the caudate putamen, whereas no significant change in dynamin immunoreactivity was observed in the −/− mice. Strikingly, in this mice strain, the antidynamin antibody used in the Western blot revealed the presence of two bands that could correspond to different dynamin isoforms or different states of phosphorylation of the protein.

The time course of dynamin overexpression was also evaluated following chronic morphine treatment using Western blot analyses. As shown in Fig. 5, in CD_1 mice, morphine-induced enhancement of dynamin immunoreactivity decreased progressively 1, 2, and 4 days following the last morphine injection. After 4 days, the dynamin immunoreactivity levels in the caudate putamen were similar for morphine-treated mice and control animals.

Translocation of Dynamin Immunoreactivity from Intracellular Pools to the Plasma Membrane in Brain of Morphine-Dependent Rats. In good agreement with the results obtained in mice, no modification of the total amount of dynamin immunoreactivity, evaluated by quantitative Western blot using purified dynamin, was observed in the whole brain homogenate.

We have evaluated the distribution of dynamin in highly purified SPM and MI fractions prepared from the brain of rats subjected to chronic morphine treatment and from controls. Both subcellular fractions were prepared using the fractionation technique (Szücs and Coscia, 1992). The results show that, in animals chronically treated with morphine, the levels of dynamin immunoreactivity decreased by about 20% in the MI, with a concomitant increase in SPM. Similar results were observed in less purified fractions: P_2 and S_2 (Fig. 6A).

These results were only observed after chronic morphine treatment. Indeed, acute administration of the alkaloid did not induce modifications in the distribution of dynamin immunoreactivity in the P_2 and S_2 fractions as compared with control rats (Fig. 6B).

Control of Drug Dependence Induced by Chronic Morphine Treatment. Opioid dependence was evaluated by measuring the withdrawal syndrome after administration of the opioid antagonist naloxone in mice and rats chronically treated with morphine in the conditions leading to selective μ-related up-regulation of dynamin.

As expected, this treatment induced a strong physical dependence (data not shown). Thus, naloxone administration precipitated the standard behavioral signs of withdrawal (increase in jumps, paw shakes, wet dog shakes, and tremors) in morphine-treated animals but not in saline-injected control groups.

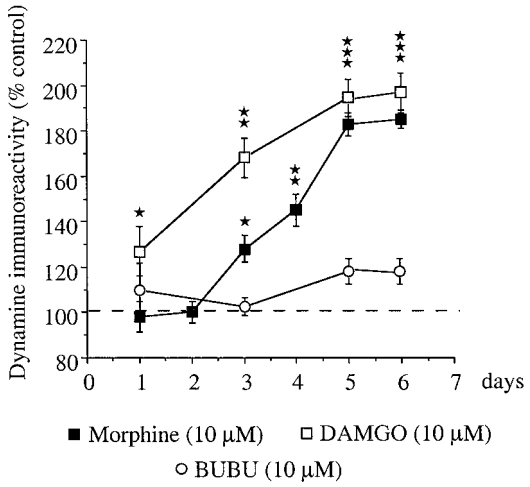

Fig. 2. Time course of the regulation of dynamin immunoreactivity in SH-SY5Y cells by chronic morphine, DAMGO, or BUBU treatment. Cells were treated with different concentrations of the μ or δ agonists for several days, and dynamin immunoreactivity was determined by Western blotting and quantified as described under *Materials and Methods*. $^{\star}P < .05$, $^{\star\star}P < .01$, and $^{\star\star\star}P < .001$ compared with control cells (C) (Newman-Keuls test).

Discussion

The first important result of the present study is that in vitro and in vivo chronic activation of μ- and δ-opioid recep-

TABLE 1
Binding parameters of [^{3}H]DAMGO and [^{3}H]DPDPE in SHSY5Y cell membranes
Cells were chronically treated with DAMGO (10 μM) or BUBU (10 μM). Results are means ± S.E. of at least three independent experiments performed in triplicate.

