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Multiple Actions of Spinophilin Regulate Mu Opioid Receptor Function

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SUMMARY

Spinophilin, a dendritic spine-enriched scaffold protein, modulates synaptic transmission via multiple functions mediated by distinct domains of the protein. Here, we show that spinophilin is a key modulator of opiate action. Knockout of the spinophilin gene causes reduced sensitivity to the analgesic effects of morphine and early development of tolerance but a higher degree of physical dependence and increased sensitivity to the rewarding actions of the drug. At the cellular level, spinophilin associates with the μ opioid receptor (MOR) in striatum and modulates MOR signaling and endocytosis. Activation of MOR by opiate agonists such as fentanyl and morphine promotes these events, which feedback to suppress MOR responsiveness. Our findings support a potent physiological role of spinophilin in regulating MOR function and provide a potential new target for the treatment of opiate addiction.

INTRODUCTION

Repeated exposure to opiates leads to addiction, characterized by drug craving, compulsive drug use, dependence, and analgesic tolerance (Koob et al., 1998; Kreek, 2001; De Vries and Shippenberg, 2002; Inturrisi, 2002). At the cellular level, opiates exert their effects via G protein-coupled receptors, primarily the μ opioid receptor (MOR) (Contet et al., 2004). Several proteins, including adenylyl cyclases, G protein receptor kinases (GRKs), β-arrestins, and regulators of G protein signaling (RGS proteins), have been shown to play a role in different aspects of opiate action by modulating the signaling duration and desensitization of MORs (Li et al., 2006; Terman et al., 2004; Marker et al., 2004; Bohn et al., 2000; Zachariou et al., 2003; Kim et al., 2006). Among the best characterized cellular

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adaptations to chronic opiate exposure are upregulation of cAMP signal transduction, changes in the rate of opioid receptor endocytosis, and altered ERK activation (Nestler and Aghajanian, 1997; Law and Loh, 1999; von Zastrow et al., 2003). However, the timing and cellular specificity of these responses in the brain have yet to be understood.

Growing evidence indicates that the actions of proteins which modulate G protein-coupled receptor signaling are dramatically affected by their binding partners and the multiprotein complexes that they form under specific physiological conditions. Consequently, molecules such as β-arrestin-2 may promote or inhibit receptor signaling depending on other factors (Wang et al., 2004). Our hypothesis is that repeated opiate exposure leads to adaptations that promote deleterious actions of opiate drugs (craving, dependence, tolerance) by preventing the formation of multiprotein complexes necessary for proper MOR signaling. Therefore, optimizing the action of proteins that are essential for the function of such complexes may delay the progress of addiction.

Spinophilin, a ubiquitously expressed, dendritic spine-enriched protein (Allen et al., 1997) interacts with several elements of the G protein-coupled receptor signaling network, including protein phosphatase 1 (PP1), RGS proteins, and dopamine D_2 receptors (Hsieh-Wilson et al., 1999; Wang et al., 2005; Smith et al., 1999). Recent studies revealed that spinophilin modulates α_2-adrenergic responses by blocking association of GRK2 with the agonist-receptor-Gβγ complex and, therefore, by antagonizing β-arrestin-2 function (Richman et al., 2001; Brady et al., 2003; Wang et al., 2004). Since MORs share many of their signal transduction pathways with α_2 receptors (i.e., both are Gi linked), it is likely that spinophilin plays a role in MOR signal transduction and desensitization and, consequently, in opiate addiction. Neurabin is a structural analog of spinophilin, with PPI and F-actin binding properties, and a similar pattern of distribution (Burnett et al., 1998; Terry-Lorenzo et al., 2002). A major difference between the two proteins is that spinophilin contains two PKA phosphorylation sites (Ser-94 and Ser-117) and a receptor binding/interacting domain (aa 169–255) that are absent from neurabin (Allen et al., 1997; Richman et al., 2001; Hsieh-Wilson et al., 1999).

The goal of the present study was to determine the influence of spinophilin on the acute and chronic actions of opiates. We relied on the analysis of spinophilin and neurabin knockout mice (Feng et al., 2000; Allen et al., 2006) in a series of behavioral paradigms. We show that spinophilin, but not neurabin, plays a prominent physiological role in regulating behavioral responses to opiates by opposing the development of dependence and tolerance and by decreasing sensitivity to the rewarding actions of the drugs. We also show that spinophilin affects MOR functional responses by promoting endocytosis and by inhibiting MOR signaling. Furthermore, our data indicate that opiate drugs induce association of spinophilin with MOR in striatum by promoting the formation of complexes between spinophilin and GRK2, RGS9-2, and Gβ subunits, signal transduction molecules that are essential for MOR signaling and desensitization.

