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Molecular Components of Tolerance to Opiates in Single Hippocampal Neurons

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ABSTRACT

We examined the effect of acute and chronic opioid treatment on synaptic transmission and μ-opioid receptor (MOR) endocytosis in cultures of naïve rat hippocampal neurons. Opioid agonists that activate MOR inhibited synaptic transmission at inhibitory but not excitatory autapses. [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO), morphine, and methadone were all effective at blocking inhibitory transmission. These same drugs also reduced the amplitude of voltage-dependent Ca^{2+} currents in inhibitory but not excitatory neurons. Chronic treatment with all three opioids reduced the subsequent effects of a challenge with either the same drug or one of the others in individual autaptic neurons. Chronic treatment with DAMGO or methadone produced internalization of enhanced yellow fluorescent protein-tagged MOR expressed in hippocampal neurons within hours, whereas morphine produced internalization much more slowly, even when accompanied by overexpression of β-arrestin-2. We conclude that DAMGO, methadone, and morphine all produce tolerance in single hippocampal neurons. Morphine-induced tolerance does not necessarily seem to involve receptor endocytosis.

Opioid drugs have been used widely for thousands of years for the control of various ailments such as pain, diarrhea, and cough. Opioid drugs have also been widely used for their subjective effects, and abuse of these substances constitutes an extremely serious social problem throughout the world. The therapeutic and subjective effects of opioid drugs are a manifestation of the activation of an endogenous system of opioid peptides and their receptors that are found throughout the central and peripheral nervous systems (Terenius, 2000). The effects of opioids on neurons have been widely studied. Activation of opioid receptors, which are all members of the G protein-coupled receptor (GPCR) family, produces effects on a number of enzymatic cascades and ion channels, resulting in alterations in neuronal excitability and synaptic communication (Massotte and Kieffer, 1998). Rapid inhibition of neurotransmitter release and neuronal hyperpolarization are thought to be responsible for the major therapeutic effects of opioid drugs. However, defining the molecular basis for chronic opioid-induced phenomena such as tolerance, dependence, and withdrawal has been much more difficult. Presumably, these effects of opioids also result in some way from the activation of opioid receptors (Harrison et al., 1998; Roth et al., 1998). One important line of investigation has focused on the ability of opioid agonists to promote opioid receptor endocytosis, a phenomenon commonly associated with the effects of agonists on GPCRs. One particularly interesting observation has been that opioid agonists differ greatly in their ability to promote μ-opioid receptor (MOR) endocytosis. Thus, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) and etorphine, for example, produce extensive receptor endocytosis, whereas morphine does not (McConalogue et al., 1999; Sternini et al., 2000). These differential effects were originally attributed to differences in drug efficacy. However, recent observations have dissociated the two phenomena. Thus, morphine is nearly as efficacious as DAMGO in its ability to activate G protein-coupled inwardly rectifying potassium channel (K^+) channels in a heterologous expression system, and yet it produces much less receptor endocytosis, whereas methadone produces extensive receptor endocytosis but is clearly less efficacious than DAMGO or morphine in terms of its ability to activate effectors such as G protein-coupled inwardly rectifying potassium channels (Whistler et al., 1999). On the basis of these observations, Whistler et al. (1999) hypothesized that the inability of morphine to produce receptor endocytosis is actually the key to its increased propensity to produce physiological tolerance. Thus, morphine will continue to signal through MOR, whereas drugs such as methadone will be protected from

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ABBREVIATIONS: GPCR, G protein-coupled receptor; MOR, μ-opioid receptor; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; GABA, γ-aminobutyric acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; IPSC, inhibitory postsynaptic current; PCR, polymerase chain reaction; GFP, green fluorescent protein; EYFP, enhanced yellow fluorescent protein; 3-D, three-dimensional; GRK, G protein-coupled receptor kinase; U69,593, (5,7,8)-(+)-*N*-methyl-*N*-(7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl)-benzeneacetamide.

doing so by receptor endocytosis. According to this model, long-term activation of MOR by morphine must produce physiological tolerance at some point downstream of receptor internalization.

To further examine these various hypotheses, we have carried out studies on the effects of different opioids on synaptic transmission in cultures of rat hippocampal pyramidal neurons. Our results indicate that tolerance to the effects of morphine occurs in the absence of MOR endocytosis.

Materials and Methods

Cell Culture. Primary nonautaptic hippocampal and autaptic neuronal cultures were prepared as described previously (Bushell et al., 1999), although in the present study, the hippocampi were dissected from fetal Holtzmann rats at 20 days' gestation. Hippocampal cultures made at 20 days' gestation have an increased number of GABA-ergic neurons. Hence, because in the current study we investigated the effects of opioid agonists on both glutamatergic and GABA-ergic neurons, all experiments were performed on these cultures.

Electrophysiology. For all experiments, the whole-cell, patch-clamp technique was used on hippocampal cultures of 9 to 16 days in vitro. Patch pipettes (3–5 MΩ) were filled with an internal solution containing either 100 mM KCl for evoked or 100 mM CsCl for Ca^{2+} experiments and 1 mM $MgCl_2$, 10 mM HEPES, 10 mM BAPTA, 3.6 mM Mg-ATP, 0.1 mM GTP, 14 mM creatine phosphate, and 50 units/ml creatine phosphokinase (all Sigma, St. Louis, MO), which was adjusted to pH 7.2 with the respective hydroxide solution, osmolarity range 290 to 300 mOsM. For evoked experiments, cells were transferred to a recording chamber that was continuously perfused with bathing medium containing 140 mM NaCl, 10 mM HEPES, 10 mM glucose, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM KCl (all Sigma); the osmolarity was approximately 310 mOsM and pH was 7.4. For Ca^{2+} current experiments, Ba^{2+} was used as the charge carrier; hence, the bathing medium contained 151 mM tetraethylammonium-Cl, 10 mM HEPES, 10 mM glucose, 5 mM $BaCl_2$, and 1 mM $MgCl_2$ (all Sigma), again 310 mOsM and pH 7.4. Autapses were identified electrophysiologically as either glutamatergic or GABA-ergic before application of the Ba^{2+}/tetraethylammonium bathing medium. All data were acquired using an Axopatch-1D amplifier and digitized via a Digidata 1200 interface (both Axon Instruments, Foster City, CA). All experimental data, sampled at either 5 or 10 kHz and filtered at 2 kHz, were acquired on-line by using pClamp 7.0 (Axon Instruments) and stored for later analysis on a Pentium computer. Series resistance observed during all recordings was <10 MΩ, with 80% compensation, 10-μs lag, applied in the Ba^{2+} current experiments. Excitatory postsynaptic currents/IPSCs were evoked from autaptic hippocampal cultures by means of a depolarizing step (70 mV, 2 ms, 0.033 Hz) from the holding potential (−60 mV), whereas Ba^{2+} currents were obtained by means of a depolarizing step (90 mV, 200 ms, 0.033 Hz) from the holding potential (−80 mV). Evoked excitatory postsynaptic currents/IPSCs and Ba^{2+} currents were analyzed using pClamp7 software. Data were statistically analyzed using a paired t test and were considered significant if $p < 0.05$. Data are expressed as mean ± S.E.M.