Treatments	[^{3}H]DAMGO		[^{3}H]DPDPE	
	K_i	B_{max}	K_i	B_{max}
	nM	*fmol/mg protein*	*nM*	*fmol/mg protein*
Control	0.62 ± 0.20	78.71 ± 2.62	0.89 ± 0.43	21.78 ± 3.19
BUBU (5 days)	0.78 ± 0.69	75.45 ± 4.58	1.16 ± 0.46	5.56 ± 2.04*
DAMGO (5 days)	0.86 ± 0.54	49.86 ± 2.56*	0.97 ± 0.59	25.21 ± 6.16

*$P < .01$ compared to control.

tors lead to distinct intracellular changes in dynamin levels. These changes could be related to behavioral experiments showing that repeated stimulation of μ receptors induced a strong dependence, whereas chronic activation of δ receptors produced weak addictive effects (Cowan et al., 1988; Maldonado et al., 1990).

The increase in dynamin immunoreactivity only observed following chronic stimulation of the μ-opioid receptor in SH-SY5Y cells (Fig. 2) is not due to differences in initial events following receptor stimulation. Indeed, μ- and δ-opioid receptors are structurally homologous receptors belonging to the same family of G protein-coupled binding sites, and their activation was shown to trigger similar effects on several intracellular effectors (adenylyl cyclase, ion channels, and others) (review in Childers, 1991) after acute and repeated stimulation. Accordingly, in this study DAMGO and BUBU induced inhibition of adenylyl cyclase in naive cells, and both agonists produced desensitization and down-regulation of μ- and δ-opioid receptors, respectively, after chronic treatment (Tables 1 and 2). It is therefore possible to conclude that both receptors interact with identical G proteins that belong to pertussis toxin-sensitive G_i and G_o subtypes. However, there are several members of the G_i and G_o families, consisting of G_{i1}, G_{i2} and G_{i3}, and two splice variants of G_o:G_{o1} and G_{o2} (Hepler and Gilman, 1992). This G protein heterogeneity could account for the difference observed in this study on

TABLE 2

Inhibition of adenylate cyclase activity after long-term exposure (24 h) of SHSY5Y cells to DAMGO (10 μM) or BUBU (10 μM)

Results represent the maximal inhibition observed in presence of DAMGO (10 μM) or DPDPE (10 μM) compared to basal activity, mean ± S.E. of three independent experiments performed in triplicate.

Treatments	Adenylate Cyclase Inhibition by	
	DAMGO	DPDPE
	%	
Control	41.33 ± 4.48	47.33 ± 5.3
DAMGO	4.67 ± 2.72*	35.02 ± 6.66
BUBU	36.23 ± 6.54	6.33 ± 5.25*

*$P < .01$ compared with control cells.

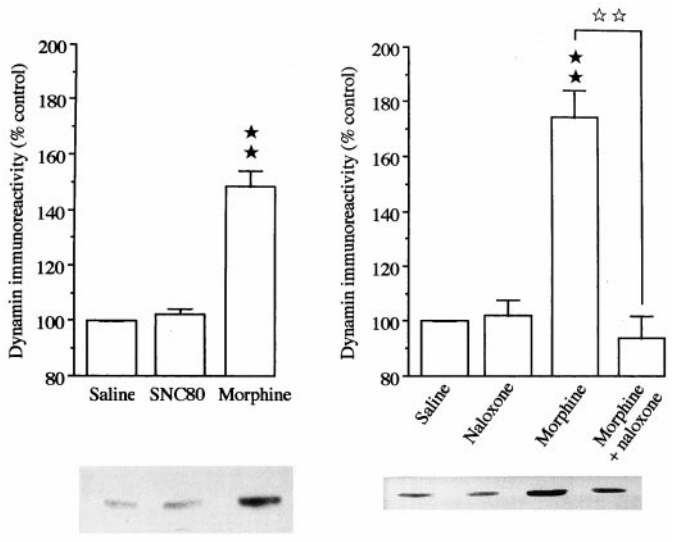

Fig. 3. Regulation of dynamin immunoreactivity in CD_1 mice caudate putamen by chronic treatment (5 days) with morphine, naloxone, morphine plus naloxone, or the δ agonist SNC 80. Quantification of the dynamin immunoreactivity (representative immunoblots shown under the histograms) was determined as described under *Materials and Methods*. ★★$P < .01$ compared with control animals (saline). ☆☆$P < .01$ compared with morphine-treated mice (Newman-Keuls test).