RESULTS

Regulation of Spinophilin Levels by Opiate Exposure In Vivo

As a first step to investigate the role of spinophilin in physiological responses to opiates, we administered several MOR agonists to mice, at doses which produce maximal analgesic responses in behavioral tests, and monitored spinophilin levels in the nucleus accumbens, the ventral portion of the striatum which is important for many opiate actions. Table 1 shows regulation of spinophilin levels, measured by immunoblotting, in the nucleus accumbens two hrs after administration of different MOR agonists. Spinophilin is downregulated in this brain region by acute morphine (20 mg/kg, i.p.) administration. A similar effect was

observed in response to acute administration of other opioid agonists, such as methadone (20 mg/kg, i.p) and fentanyl (0.25 mg/kg, s.c.). Our initial working hypothesis was that downregulation of spinophilin following acute opiate administration is one of the cellular adaptations that contribute to MOR desensitization. Interestingly, exposure to opiates has no effect on neurabin levels and administration of the α_2-adrenergic agonist clonidine does not affect spinophilin levels in the nucleus accumbens at the 2 hr time point (Table 1). In contrast to the effects of acute morphine exposure, chronic morphine has no effect on spinophilin or neurabin levels in this brain region (not shown). Loss of spinophilin regulation by chronic morphine may be interpreted as one of the adaptive changes that follow continuous morphine use.

Behavioral Responses to Opiates in Spinophilin Knockout Mice

To gain insight into the influence of spinophilin on opioid receptor function, we investigated morphine analgesia in the 52°C hot plate assay in mice lacking the spinophilin gene. As shown in Figure 1A, spinophilin mutants are less sensitive to low doses of morphine in this assay compared to their wild-type littermate controls. The spinophilin knockout mice are similarly less sensitive to the analgesic actions of methadone and fentanyl in this assay (Figures 1B and 1C). These findings suggest that spinophilin may be a positive regulator of analgesic responses to opiate drugs. We next tested the effect of spinophilin knockout on analgesic responses to a nonopiate drug, namely, the α_2-adrenergic receptor agonist, clonidine. It has been shown previously that spinophilin is a *negative* modulator of α_2-adrenergic receptor responses (Wang et al., 2004). Consistent with these earlier data, we found that spinophilin mutant mice are *more* sensitive to the analgesic effects of clonidine (Figure 1D). These findings show that spinophilin modulates analgesic responses in a receptor selective manner: it is a positive modulator of MOR actions, but a negative modulator of α_2-adrenergic actions.

To assess the role of spinophilin in the development of analgesic tolerance, we used the 52°C hot plate assay and monitored morphine effects once a day for 4 consecutive days. Under these conditions, mice show a gradually reduced response to morphine due to development of tolerance and, by day 4, they show no analgesic response to a constant dose of the drug. Interestingly, spinophilin knockout mice develop maximal tolerance following a single exposure to morphine, as they show no response to the drug by day 2 of the hot plate assay (Figure 1E). To confirm that this is a morphine-related phenotype, we used the same paradigm and administered the α_2 adrenergic agonist, clonidine. In contrast to what we observed with morphine, analgesic responses to clonidine (0.4 mg/kg) remained unchanged in both genotypes over 3 days of testing (% MPE for wild-type, day 1 = 25 ± 7, day 2 = 23 ± 3, day 3 = 39 ± 10; %MPE for knockout, day 1 = 58 ± 7, day 2 = 64 ± 3, day 3 = 70 ± 10). Notably, neurabin knockout mice develop tolerance to morphine at the same rate as their wild-type littermate controls (Figure 1F). These latter data show that rapid development of tolerance is not a general feature of depletion of proteins containing PP1 interacting/F-actin binding domains. Furthermore, the facilitated tolerance seen in spinophilin knockout mice does not reflect a general learning phenomenon, since initial analgesic responses are reduced and the mice show no abnormality in the Morris water maze (not shown).

In addition to tolerance, chronic exposure to opiates causes severe physical dependence. The role of spinophilin in opiate physical dependence and withdrawal was evaluated using a standard morphine withdrawal paradigm. Mice were treated chronically with increasing doses of morphine, and withdrawal was precipitated by administration of the opioid receptor antagonist, naloxone (1 mg/kg, s.c.). Several signs of opiate withdrawal were monitored for 25 min after naloxone administration. We found that the intensity of withdrawal is ~2-fold higher in spinophilin knockout mice compared to their wild-type controls for most of the signs monitored (Figure 2A). In contrast, no significant difference in opiate withdrawal

behavior was observed between neurabin knockout and wild-type mice. In fact, for some of the withdrawal signs, there was a trend for milder withdrawal in the neurabin mutants. However, this effect was significant only for weight loss (e.g., jumps for wild-type = 76.2 ± 34, for knockout = 22 ± 14; wet dog shakes for wild-type = 6 ± 2.6, for knockout = 3.6 ± 1.1; diarrhea for wild-type = 9 ± 1, for knockout = 5 ± 1.8; weight loss for wild-type = 3.9 ± 0.54, for knockout = 1.78 ± 0.38*, n = 7–8 per group, $p < 0.05$, two way ANOVA followed by Bonferroni test). These data suggest that spinophilin plays an important physiological role opposing the development of morphine dependence.