Neuronal Transfection. Hippocampal cultures of 7 to 10 days in vitro were used in all transfections. The cultures were transiently transfected using the polycationic lipid transfection method. In brief, 2 μl of a 10× diluted stock of polyethylenimine (Sigma) was mixed with 98 μl of 0.15 M NaCl and mixed thoroughly. To this, the equivalent of 2 μg of cDNA was added, mixed thoroughly, and left to stand for 10 min. This mixture was then added directly to neurons on a glass coverslip in a 35-mm culture dish with conditioned media. This was then spun at 1200 rpm for 10 min in a centrifuge. After this centrifugation, the neurons were returned to the incubator for 4.5 to 5 h. The neurons were then removed from the conditioned media and returned to the glial feeder layer from which they were initially removed. In the case of the MOR, trichostatin A (200 ng/ml), a histone deacetylase inhibitor that has been shown to increase gene expression, was required for the visualization of this fluorescently tagged receptor. The neurons were then visualized 24 to 48 h after transfection.

Synthesis of μ-Receptor and β-Arrestin Fluorescent Constructs. Total RNA was prepared from whole adult female Holtzmann rat brains with the use of TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was obtained from 5 μg of total RNA with the use of Superscript II reverse transcriptase and was primed with 100 ng of random primers. The rat β-arrestin-2 was amplified from 3 μl of rat brain cDNA by using TakaRa LA Tag DNA polymerase (Pan Vera, Madison, WI) with the primer; the forward primer was 5′-TGGAATTAAGCTTGCCGCCACCATGGGT-3′ and the reverse primer was 5′-CGGCTTTTCCACCGGTAACTGGTC-3′. After heating at 98°C for 3 min, PCR amplification was carried out for 35 cycles: 98°C for 20 s, 56°C for 1 min, and 72°C for 2 min. The amplified fragment was purified and cloned into pCR3.1 vector (Invitrogen). The resulting clones were verified by restriction analysis and by using the dRhodamine Terminator cycles sequencing kit (PerkinElmer Life Sciences, Boston, MA) automated analysis DNA sequencing. The *Hin*dIII/AgeI fragment of clone was ligated into pEYFP-Nl (CLONTECH, Palo Alto, CA) and also was ligated into pECFP-N1.

The rat μ-opioid receptor was amplified from 3 μl of rat brain cDNA by using *Pfu* DNA polymerase (Promega, Madison, WI) with

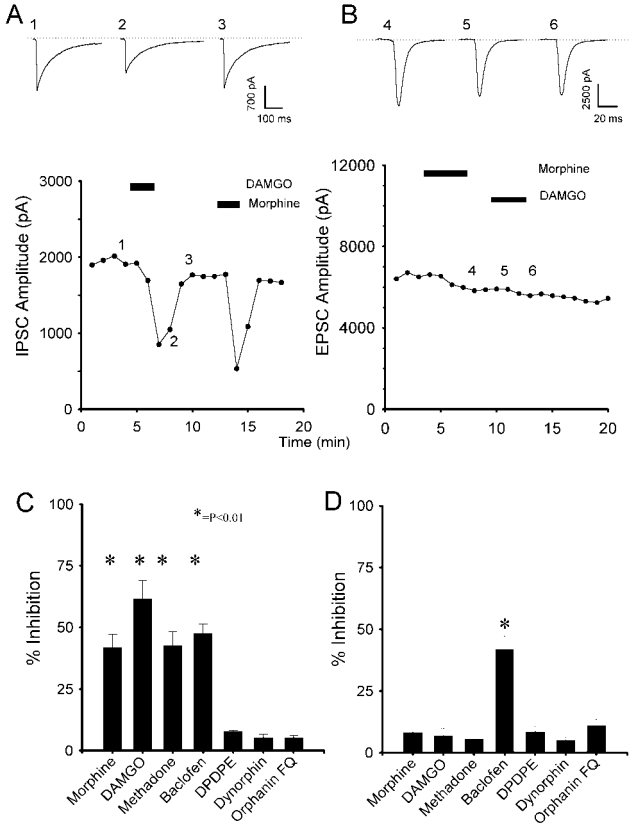

Fig. 1. Opioid inhibition of synaptic transmission at inhibitory and excitatory autapses. Acute application of opiates inhibited synaptic transmission in voltage-clamped inhibitory neurons (A and C) but not at excitatory synapses (B and D). A, inhibition of IPSCs by DAMGO. Representative current traces are shown before, during, and after DAMGO application (1, 2, and 3, respectively). Morphine also inhibited the current as shown in the time course. B, lack of effect of morphine and DAMGO in excitatory hippocampal autaptic neurons. Summaries of synaptic inhibition are shown in C and D for inhibitory and excitatory autapses. Although baclofen was effective at both types of synapses, MOR agonists were only effective at inhibitory autapses.

the primer; the forward primer was 5′-GCGGCCGCCACCATGGACAGCAGCACCGGCCCAGGGAACACC-3′ and the reverse primer was 5′-GTTTAAACGGCAATGGAGCAGTTTCTGCCTCCA-3′. After heating at 96°C for 3 min, PCR amplification was carried out for 35 cycles: 96°C for 30 s, 56°C for 30 min, and 72°C for 2 min. The amplified fragment was purified and ligated into vector W7-pcDNA3.1(−) with *Eco*RV. The resulting clones were verified by restriction analysis and automated DNA sequencing (PerkinElmer Life Sciences), then amplified μ-opioid DNA plasmid using *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA); the forward primer was 5′-AATTCAAAGCTTGCCGCCACCATGGACAGCACC-3′ and the reverse primer was 5′-GAATTCTACCGGTCCCGGCAATGGAGCAGTTTCTGC-3′ under PCR conditions as described above. The PCR product was digested with the *Hin*dIII/Age1 and gel purification (QIAGEN, Valencia, CA), which was ligated into pEYFP-N1.

Internalization. GFP-tagged proteins were visualized using a Fluoview laser scanning confocal microscope with excitation at 488 nm (6% intensity) and 510- to 540-nm emission filters on an Olympus inverted microscope. Because of the relatively poor excitation of cyan fluorescent protein by the 488 laser line, we used EYFP-tagged construct of MOR and β-arrestin-2. GFP-positive neurons were sectioned optically using a 60× objective (numerical aperture, 1.4) at 0.3-nm steps. The 3-D reconstructions were created and analyzed using Metamorph software (Universal Imaging, West Chester, PA).

Drugs. All drugs were diluted from stock solutions that were at least 1000× the final concentration. The drugs used for electrophysiological experiments were diluted in the bathing medium and added via the perfusate. The drugs used were DAMGO, morphine, [D-Pen^2,D-Pen^5]-enkephalin, dynorphin, U69,593 (Upjohn, Kalamazoo, MI), and orphanin FQ. Trichostatin A was obtained from Sigma.