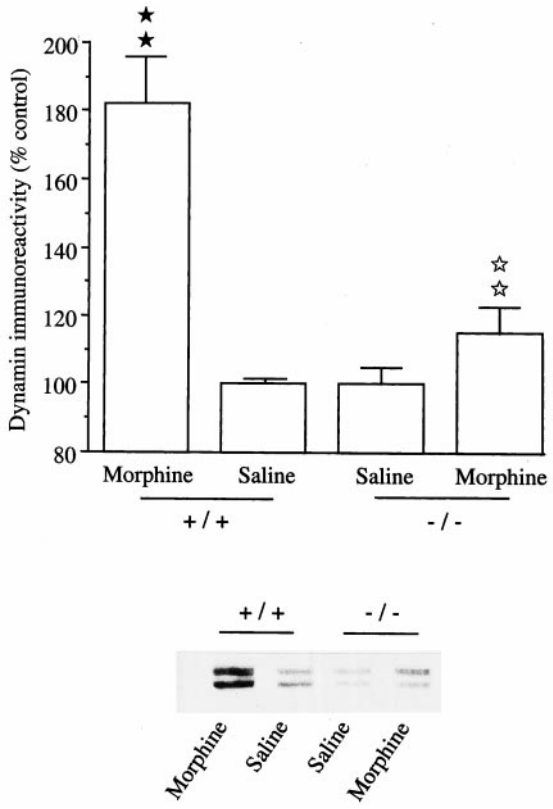

Fig. 4. Changes in dynamin expression in the caudate putamen of 129/Sv mice following chronic morphine treatment (5 days): wild type (+/+) and mutant (−/−) lacking the μ-opioid receptor gene. Quantification of the dynamin immunoreactivity determined as described under *Materials and Methods* and representative immunoblot. ★★$P < .01$ compared with control animals (saline) and ☆☆$P < .01$ compared with wild-type mice, which received chronic morphine treatment (Newman-Keuls test).

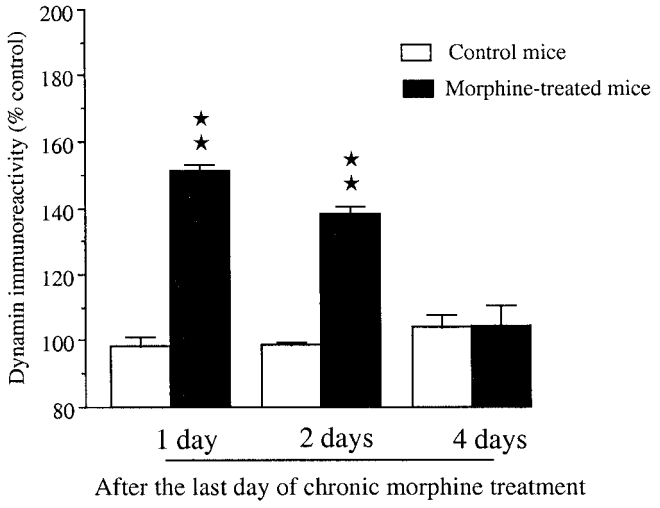

Fig. 5. Time course of the reversal of dynamin up-regulation induced by chronic morphine treatment in the caudate putamen of CD_1 mice. Animals were treated for 5 days with morphine as described in *Materials and Methods*, and sacrificed 1, 2, and 4 days after the last morphine injection. Quantification of the dynamin immunoreactivity was determined as described under *Materials and Methods* by quantitative immunoblots. ★★$P < .01$ compared with control animals (saline) (Dunnett's test).

intracellular expression of dynamin. This hypothesis is supported by photolabeling experiments performed by Laugwitz et al. (1993) that have shown profound differences in coupling of μ- and δ-opioid receptors to pertussis toxin-sensitive G proteins in SH-SY5Y cells. Although the δ-opioid receptor seems to be preferentially coupled to the G_{i1} protein, the μ-opioid receptor could be more efficiently linked to G_{i3}. Besides, G_o subtypes were also shown to be differentially recruited by the two opioid receptors (Laugwitz et al., 1993). Thus, differential coupling of μ- and δ-opioid receptors to G protein subtypes may be at the basis of the differences observed in this study regarding dynamin expression in SH-SY5Y cells.

The maximum effects induced by morphine or DAMGO on the increase of dynamin immunoreactivity in SH-SY5Y cells were similar. Nevertheless, the μ-selective agonist DAMGO induced a significant effect after 1 day of treatment, whereas morphine induced the same effect after 3 days. The difference observed between both agonists after chronic treatment of SH-SY5Y cells on dynamin immunoreactivity could be due to the respective ligand efficacy, as it has been shown that DAMGO is a potent full agonist, whereas morphine is a partial agonist. This hypothesis is in good agreement with a recent study showing the occurrence of a good fit between the efficacies of opiates in μ-receptor activation and desensitization (Yu et al., 1997).