We next examined the influence of spinophilin on the rewarding actions of morphine using the place preference paradigm, in which animals learn to prefer an environment paired with drug exposure. In this test, knockout of spinophilin increases the sensitivity of animals to morphine reward (Figure 2B). No differences were observed between neurabin knockout mice and their wild-type controls in this paradigm—place preference scores (in s) for wild-type were as follows: saline = −64 ± 57, morphine (5 mg/kg, i.p.) = 284 ± 67, morphine (10 mg/kg, i.p.) = 390 ± 69; knockout were as follows: saline =−68 ± 24,morphine (5 mg/kg, i.p.) = 240 ± 39, morphine (10mg/kg, i.p.) = 272 ± 37. The opposite phenotype was observed using a model of spinophilin overexpression in the nucleus accumbens. C57Bl/6 mice overexpressing spinophilin in the nucleus accumbens 3 weeks after bilateral stereotaxic injection of an AAV-spinophilin vector (which causes a 78% ± 23% increase in spinophilin protein levels selectively in this brain region determined by immunoblotting), show reduced sensitivity to morphine place conditioning (5 mg/kg, s.c.) compared to AAV-GFP injected control mice (Figure 2C). These data specifically relate the altered behavioral sensitivity to morphine seen in spinophilin knockout mice to the nucleus accumbens per se and argue against the involvement of a developmental abnormality in this phenomenon.

Finally, we examined the influence of spinophilin in the locomotor activating effects of morphine (10 mg/kg, s.c.). In this test, deletion of the spinophilin gene results in increased locomotor responses to acute and repeated morphine administration (Figure 2F). In contrast, viral-mediated overexpression of spinophilin in the nucleus accumbens does not affect the acute locomotor activating effect of morphine but prevents the development of locomotor sensitization to repeated drug exposure (Figure 2E).

Deletion of the spinophilin gene does not alter opioid receptor levels in nucleus accumbens, as determined by specific binding of [^{3}H]-DAMGO, a highly selective MOR ligand, in striatal membranes of wild-type and knockout mice: wild-type = 109.15 ± 15 fmols/mg protein, knockout = 99.9 ± 13 fmols/mg protein. In addition, MOR mRNA levels (measured using real time PCR and expressed as fold change over control) in this brain region are unaffected by the loss of spinophilin: wild-type = 1 ± 0.06 and knockout = 0.85 ± 0.26. Likewise, striatal levels of proteins involved in MOR responses, such as RGS9-2, RGS4, β-arrestin-2, and Gβ5, determined by immunoblotting, are also not different in nucleus accumbens between wild-type and knockout mice (not shown).

Influence of Spinophilin on Cellular Responses to Opiates In Vitro and In Vivo

We used coimmunoprecipitation to investigate the effect of opiate drugs on the formation of complexes between spinophilin and MOR in mouse striatum. These studies revealed that under basal conditions MORis associated with spinophilin and that this association is greatly enhanced 30 min after s.c. administration of the MOR agonists, fentanyl or morphine (Figure 3A). We also found that activation of MOR promotes the association of spinophilin with GRK2 (Figure 3B) and with Gβ5 (Figure 3C). In fact, exposure to opiates promotes the formation of spinophilin complexes with all Gβ subunits in striatum (not shown). As Gβ5 is a binding partner of the striatal-enriched protein RGS9-2, we examined whether RGS9-2 is also coprecipitated with spinophilin after stimulation of MOR. As expected, the spinophilin-

RGS9-2 complex is greatly enhanced in striatum following fentanyl or morphine treatment (Figure 3D).

We next investigated the effect of loss of spinophilin on signal transduction events that follow activation of MOR. For this purpose, we monitored how the loss of spinophilin affects MOR endocytosis, inhibition of adenylyl cyclase, and ERK phosphorylation, all three being well-characterized responses to acute MOR activation (Nestler and Aghajanian, 1997; Law and Loh, 1999; von Zastrow et al., 2003; Eitan et al., 2003; Muller and Unterwald, 2004). We first monitored the effect of morphine on ERK phosphorylation and inhibition of cAMP formation in striatum of spinophilin knockout mice and their wild-type littermates. As expected, systemic administration of morphine increases phosphoERK levels in wild-type striatum at 20 min but causes a significantly greater induction of phosphoERK in the absence of spinophilin (250% of saline in striatum of knockout mice versus 150% of saline observed in wild-type controls; Figure 4A). As a control, we monitored phosphoERK levels in striatum from spinophilin wild-type and knockout mice following saline or clonidine (0.4 mg/kg) administration. Contrary to observations with morphine, clonidine had no effect of on ERK phosphorylation in striatum in wild-type (123% ± 20%) or spinophilin knockout (86% ± 11%) mice.