Results

Inhibition of Synaptic Transmission and Ba^{2+} Currents by Various Opioid Agonists. We first investigated the effects of various opioid agonists on synaptic transmission at cultured hippocampal autapses. In the case of GABAergic synaptic transmission, agonists at MORs [i.e., DAMGO (1 μM), morphine (10 μM), and methadone (5 μM)] inhibited transmission, whereas agonists selective for δ-, κ-, and orphan-opioid receptor receptors [e.g., [D-Pen^2,D-Pen^5]-enkephalin (1 μM), dynorphin (1 μM), and orphanin FQ (500 nM)] were ineffective (Fig. 1, A and C). In contrast, agonists for all subtypes of opioid and orphan opioid receptors failed to produce any inhibition of glutamatergic synaptic transmission (Fig. 1, B and D). In addition to the effects of opioid agonists, the GABA-B agonist baclofen inhibited synaptic

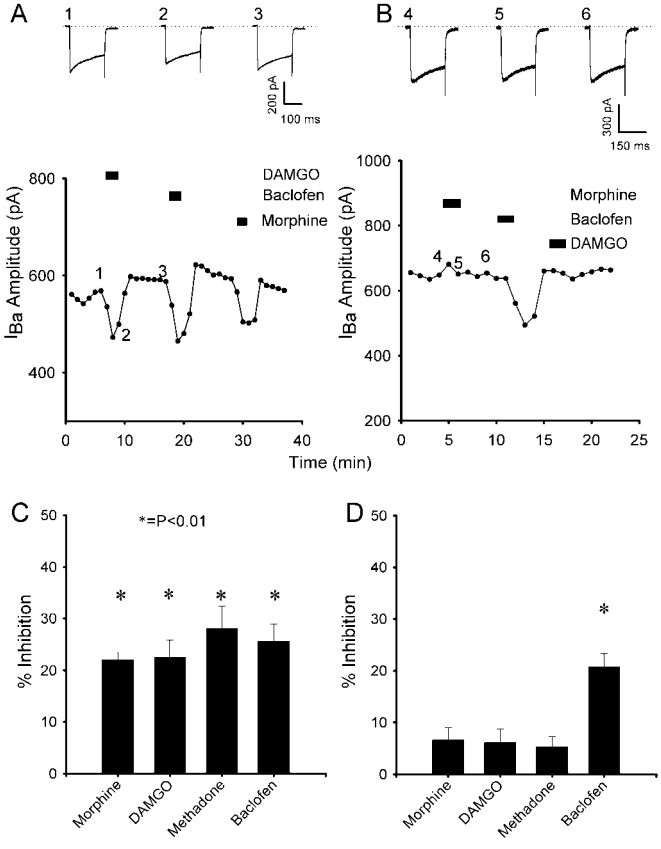

Fig. 2. Opioid inhibition of voltage-gated Ca^{2+} currents. Autaptic neurons were scored as inhibitory or excitatory before these experiments. Acute application of opiates inhibited synaptic transmission in voltage-clamped inhibitory neurons (A and C) but not at excitatory synapses (B and D. A, inhibition of I_{Ba} by DAMGO. Representative current traces are shown before, during, and after DAMGO application (1, 2, and 3, respectively). Baclofen and morphine also inhibited the current as shown in the time course. B, lack of effect of morphine and DAMGO in excitatory hippocampal autaptic neurons. The time course shows baclofen inhibited I_{Ba} in these neurons. Summaries of inhibition are shown in C and D for inhibitory and excitatory hippocampal neurons. Although baclofen was effective at both types of synapses, MOR agonists were only effective in inhibitory neurons.

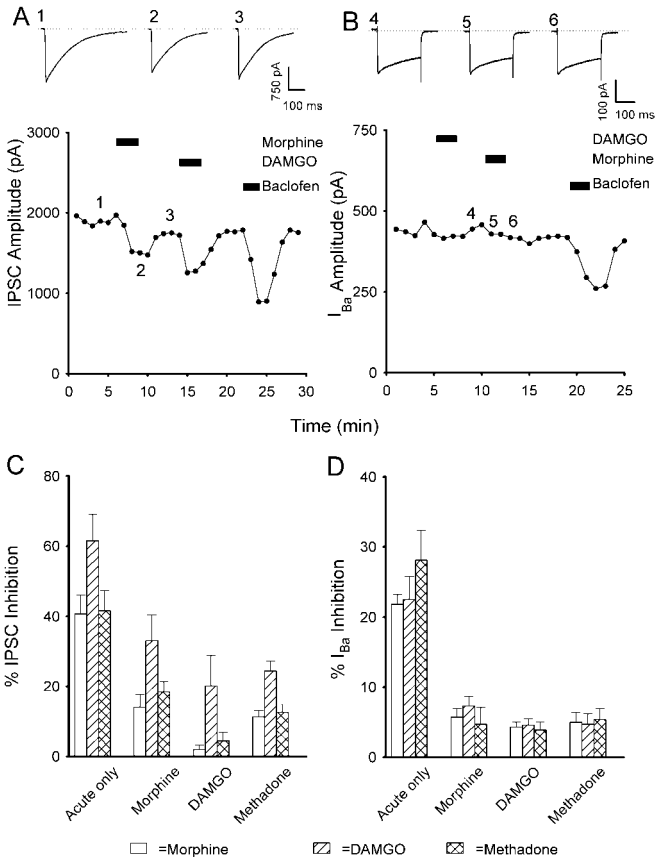

Fig. 3. Development of tolerance in single autapic interneurons. Pretreatment of inhibitory autaptic hippocampal neurons with DAMGO (1 μM) for 48 h diminished the inhibition of the IPSC and I_{Ba} (A and B). Baclofen inhibition was not affected by DAMGO pretreatment. C and D, summary of IPSC inhibition produced by acute (control) application of morphine, DAMGO, and methadone and after pretreatment with each agonist (morphine, DAMGO, and methadone bar clusters) for IPSCs and I_{Ba}, respectively. Self- and cross-desensitization is shown for each drug for each pretreatment condition within the bar clusters. Pretreatment with any opiate, including morphine, produced reduced inhibition of IPSCs and I_{Ba} by all opiates.

transmission in both inhibitory and excitatory autapses (Fig. 1).

The effect of the same agonists on the I_{Ba} in identified GABA-ergic and glutamatergic autaptic neurons was also investigated. In agreement with the observed effects on synaptic transmission, only MOR agonists were able to inhibit I_{Ba} in identified GABA-ergic autapses. All other agonists tested on GABA-ergic and glutamatergic autapses did not inhibit the observed Ba^{2+} currents (Fig. 2). In contrast, baclofen inhibited the I_{Ba} in both inhibitory and excitatory autapses (Fig. 2). These data suggest that of the known opioid receptors, only MOR receptors are expressed in these cultures, and these are specifically located on GABA-ergic neurons, as suggested previously (Simmons and Chavkin, 1996).

Do These GABA-ergic Autapses Exhibit Tolerance after Prolonged Exposure to MOR Agonists? We next investigated whether we could induce tolerance to the various MOR agonists in single autaptic neurons. After a 48-h exposure to DAMGO (1 μM), morphine (10 μM), or methadone (5 μM), application of any of the three agonists resulted in a reduced inhibition of synaptic transmission compared with that observed under control conditions (Fig. 3). In contrast, chronic exposure to any of these opioids did not reduce the effects of baclofen.