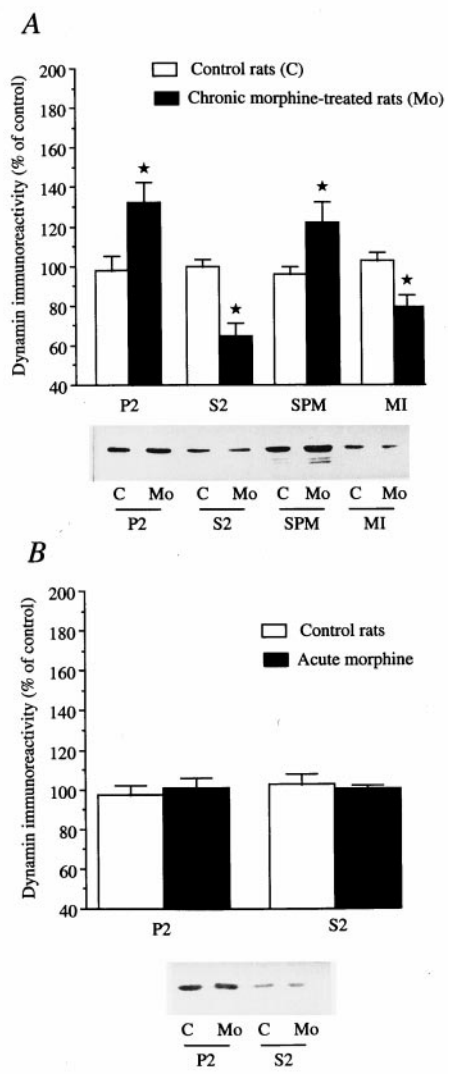

Fig. 6. Distribution of dynamin immunoreactivity in subcellular fractionation of rat brains. A, rats were treated for 5 days with increasing doses of morphine and sacrificed on the 6th day. Preparation of P2, SPM, S2 and MI and determination of quantification of the dynamin immunoreactivity were as described under *Materials and Methods* and representative immunoblot. $^{\star}P < .05$ compared with control rats (saline) (Dunnett's test). B, rats were treated with 10 mg/kg morphine (s.c.) and sacrificed 2 h later.

The physiological relevance of the results obtained in vitro has been confirmed in vivo using CD_1 mice. The caudate putamen has been selected owing to the high level of μ-opioid receptors present in this brain structure. Chronic treatment with the δ-selective agonist SNC 80 (Calderon et al., 1994) did not modify dynamin immunoreactivity in the caudate putamen as compared with naïve mice, whereas a large increase in protein levels was observed following chronic morphine administration, which was totally abolished in animals chronically treated with morphine plus naloxone. Moreover, the in vivo selective involvement of μ-opioid receptor in morphine-induced up-regulation of dynamin has been confirmed using mice lacking the μ-opioid receptor gene (Matthes et al., 1996). Indeed, in wild-type animals (+/+), chronic morphine treatment increased dynamin immunoreactivity in the caudate putamen, whereas no modification was observed in μ-receptor-deleted mice (−/−) (Fig. 4). This up-regulation of dynamin immunoreactivity appears to be selectively dependent on the presence of μ-opioid receptors, because no change in protein levels was observed in rat cerebellum, which is devoid of opioid binding sites. Furthermore, the dynamin immunoreactivity was not significantly altered by chronic morphine treatment when the whole brain was examined, according to the weak proportion of opioid receptors in the whole brain, and the regioselective modifications commonly observed (e.g., protein kinase A activity, adenylyl cyclase activity) after chronic morphine (review in Nestler and Aghajanian, 1997).

The relevance of the selective modifications of dynamin observed in the present study following chronic μ-opioid receptor activation with the phenomenon of dependence is supported by the well known physical and psychic dependence, induced by μ but not δ agonists (Cowan et al., 1988; Maldonado et al., 1990). We have verified that, in the conditions leading to dynamin overexpression, the chronic morphine treatment triggered, as expected, naloxone-precipitated signs of withdrawal syndrome that illustrate the occurrence of opiate dependence. Moreover, in mice lacking the μ-opioid receptor gene, which did not exhibit physical and psychic dependence to morphine (Matthes et al., 1996) overexpression of dynamin was not observed.