In striking contrast to regulation of ERK, spinophilin is required for opioid inhibition of cAMP formation in striatum: the MOR agonist DAMGO reduces cAMP formation in striatal homogenates of wild-type mice, an effect not observed in extracts of knockout mice (Figure 4C).

To evaluate the role of spinophilin in MOR endocytosis, we first monitored the rate of MOR internalization following exposure to opiate drugs in the presence or absence of spinophilin in PC12 cells. MOR internalization was quantified using ELISA in PC12 cells transiently transfected with epitope-tagged MOR (HA-MOR), along with a spinophilin plasmid or empty vector. Under control conditions, application of the opioid peptide DAMGO leads to fast internalization of MOR, while morphine application leads to delayed MOR internalization. As shown in Figure 5A, overexpression of spinophilin promotes morphine-induced MOR internalization so that the receptor is internalized within 30 min. Similar results were observed using immunofluorescence to directly visualize HA-MOR in PC12 cells (Figure 5B).

We next monitored MOR endocytosis in cultured mouse embryonic fibroblasts (MEFs) from spinophilin wild-type and knockout embryos using ELISA. We used MEF cultures and not cultured striatal neurons, because homozygous spinophilin knockout mice do not breed well and, while we were able to obtain sufficient quantities of MEF cells from embryos of heterozygous crosses, it was not possible to derive sufficient quantities of striatal neurons. MEF cells were infected with a herpes simplex virus (HSV) vector expressing HA-MOR (HSV-HA-MOR) (Haberstock-Debic et al., 2003), and drug treatments were applied 24 hr later. Similar to what was observed previously in striatal cultures (Haberstock-Debic et al., 2005), both morphine and DAMGO induce MOR internalization within 30 min in MEF cells. Interestingly, endocytosis is observed within 30 min in wild-type MEFs, but not in MEFs from spinophilin knockout mice (Figure 5C). In addition, MEF cells from spinophilin wild-type and knockout mice were infected with HSV-HA-MOR, and MOR cellular localization was monitored 24 hr later using immunofluorescence. In wild-type MEFs, MOR is internalized 30 min following morphine treatment (10 μM), however, no internalization is observed in MEFs from spinophilin knockout embryos at this time point (Figure 5D).

We also determined the action of spinophilin in MOR endocytosis in wild-type striatal neurons in primary culture. To knock down spinophilin expression in the neurons, we

transfected striatal cultures with a small interfering RNA (siRNA) directed against spinophilin or a control siRNA as described by Wang et al. (2004). We found that this treatment is effective in reducing spinophilin protein levels by >75% (24 ± 2 of control; Figure 6A) as determined by immunoblot analysis. The cells were then infected with HSV-HA-MOR and 24 hr later, HA-MOR localization was monitored using immunofluorescence. As shown in Figure 6B, under these conditions, administration of 10 μM morphine causes robust MOR internalization at 30 min in control, but internalization is significantly delayed in spinophilin siRNA transfected neurons (mean number of HA-MOR infected cells in which MOR showed intracellular localization at 30 min: control siRNA group = 65.9 ± 6.7, spinophilin siRNA group = 34 ± 1.8).

DISCUSSION

Our study provides evidence for an important physiological role of spinophilin in the regulation of MOR signaling and behavioral responses to opiate drugs of abuse. Deletion of the spinophilin gene causes reduced analgesic effects of acute morphine but enhanced adaptations to repeated morphine exposure, including increased morphine dependence, place conditioning, locomotor sensitization, and analgesic tolerance. These diverse effects of spinophilin may result not only from its broad distribution in the brain, but also from its multiple actions at the cellular level. Thus, spinophilin modulates MOR responsiveness via complex mechanisms that promote MOR internalization and recycling as well as MOR inhibition of adenylyl cyclase but oppose MOR activation of ERK. Our findings offer new insight into the cellular events underlying chronic opiate action and point to spinophilin as a promising new target for the optimization of the analgesic actions of opiate drugs with reduced drug tolerance and dependence.