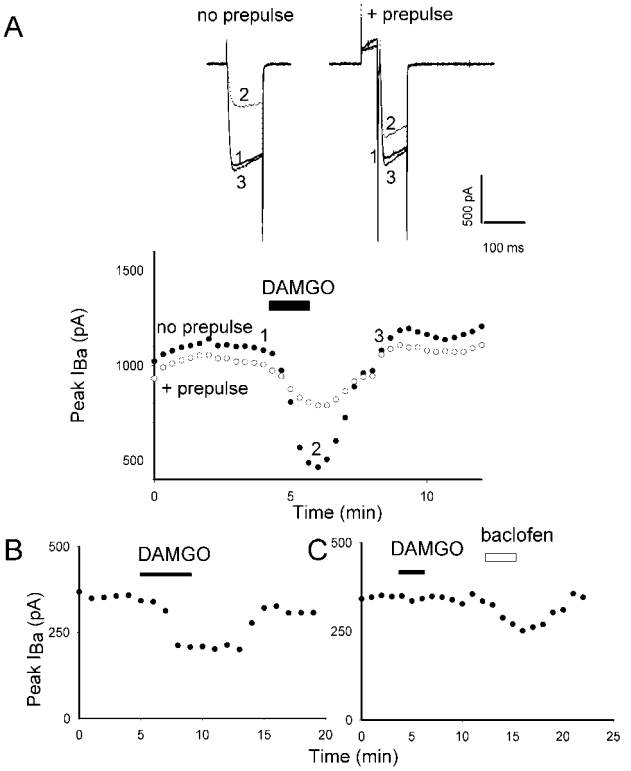

Fig. 4. EYFP-tagged MOR inhibits N-type Ca^{2+} channels in transiently transfected G1A1 cells and I_{Ba} in transfected neurons. Voltage-clamp experiments were performed on G1A1 cells stably expressing N-type Ca^{2+} channels and transfected with the EYFP-MOR. Application of 1 μM DAMGO reversibly inhibited I_{Ba} as shown in A (●). The receptor was shown to be fully functional because the inhibition was partially relieved by strong depolarizing prepulses (A, ○), indicating a voltage dependence of inhibition. Transfection of hippocampal neurons also revealed the tagged receptor to be functional. DAMGO (1 μM) reversibly inhibited I_{Ba} in five of six visually identified transfected neurons (B). In contrast, I_{Ba} was inhibited in only one of six neurons from cells recorded blindly (C).

We also examined the effects of chronic opioid treatment on the inhibition of the I_{Ba}. We treated cells with morphine, methadone, or DAMGO for 48 h and then examined the effect of a challenge dose of drug. As with synaptic transmission, we found that treatment for 48 h with any of the three drugs greatly reduced their ability to inhibit the I_{Ba} (Fig. 3). As with the experiments on synaptic transmission, treatment with any of the opioid agonists for 48 h had little effect on the ability of the GABA-B receptor agonist baclofen to inhibit the I_{Ba}. We also investigated the induction of tolerance after a short-term (1–2 h) exposure of the neurons to DAMGO (1 μM), morphine (10 μM), or methadone (5 μM). In contrast to the long-term exposure, inhibition of the I_{Ba} was still observed after the application of these three agonists, albeit to a lesser extent than under control conditions [19.3 ± 0.9% (n = 4), 16.6 ± 0.9% (n = 4), and 16.7 ± 2.2% (n = 4) for DAMGO, morphine, and methadone, respectively, after DAMGO treatment; 15.6 ± 1.9% (n = 4), 9.1 ± 1.0% (n = 5), and 16.3 ± 2.3% (n = 5), respectively, after morphine treatment; and 13.4 ± 0.9% (n = 5), 22.3 ± 1.4% (n = 4), and 19.7 ± 0.9% (n = 4), respectively, after methadone treatment). In conclusion, we clearly observed tolerance to all three opioid agonists after long-term exposure, and to a lesser extent after short-term exposure, and this tolerance was homologous.

Opioid Agonist Effects on MOR Internalization. We next examined the effects of each opioid on the state of μ-receptor endocytosis. To do this, we synthesized a MOR

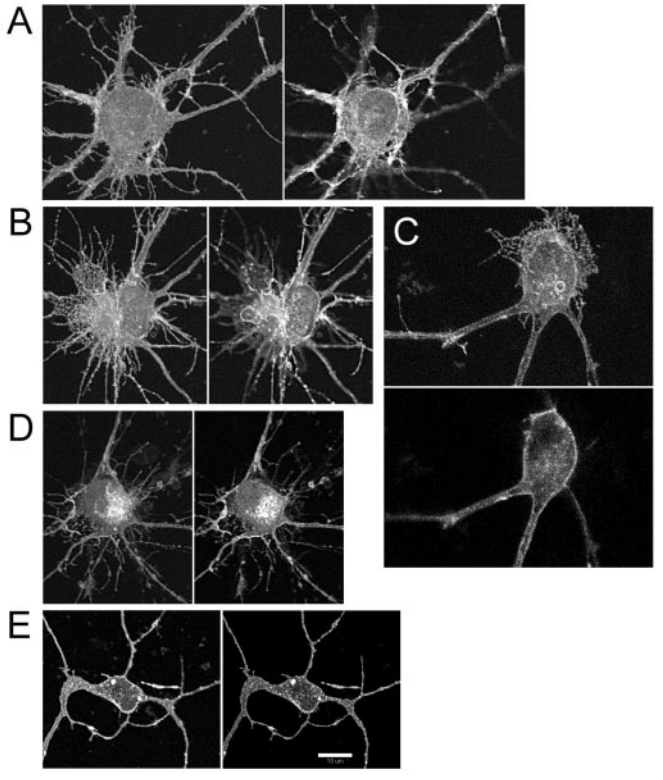

Fig. 5. Fluorescence distribution of EYFP-tagged MOR in hippocampal neurons expressing native (wt) β-arrestin-2. The 3-D reconstructions of confocal images are shown on the left and single, near-equatorial plane images are shown on the right for each example. Images have been filtered with unsharp masking to display more detail. Neurons are 48 h post-transfection and are untreated. Although most neurons had primarily uniform cell surface distributions of MOR, some neurons had limited numbers of internal clumps (D and E).

tagged with EYFP on the C terminus. We found that this did not effect the ability of the receptor to signal by expressing it in G1A1 cells expressing the N-type Ca^{2+} channel α_{1B}. Addition of DAMGO to such cells produced inhibition of the I_{Ba} in the same manner as with the wild-type opioid receptor (Fig. 4). We then transfected the receptor into cultures of hippocampal neurons. The transfection technique that used affords a low percentage of transfected cells (i.e., <1%). We found that in recordings from six fluorescent cells, five showed inhibition of the I_{Ba} when DAMGO was added (Fig. 4), whereas this occurred in only one cell when we recorded from transfected cultures "blindly". Because the majority of the cells in the cultures are excitatory glutamatergic neurons, and because the MOR is normally found only on GABAergic neurons (see above), it is clear that the majority of the transfected cells are excitatory neurons and that the EYFP-tagged MOR couples to Ca^{2+} channels in these cells.