Dynamin represents a previously unknown target of chronic morphine action, and its up-regulation in some brain regions may be an important component of the increased neuronal plasticity reported to be at the basis of morphine addiction (Nestler and Aghajanian, 1997). Dynamin has been proposed to induce the formation of constricted necks of coated pits and endocytosis of receptor-charged vesicles (review in McClure and Robinson, 1996). Through this function, dynamin could regulate the level of expression of different membrane receptors and participate very likely in the observed internalization of μ- and δ-opioid receptors. However, the translocation of dynamin from cytoplasmic pools to syn-

aptic membranes in morphine-dependent rats observed in the present study (Fig. 6) could also be responsible of the activation of various cellular signaling pathways. Thus, dynamin may be recruited from its intracellular pool by the $\beta\gamma$ subunits, which are released from morphine-induced G_i/G_o dissociation and have the ability to bind the pleckstrin homology domain present on dynamin (Liu et al., 1997). Such a process should be similar to the interaction of G protein $\beta\gamma$ subunits with the pleckstrin homology domain of the β-adrenergic receptor kinase (Touhara et al., 1994). Interestingly, the density of G_β subunits is markedly increased in brains of opioid addicts and morphine-dependent rats (Escriba et al., 1994). At the synaptic membranes, dynamin may interact by its proline-rich domain with several proteins containing SH3 domains, such as Src kinase (Foster-Barber and Bishop, 1998). This could contribute to Src activation with subsequent tyrosine phosphorylation of several intracellular targets, including receptor tyrosine kinases or adaptator proteins such as Shc. Once phosphorylated, the receptor tyrosine kinase would provide docking sites for the SH2 domain of Shc and Grb2 molecules, resulting in the recruitment of the Ras guanine nucleotide exchange factor (Sos) (Vidal et al., 1998). The subsequent activation of Ras would initiate a phosphorylation cascade leading to mitogen-activated protein (MAP) kinase activation (review in Chardin et al., 1995). This model is in agreement with several studies showing that G_i-coupled receptors mediate $G_{\beta\gamma}$ subunit-dependent MAP kinase activation through a pathway involving the Src family of tyrosine kinase and the protein Ras (Hordijk et al., 1994; Luttrell et al., 1996). Moreover, it is now well documented that the MAP kinase pathway is activated upon chronic morphine treatment (Ortiz et al., 1995; Schulz and Höllt, 1998) and that opioid modulation of MAP kinase activity is Ras-dependent (Belcheva et al., 1998).

On the other hand, largely based on studies of antimicrotubular drugs effects on cell morphology and secretion, it has been shown that intact microtubules are required to preserve the normal structure and function of the Golgi complex (review in Thyberg and Moskalewski, 1999). The latter is composed of cisternal stacks that function in processing and sorting of proteins en route from the endoplasmic reticulum to lysosomes, secretory vacuoles, and the cell surface. As dynamin has a microtubule cross-linking activity and may play a role in the axonal transport (Obar et al., 1990; Scaife and Margolis, 1990), it could be suggested that the decrease of dynamin observed in the MI fraction reflects an alteration of microtubules. After drug-induced disruption of microtubules, the Golgi stacks were shown to be disconnected from each other (review in Thyberg and Moskalewski, 1999). This alteration could contribute to the neuronal changes observed after repetitive administration of μ-opioid agonists. This hypothesis is supported by previous data showing that chronic morphine treatment affect the neurofilaments and is associated with decreased rates of axonal transport and reduction in axonal caliber (Hoffman et al., 1984; Beitner-Johnson et al., 1992; Sklair-Tavron et al., 1996). Translocation of dynamin and reduction of neurofilaments could contribute to reduced levels of proteins in nerve terminals and, thus, to impaired nerve function. This plasticity may be at the basis of long-lasting changes produced by opioid drugs, resulting in behavioral sensitization, which remains for months to years, as already suggested for other drugs of abuse (Robinson and Becker, 1986), thus contributing to drug-induced behavioral modifications defining an addicted state (Segal and Schukit, 1983; Robinson and Berridge, 1993).

Acknowledgments

The contribution of G. Fabian, E. Kicsi, B. Bozo, and I. Németh in preparing subcellular fractions of rat brains for some preliminary experiments is greatly acknowledged.

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