Several studies have implicated signal transduction proteins in particular aspects of opiate action. Behavioral characterization of GRK3 knockout mice reveals that GRK3 is involved in analgesic tolerance but does not affect other aspects of morphine action (Terman et al., 2004). Studies of genetic mutant mice suggest that RGS9-2 and β-arrestin-2 each act as a negative modulator of morphine's effects (Bohn et al., 2000; Zachariou et al., 2003). RGS9-2 potently suppresses morphine reward, analgesia, and physical dependence, whereas β-arrestin-2 mainly affects analgesic responsiveness and tolerance but not physical dependence. The fact that β-arrestin-2 mutants show increased sensitivity to the analgesic effects of morphine but normal responses to other opioid drugs (Bohn et al., 1999, 2000, 2004), indicates that signal transduction molecules beyond β-arrestin-2 modulate MOR analgesic responses. Our data suggest that spinophilin is one such additional modulator of MOR function, one that regulates MOR desensitization and affects the actions of all opiate analgesic drugs. Unlike the other signal transduction proteins studied to date, genetic ablation of spinophilin causes opposite effects on morphine reward and dependence versus analgesic responsiveness, a phenotype that mimics several features of addiction (exacerbated withdrawal, locomotor sensitization, place conditioning, and tolerance). The only other protein known to mediate this complex phenotype is the transcription factor, ΔFosB, acting in the nucleus accumbens (Zachariou et al., 2006). However, knockout of spinophilin does not alter ΔFosB levels in this brain region, nor does ΔFosB regulate spinophilin expression (Zachariou et al., 2006), suggesting that spinophilin and ΔFosB act through distinct mechanisms. Future studies are needed to determine whether modulation of dopamine receptor responsiveness by spinophilin, or possible spinal cord-specific actions of this protein, also contribute to the opiate phenotype.

Our data demonstrate an essential role of spinophilin in regulating the rate of MOR internalization: reducing spinophilin levels with siRNA in cultured striatal neurons delays internalization. Similar effects on MOR internalization are observed in cultured MEF cells

from spinophilin knockout embryos, whereas overexpression of spinophilin in cell lines promotes MOR internalization. The facilitation of MOR internalization by spinophilin is particularly dramatic for morphine, which is normally associated with much slower receptor internalization in cultured cells compared to that induced by opioid peptides (Keith et al., 1998; Whistler and von Zastrow, 1998; Whistler et al., 1999). Indeed, the high levels of expression of spinophilin in striatum could explain the rapid internalization of MOR observed in response to morphine in striatal neurons in vivo compared to cultured cells in vitro (Haberstock-Debic et al., 2003).

Earlier in vitro studies indicated that the rate of MOR endocytosis is a critical factor controlling the development of analgesic tolerance (Keith et al., 1998; Whistler and von Zastrow, 1998; Whistler et al., 1999), with reduced endocytosis associated with exacerbated tolerance. Thus, our findings that knockout of spinophilin reduces MOR internalization and facilitates tolerance (and other adaptations to repeated morphine exposure) are consistent with this model. Several more recent studies have provided important information on the molecular determinants of MOR endocytosis, as well as information on its occurrence in vivo (Haberstock-Debic et al., 2003, 2005; Arttamangkul et al., 2006; Zhao et al., 2006). It will therefore be important in future studies to focus on interactions between spinophilin and other regulators of MOR trafficking and on local manipulation of spinophilin in particular brain regions, to determine the mechanism via which this protein modulates MOR function.

Our behavioral studies reveal that spinophilin knockout mice show increased sensitivity to the rewarding and locomotor sensitizing actions of morphine. We speculate that this increased sensitivity could be interpreted as earlier development of motivational dependence and are therefore consistent with our data on physical dependence. The increased sensitivity observed in the place preference test may also be related to interactions between spinophilin and PP1; however, if this were the case, this phenotype might be expected to be observed in neurabin knockout mice as well, as neurabin is also a ubiquitous PP1 interacting protein. Another possible mechanism via which spinophilin could modulate sensitivity to the rewarding actions of morphine would involve interactions with G protein Gβ subunits. Earlier reports indicated that spinophilin interacts with the βγ complex (Brady et al., 2003; Wang et al., 2004). We show here that exposure to opiate drugs leads to the rapid association of spinophilin with Gβ5. Gβ5 is one of the most abundant G protein β subunits in brain and, in particular, is enriched in striatum. Gβ5 is also an essential binding partner for RGS9, as it contributes to the protein's stability (Chen et al., 2003). The striatal-enriched protein, RGS9-2, is considered a key regulator of MOR responsiveness (Zachariou et al., 2003; Psifogeorgou et al., 2007) and, as expected, it is found in the same complex with spinophilin after activation of MOR. This is particularly interesting since lack of either RGS9 or spinophilin increases sensitivity to morphine reward and leads to more severe dependence. The fact that spinophilin knockout mice show decreased sensitivity to morphine's analgesic effects, whereas RGS9 knockout animals show increased sensitivity, indicates that modulation of morphine analgesia by spinophilin may involve distinct actions at the cellular level or actions beyond the nucleus accumbens and dorsal striatum.