The EYFP-MOR expression was associated with cellular membranes, and the majority of fluorescence was associated with the plasma membrane. This typical distribution was not altered by cotransfection of neurons with wild-type β-arrestin-2. The MOR localization varied from cell to cell with expression limited to the plasma membrane or additionally to membranes within the cytosol (Fig. 5, A–C). The membrane distribution did not correlate to expression levels based on fluorescence intensities (data not shown). Surface membrane association is most evident in the single optical sections made through the dendrites in Fig. 5, A to C. In addition to membrane localization, EYFP was sometimes detected in vesicle-like structures that were perinuclear (Fig. 5D) or dispersed throughout the soma and neurites (Fig. 5E) in addition to the cell surface.

The EYFP-MOR exhibited large changes in distribution after treatment with different opiates. It should be noted that most neurons did not exhibit much receptor internalization at early time points (≤2 h) without the expression of β-ar-

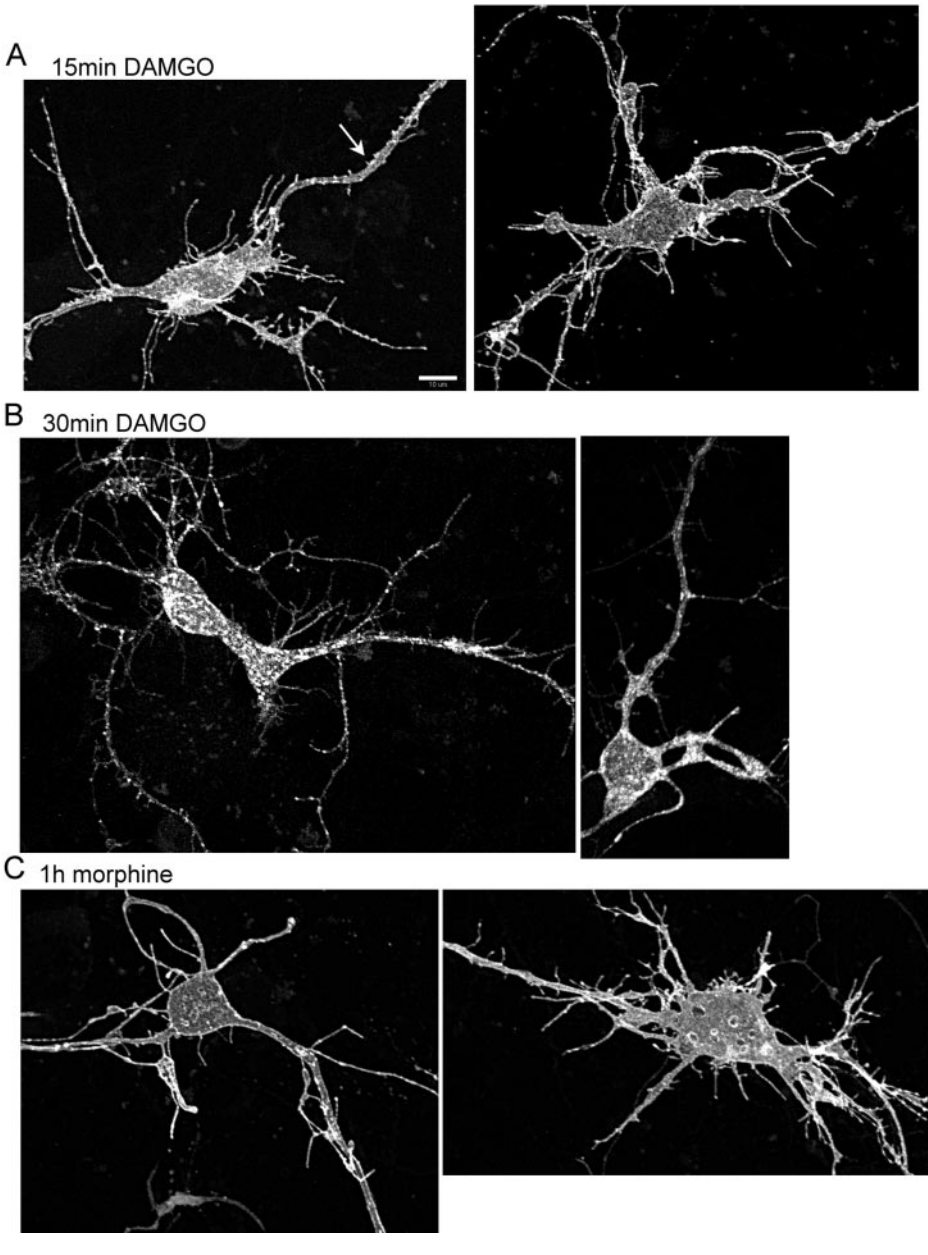

Fig. 6. Early time course of EYFP-MOR internalization in hippocampal neurons after treatment with DAMGO (1 μM). Neurons were cotransfected with EYFP-MOR and β-arrestin-2, 48 h before imaging. Cells were fixed with 4% paraformaldehyde for 20 min to preserve receptor distribution during confocal imaging. MOR clumping becomes evident at 15 min after DAMGO administration, with puncta forming in neurites and at base of spine-like projections (e.g., arrow). Large puncta are present along cell surfaces after 30 min (B) and some puncta have moved into the cytosol (data not shown). In contrast, a 1-h treatment with morphine (10 μM) results in a predominantly uniform surface distribution of EYFP-MOR (C). Scale bar, 10 μm.

restin-2. For this reason, we routinely cotransfected β-arrestin-2 with MOR. Changes in MOR distribution were evident in as little as 15 min after DAMGO application (Fig. 6A). Bright puncta were localized throughout the somatic cytosol and in the neurites, including spines (Fig. 6A, arrow). The diameter and number of puncta increased at 30 min (Fig. 6B). Most puncta were still located near the surface of the neuron. In sharp contrast, most of the MOR remained on the surface even at 1 h after application of morphine (Figs. 6C and 10; see below). Four examples of neurons treated with DAMGO for 1 h are shown in Fig. 7. The size of the puncta was similar to those seen at 30 min. Most neurons showed mainly internal EYFP distributions and relatively little fluorescence remained on the cells surface (one exception is shown in Fig. 7C). Orthogonal projections through reconstructions of DAMGO-treated neurons (1 h) are shown in Fig. 8. The X-Z and Y-Z profiles reveal that most of the MOR was internalized. This internalization was effectively blocked by coapplication of naloxone with most of the receptor uniformly distributed over the surface (Fig. 7E).