Modified ERK phosphorylation and cAMP activity in the brain have been associated with regulation of morphine reward and dependence (Nestler, 2001; Valjent et al., 2006). Opioids can increase or decrease ERK phosphorylation depending on the brain region involved (Eitan et al., 2003; Muller and Unterwald, 2004), but little information is available concerning the influence of ERK signaling in the nucleus accumbens on behavioral responses to morphine. Previous work showed that spinophilin accelerates ERK phosphorylation in cultured MEF cells after activation of α_2 receptors (Wang et al., 2005). Interestingly, in striatum, knockout of spinophilin enhances morphine-induced increases in phosphoERK. Further work is needed to explore the behavioral consequences of spinophilin

regulation of ERK function in this brain region. Our studies also demonstrate that knockout of spinophilin reduces the ability of morphine to inhibit adenylyl cyclase in striatum, an effect which would be expected to influence behavioral responses to opiate drugs (Barrot et al., 2002). It is striking that loss of spinophilin impairs MOR signaling via adenylyl cyclase but enhances MOR signaling via ERK. Opiate inhibition of adenylyl cyclase is thought to represent a direct effect of Gi on the enzyme, whereas the molecular pathway by which Gi-linked receptors activate the ERK pathway has remained incompletely understood. The opposite effects of spinophilin on these signaling pathways could provide unique insight into understanding MOR regulation of cAMP and ERK signaling.

In summary, we show that spinophilin opposes the consequences of repeated opiate exposure and that these actions could be achieved by promoting MOR internalization and recycling and by modulating signal transduction events that follow opiate activation of MOR. Spinophilin regulation of MOR is very different from that observed previously for other receptors. These findings thereby contribute to our understanding of receptor-selective functions of spinophilin and provide information on new molecular elements that regulate MOR responsiveness. The molecular actions of spinophilin suggest that it could serve as a new pharmacological target to promote opiate analgesia, while preventing cellular events that lead to addiction after repeated exposure to these drugs.

EXPERIMENTAL PROCEDURES

Mouse Breeding and Genotyping

All spinophilin and neurabin mutant mice used in this study (Feng et al., 2000; Allen et al., 2006) were generated from breedings of heterozygous spinophilin or neurabin knockout mice. For all behavioral assays, we used naive 2-monthold male knockout mice and their wild-type littermates. For immunoblotting analysis, striata from 2-month-old C57Bl/6 mice were extracted 2 hr after morphine treatment or 3 days after s.c. implantation of 25 mg morphine pellets as described (Zachariou et al., 2003, 2006). Animals were housed in a 12 hr dark/light cycle room according to the animal care and use committees of UT Southwestern Medical Center and the University of Crete.

Place Conditioning, Analgesia, Locomotor Activity, and Morphine Withdrawal Assays

An unbiased place conditioning procedure was performed as described in earlier studies (Zachariou et al., 2006). Analgesia was measured using a 52°C hot plate test, as described before (Zachariou et al., 2006). Data are expressed as %maximal possible effect (MPE = [Latency-baseline]/[cutoff latency]). For opiate withdrawal assays, mice were injected with increasing morphine doses as described (Zachariou et al., 2003), and withdrawal was precipitated after the third day of injections using naloxone (1 mg/kg, s.c., Sigma). Withdrawal signs were monitored for 25 min after naloxone administration. For the morphine locomotor sensitization assays, mice were placed in locomotor activity chambers as described earlier (Allen et al., 2006) and ambulatory activity was monitored for 30 min after, saline (days 1–3) or morphine (days 4–9) injection.

Cell Culture and Immunocytochemistry

PC12 cells were grown in poly-lysine-coated coverslips in 6-well plates in DMEM containing 10% fetal bovine serum, 5% horse serum, and 1% Pen Strep (Invitrogen). Transfections with Spinophilin, HA-MOR, or control pcDNA3 constructs were performed using Lipofectamine 2000 in Optimem (Invitrogen). Mouse embryonic fibroblasts from spinophilin wild-type and spinophilin knockout day 14 embryos were grown in DMEM in 20% fetal bovine serum and 1% Pen Strep (Invitrogen). For immunohistochemical studies cells, were grown in four-chamber polystyrene vessel tissue culture-treated glass slides (BD

Biosciences) and infected with HSV HA-MOR virus (1:1000) in serum- free DMEM. Cells were treated with morphine (10 μM) or vehicle 24 hr following transfection and then fixed in 4% formaldehyde in phosphate-buffered saline (PBS) and incubated with IMF saponin (0.25% cold water fish gelatin, 0.02% saponin, 0.01% Na Azide in PBS) for 20 more min. Cells were then incubated with an anti-HA mouse monoclonal antibody (1:1000, Sigma) for 45 min, washed three times with PBS, then with Cy3-conjugated donkey anti-mouse secondary antibody (1:500, Jackson Immunoresearch) for 20 min. Images were acquired using a Leica confocal microscope with a 60× oil objective.

Quantitative Internalization Assay (ELISA)

PC12 cells, transfected with HA-MOR and Spinophilin (or control) constructs or MEF cells infected with HSV-HA-MOR virus were incubated with mouse anti-HA antibody (Sigma-Aldrich) for 2 hr in Optimem at 4°C, as described before (Psifogeorgou et al., 2007). They were next treated with 5 μM DAMGO or 10 μM morphine in Optimem, for 30 min at 37°C and incubated for 2 more hr with peroxidase-conjugated anti-mouse antibody (1:1000, Jackson Immunoresearch). Plates were developed with ABTS (2,2-Azino-di-(3-ethylbenzthiazoline-6-sulfonic acid (Sigma). Ten to thirty minutes later, 200 μl substrate of each sample was transferred to a 96-well plate and analyzed at 405 nm in a micro-plate reader.