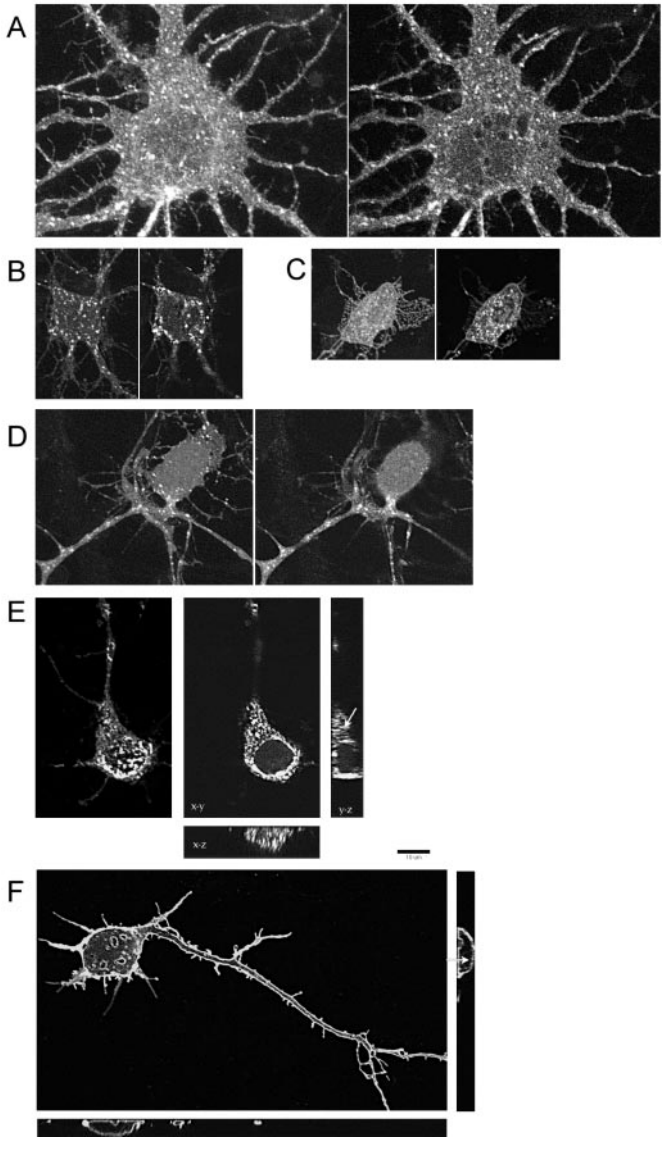

Fig. 7. MOR internalization at 1-h DAMGO treatment and blocked by naloxone. Confocal reconstructions of the entire neuronal soma are shown on the left and near-equatorial planes are shown on the right for each representative neuron. Most of the EYFP-MOR fluorescence is localized to intracellular puncta, but a few neurons also retain a pool in the cell surface (C). Orthogonal (X-Z and Y-Z) projections through the 3-D reconstructed neuron shown in E (left) reveal that most of the MOR is within internal clusters (e.g., arrow) and little is evident on the edges in any projected view. F, shows that coapplication of naloxone (3 μM) blocked the internalization, leaving nearly all of the MOR uniformly distributed on the surface (e.g., arrow). The circular regions within the soma are the result of optically sectioning through dimples within the cell membrane (see X-Z and Y-Z projections).

Treatment with methadone produced a similar redistribution of MOR as that observed with DAMGO (Fig. 8). Although most neurons displayed internalized fluorescence clumps, it was also apparent that most neurons retained a pool of cell surface receptor at 2 h (Fig. 8, B–F). Puncta had similar size and distribution as those obtained with DAMGO.

Morphine produced little or no MOR internalization in hippocampal neurons at 1 h of treatment. Notably smaller internal puncta were visible in some neurons, but the majority of the MOR was located on the cell surface. Prolonged morphine treatment, however, did result in significant receptor internalization in hippocampal neurons. At 60 h, most of the receptor was internalized (Fig. 9, E and F). These puncta were much smaller than those produced by DAMGO or methadone. Although we were unable to fully assess the level of receptor remaining on the cell surface from confocal images, the cell surface appeared largely devoid of GFP fluorescence at late time points after morphine treatment.

Effects of Opioids on β-Arrestin-2 Distribution. Uncoupling of GPCRs from signaling through heterotrimeric G proteins requires the binding of β-arrestin-2. We therefore examined the effects of acute and chronic opioid treatment on the distribution of β-arrestin-2. To do this, we cotransfected cells with the native MOR (untagged) and EYFP-tagged β-arrestin-2.

EYFP-β-arrestin-2 expression was limited to the cytosol; the nuclear volume was devoid of fluorescence (Fig. 10). The fluorescence was generally uniform, but vesicle-like puncta were also found in some neurons. Two examples of EYFP-β-arrestin-2–expressing neurons are shown in Fig. 10A. The 3-D reconstructions are shown on the left; near-equatorial single optical sections are shown on the right. Occasionally, linear fluorescent stripes appeared to run across the nuclear space (Fig. 10, A and B), perhaps representing folds in the nuclear envelope. Increased EYFP fluorescence was often located in the perinuclear region (Fig. 10, B and D). This may represent protein in lysosomes or aggresomes awaiting degradation and could be observed in control and opiate-treated cells. β-Arrestin-2 remained relatively uniformly dispersed in neurons treated with DAMGO and methadone (Fig. 10, B and C) and actually appeared more uniform after morphine treatment (Fig. 10D).

Discussion

The molecular basis for opiate tolerance and dependence remains poorly understood despite widespread investigation in several preparations, including the hippocampus (Fan et al., 1999; Lu et al., 2000). Numerous cellular mechanisms may be involved in these phenomena, including activation of kinases (e.g., G protein-coupled receptor kinases, cAMP-dependent kinases, mitogen-activated protein kinase, Ca^{2+}/calmodulin kinases, protein kinase C), MOR internalization/

sequestration/down-regulation, up-regulation of *N*-methyl-D-aspartate receptors, ion channel modulation, nitric oxide production, and compensatory changes in neurocircuitry (Mao et al., 1995; Mansouri et al., 1997; Pitcher et al., 1998; Mayer et al., 1999; Wang and Sadée, 2000). Many investigations of these phenomena have used both heterologous and in vivo expression systems. Unfortunately, these two different approaches have resulted in somewhat contrasting conclusions. For example, morphine causes relatively little internalization of expressed MOR (Keith et al., 1996; Whistler et al., 1999), whereas binding studies in vivo have revealed down-regulation of the receptor binding after prolonged exposure to morphine (Díaz et al., 2000; Shen et al., 2000; Tao et al., 2000).

Adding to this complexity is the differing capacity of opiates to produce tolerance in vitro. For example, Whistler et al. (1999) have reported that morphine treatment did not produce uncoupling or endocytosis, whereas DAMGO was effective. Indeed, these authors proposed that the lack of endocytosis and uncoupling induced by morphine might be precisely why it produces many of its long-term effects, because it will be able to continue to signal, provoking compensatory downstream effects.

Although chronic application of opiates is expected to produce changes in all neurons expressing the appropriate receptors, recent studies have implicated the hippocampus as playing a central role in tolerance, dependence, and withdrawal (Fan et al., 1999; Lu et al., 2000). In the present study, we have investigated tolerance to opiates in cultured hippocampal neurons by using electrophysiological and imaging techniques. We have used the endogenous MOR present in inhibitory hippocampal neurons as well as cotransfected MOR and β-arrestin-2. Synaptic transmission and Ba^{2+} currents in autaptic GABA-ergic neurons were sensitive to the actions of three MOR agonists, consistent with previous data with hippocampal slices (Simmons and Chavkin, 1996). Prolonged and to a lesser extent short-term exposure of autapses to these agonists led to subsequent applications becoming less effective, a phenomenon that we interpret as a cellular correlate of tolerance. Because the experiments were performed by looking at synaptic transmission and at Ba^{2+} currents with a somatodendritic localization, the data also suggest that tolerance is not localized to the synapse alone. Previous electrophysiological studies investigating the induction of tolerance have used either primary neuronal cultures or slices from addicted animals (Connor and Christie, 1999; Connor et al., 1999; Manzoni and Williams, 1999). Our data therefore suggest that tolerance is not entirely attributable to compensatory mechanisms in neuronal networks. The observed tolerance to MOR agonists was also shown to be homologous, because the $GABA_B$ agonist baclofen was equally effective against synaptic transmission and Ba^{2+} currents before and after MOR agonist exposure. This is in agreement with previous findings in the locus ceruleus, where sensitivity to α_2-adrenoceptor agonists was unaltered after the induction of tolerance (Connor et al., 1999). Although these data indicate that tolerance is an intrinsic property of isolated GABA-ergic neurons, they do not reveal the molecular mechanisms required for its induction. We therefore used transient transfection of hippocam-

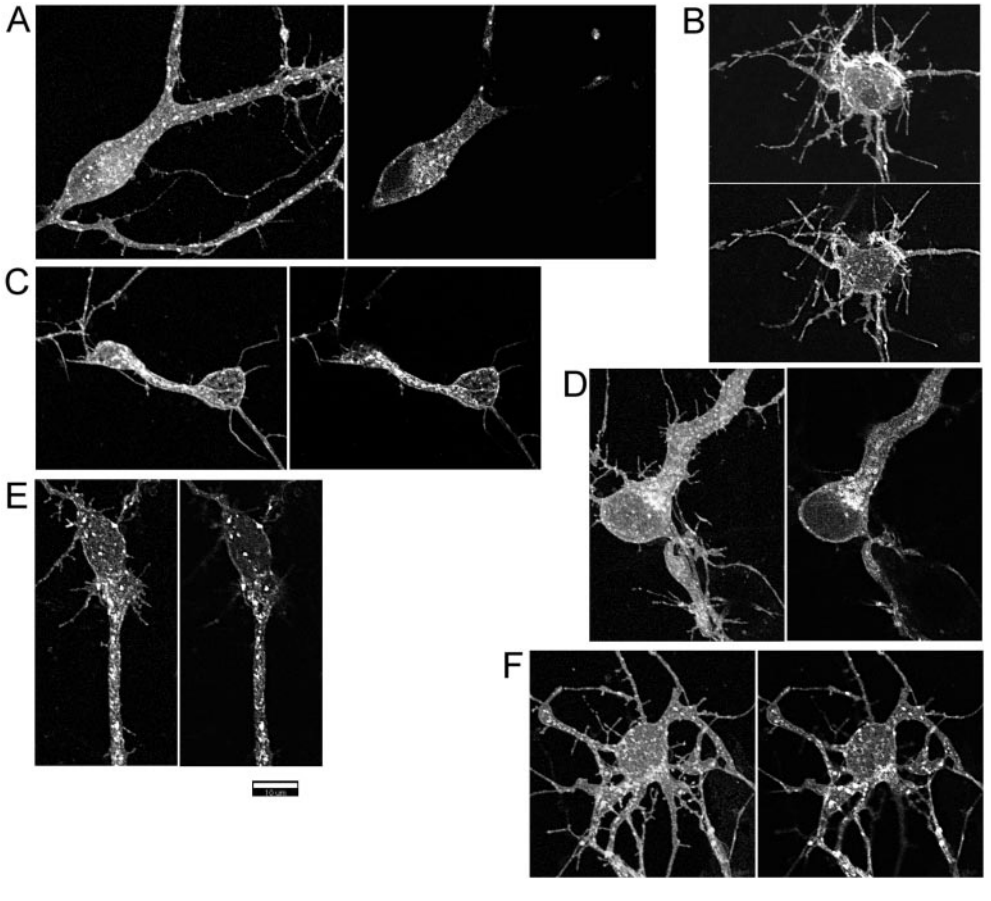

Fig. 8. MOR is internalized slower/less with methadone. Neurons are 48 h post-transfection with EYFP-MOR and β-arrestin-2. Note the abundant internal puncta and the presence of MOR on cell surface (B–F). Images are from neurons methadone treated (5 μM) for 2 h before fixation.

pal neurons to investigate the possible agonist-induced redistribution of MOR, a phenomenon previously suggested as being involved in opiate tolerance.

Redistribution of MOR from the cell surface into cytoplasmic clusters was observed at all the time points, early and late, after application of DAMGO and methadone. Morphine, on the other hand, produced little or no redistribution after short exposure times, but significant redistribution was observed after a 60-h exposure. These data may account for some of the previous differences noted between heterologous expression systems and in vivo data. The majority of experiments performed in expression systems have used relatively short exposures (minutes) to the relevant MOR agonists (Arden et al., 1995; Keith et al., 1996; Whistler et al., 1999), whereas in vivo experiments have been performed using longer exposures (Díaz et al., 2000; Shen et al., 2000; Tao et al., 2000). The issue of time course seems to be particularly relevant concerning the internalization of MOR when activated by morphine. Hence, the hypothesis that morphine produces tolerance through its inability to produce internalization may be true for short-term exposures, as shown in the present study by both electrophysiological and imaging means, whereas this may not be true for longer exposures. It is clear from our own and other studies that in the presence of sufficient β-arrestin-2, morphine-induced MOR internalization eventually becomes significant. The level of β-arrestin-2 expression may be a significant factor in determining the normal degree of tolerance observed. It is clear from data with β-arrestin-2 knock-out mice that the effects of morphine are significantly prolonged in these animals, suggesting that morphine-induced tolerance is at least partially dependent on the availability of β-arrestin-2, and involves β-arrestin-2-mediated MOR uncoupling and/or internalization (Bohn et al., 1999).

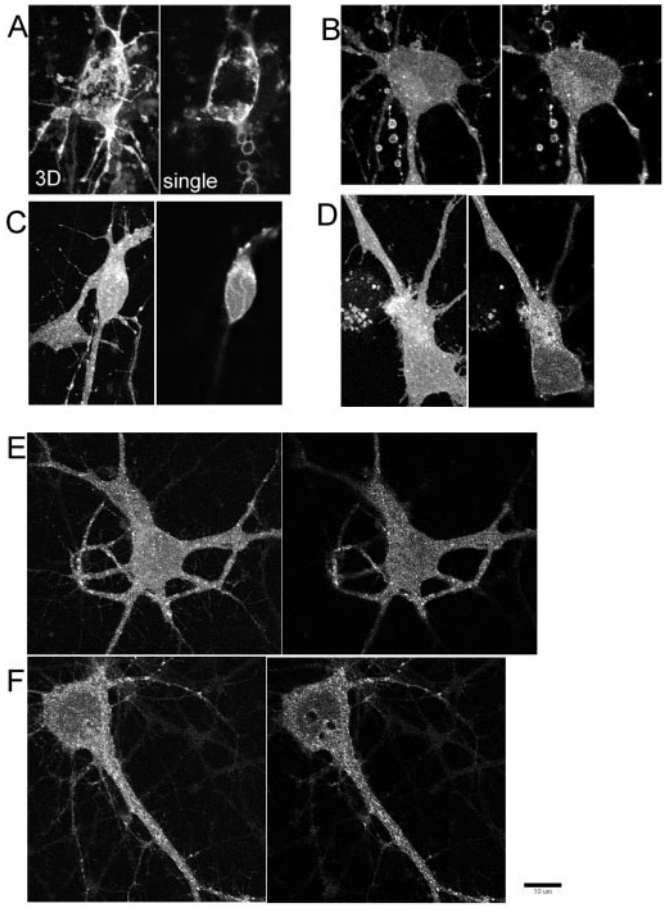

Fig. 9. Morphine treatment causes relatively little MOR redistribution at 2 h but becomes internalized after long morphine applications. A to D, four examples of neurons treated with morphine for 2 h. Although small puncta are visible in some neurons (A and D), fluorescence is evident at the cell surface. Treatment (10 μM) for 60 h produced notable internalization of MOR (E and F). The left image in each example is a 3-D confocal reconstruction of the soma and the right image is a single optical plane through the same neuron.