Coimmunoprecipitation Assays

Striatal tissues of mice treated with saline, fentanyl, or morphine for 15 or 30 min were rapidly dissected in buffer containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, and protease inhibitors. Lysates were precleared with 20 μl of G agarose beads (Roche) by 1–2 hr incubation at 4°C. Supernatants were then incubated overnight with primary antibody (anti-rabbit spinophilin, 1:1000) on a rotating platform, followed by incubation with 20 μl protein G agarose beads for 2 hr at 4°C. Beads were washed three times in lysis buffer, precipitated, and resuspended in equal volume of loading buffer. Immunoprecipitated proteins were analyzed by 10.5% SDS-polyacrylamide gel electrophoresis followed by electrotransfer to nitrocellulose membrane 0.45 μm (Bio-Rad Labs). Immunoblotting was performed with an rabbit anti-MOR antibody (Chemicon), or with a rabbit anti-Gβ5 (C terminus) antibody (W. Simonds, National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda MD) or a rabbit anti-Gβ (Santa Cruz) or rabbit anti-GRK2 (Santa Cruz, CA) or rabbit anti-RGS9-2 (S. Gold, UT Southwestern Medical Center, Dallas, TX, and T. Wensel, Baylor College of Medicine, Houston, TX) followed by incubation with HRP-conjugated anti-mouse or HRP conjugated anti-rabbit antibody (Sigma). Bands were visualized with enhanced chemiluminescence (Pierce Biotechnology).

Primary Striatal Cultures

Striata were removed from 17- 18-day-old mouse embryos. Isolated tissue was incubated for 20 min in 1 mM papain, 10 mM trypsin, 5000 U DNase I, and dissociated with gentle mechanical trituration using an eppendorf pipette. Cells were incubated in Neurobasal medium containing B-27 and l-glutamine. Transfections were performed on day 12. Transfection of siRNA (spinophilin siRNA sequence AACTCGAAGCTGGTCACTAAG) was performed using Hi-Perfect transfection reagent (QIAGEN) as described by Wang et al. (2004). A nontargeting siRNA (control nontargeting siRNA#1, Dharmacon) was used as a control. Viral infections were performed 2 days after transfection. Twenty-four hours later, cells were treated with 10 μM morphine for 30, 45, or 60 min and immunofluorescence was performed using a mouse anti-HA monoclonal antibody (1:1000), as described earlier. Cells were visualized using a Leica Microscope with a 60× objective.

Immunoblotting

Nucleus accumbens and dorsal striatal punches were dissected with a 12 gauge syringe needle from 1 mm thick coronal sections of mouse brain, and immunoblotting was performed as described (Zachariou et al., 2003). The following antibodies were used: rabbit anti-spinophilin (1:10,000 dilution; Allen et al., 1997), rabbit anti-neurabin (1:10,000 dilution; Muly et al., 2004), a mouse anti-phospho-ERK antibody (1:10,000 dilution; Sigma), a rabbit anti-RGS4 antibody (1:2000; S. Mumby, UT Southwestern Medical Center), a rabbit RGS9 antibody (1:5000; Psifogeorgou et al., 2007), a rabbit anti-Gβ5 (C terminus) antibody (1:10,000; W. Simonds, National Institute of Diabetes, Digestive and Kidney Diseases, Bethesda, MD).

cAMP and [^{3}H] DAMGO Binding Assays

Striata from naive wild-type and knockout mice were frozen at −70°C until use. Adenylyl cyclase activity from striatal membranes was measured as described by Unterwald et al. (1993) with minor modifications. Briefly, membranes (~10 μg protein/10 μl) were treated with different concentrations of DAMGO (0–10 μM) for 15 min at 30°C in an assay buffer containing a final concentration of 80 mM Tris, 10 mM theophylline, 1 mM $MgSO_4$, 0.4 mM EGTA, 30 mM NaCl, 0.25 mM ATP, 10 μM GDP (pH 7.4). Adenylyl cyclase activity was terminated by placing tubes in boiling water for 2 min. To measure the amount of cAMP formed, [^{3}H]cAMP (final concentration 4 nM) in citrate-phosphate buffer (pH 4) and 20 μl binding protein prepared from bovine adrenal glands were added to each sample. The binding reaction was allowed to reach equilibrium by overnight incubation at 4°C. The assay was terminated by rapid filtration in a Brandel machine using GF/B filters. The filters were washed three times with ice-cold 20 mM sodium phosphate buffer (pH 6) and bound radioactivity measured in a liquid scintillation counter. Radioactivity was converted to pmol of cAMP by comparison to the curve derived from the cAMP standards. For binding assays striatal membranes from wild-type or spinophilin knockout animals (~50 μg protein) were treated with 10 nM [^{3}H] DAMGO in 50 mM Tris-Cl (pH 7.4) for 1 hr at 37°C in the absence or presence of 1 μM cold DAMGO.