Although we observed internalization of MOR in the presence of overexpressed β-arrestin-2, a degree of tolerance in our electrophysiological experiments occurred with the endogenous complement of molecular components. Indeed, that MOR internalization can be observed does not necessarily mean that it is responsible for opiate-induced tolerance, as evidenced by receptor redistribution after short-term exposure to opiate agonists, whereas in electrophysiological experiments, inhibitions of I_{Ba} are still observed after a 1- to 2-h exposure. Thus, it is quite likely that a maximum response to opiate stimulation could still occur with a much smaller population of coupled MOR on the cell surface (i.e., the "spare receptor" phenomenon) (Sternini et al., 2000). If the number of spare receptors were large, then a very high percentage of the available MOR would have to uncouple or be internalized before signaling is compromised. Our studies would indicate that morphine-induced internalization does not mediate the tolerance observed after short- or long-term exposures to morphine. If however, we put our data in the context of the β-arrestin-2 knock-out results, which reveal a reduced level of tolerance to morphine (Bohn et al., 1999), it seems likely that morphine-induced β-arrestin-2 interactions or MOR internalization is normally important.

Expression of tagged-β-arrestin-2 produced a smooth cytosolic distribution in agreement with previous studies with heterologous expression systems (Barak et al., 1997; Zhang et al., 1998). At all time points investigated, little or no redistribution of the β-arrestin-2 was observed. Although there is general agreement that β-arrestin-2 is involved in the down-regulation of GPCRs, the movement of the protein seems to depend on the GPCR investigated (Groarke et al., 1999). Our data are in agreement with Zhang et al. (1998) who saw little translocation of β-arrestin-2 in the absence of overexpressed GRK2, when looking at the internalization of MOR. The role of GRKs in the desensitization/internalization of opioid receptors is well documented (Zhang et al., 1998; Li and Wang, 2001). In the present study, we have not investigated the role of GRKs in the induction of tolerance, although their involvement is likely to be critical for the desensitization/internalization of the receptor. Overexpression of β-arrestin-2 may be required to observe internalization because the majority of cells transfected were likely to be excitatory, rather than inhibitory, and thus the required molecular components for the internalization of the receptor may have been either absent or reduced.

In conclusion, we have demonstrated that tolerance to the effects of MOR agonists of different types can occur in single, naïve hippocampal neurons in vitro. Of particular importance is the observation that morphine-induced internalization of MOR can occur after chronic drug treatment. Thus, our data suggest a β-arrestin-2-mediated uncoupling/inter-

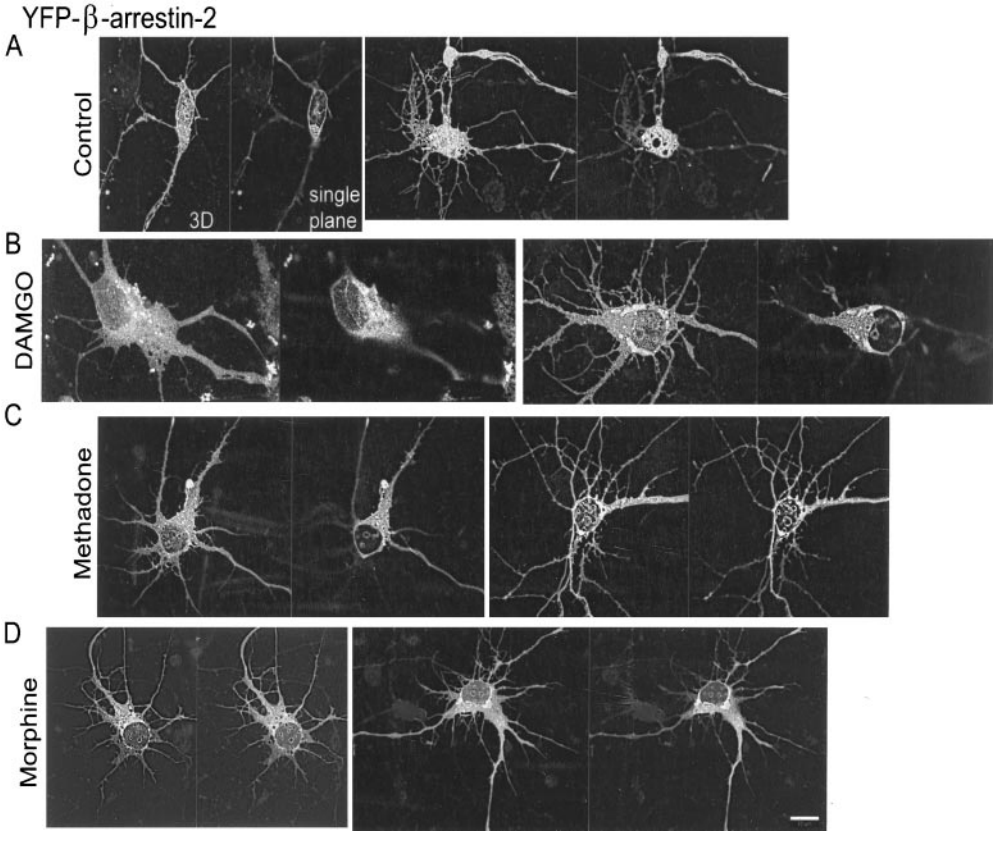

Fig. 10. Changes in β-arrestin distribution in neurons transfected with EYFP-β-arrestin-2 + wt-MOR and treated with DAMGO, morphine, or methadone for 2 h. Note the increased prevalence of the line-like stripes across the nuclear space and clumped appearance in some (DAMGO; B, right). Overall, there is little change in the pattern versus control neurons. The β-arrestin fluorescence appeared more uniform after morphine treatment (D).

nalization may play an important role in the ultimate development of morphine-induced tolerance, consistent with recent data that revealed a decreased level of tolerance in β-arrestin-2 knock-out mice (Bohn et al., 1999). Nevertheless, we have also demonstrated that a degree of tolerance can occur after relatively short treatments with morphine, when little morphine-induced MOR uptake occurs. Thus, our data suggests a β-arrestin-2-mediated uncoupling/internalization may play an important role in the ultimate development of morphine-induced tolerance, consistent with recent data obtained using β-arrestin-2 knock-out mice.

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