Viral-Mediated Gene Transfer In Vivo

AAV-spinophilin and AAV-GFP viruses were produced using a triple transfection helper free method in HEK cells and purified as described earlier (Hommel et al., 2003; Zachariou et al., 2006). Stereotaxic coordinates for AAV vector injections into the NAc were as follows: anteriorposterior +1.6 mm, lateral ± 1.5 mm, and dorsoventral −4.5 mm at an angle of 10° from the midline (relative to Bregma).

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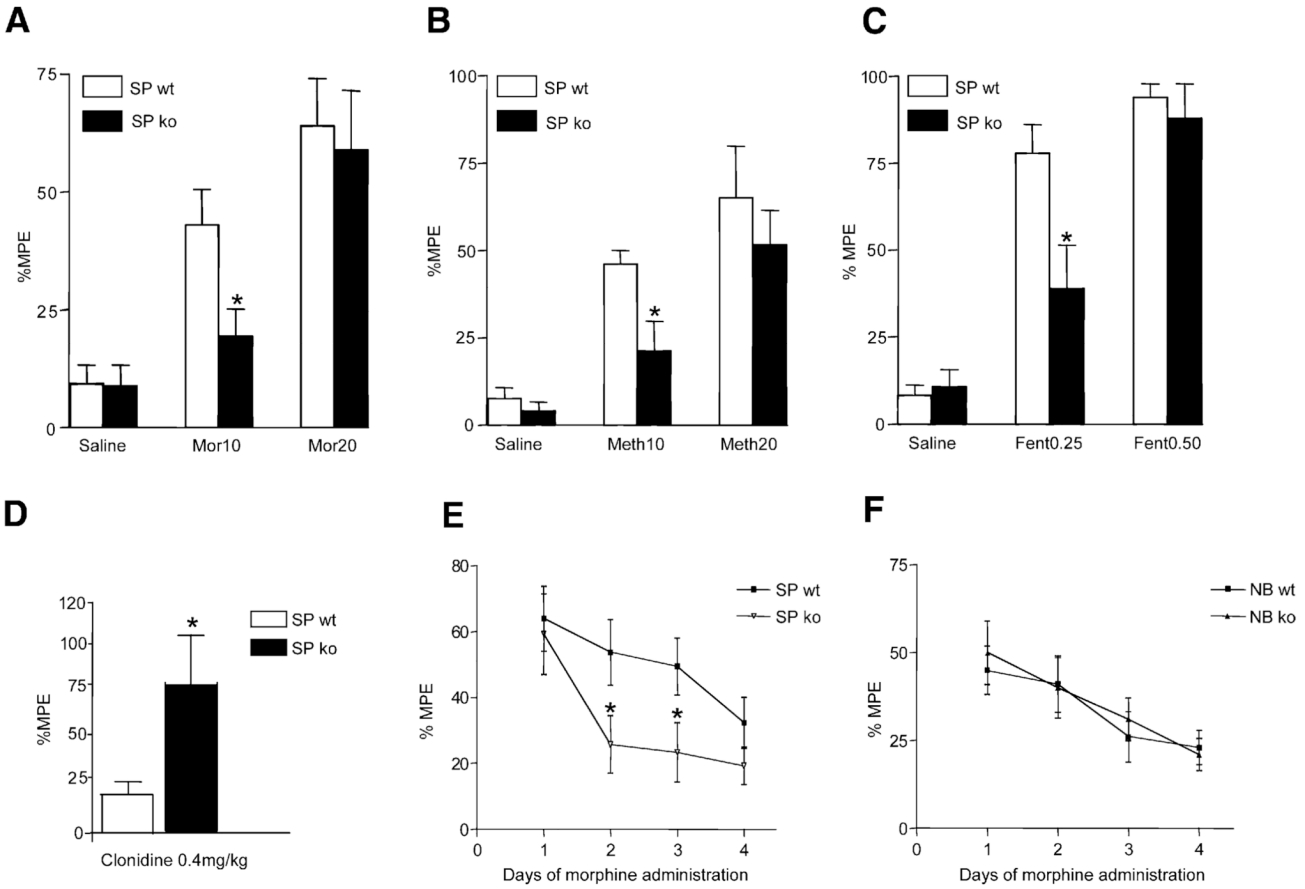

Figure 1. Spinophilin Knockout Mice Exhibit Decreased Sensitivity to the Analgesic Actions of Morphine and Accelerated Development of Analgesic Tolerance

(A) Spinophilin (SP) knockout (KO) mice show reduced analgesic responses to low morphine doses in the 52°C hot plate test compared to their wild-type littermates (SP WT).

(B and C) Dose responses to other opiate drugs, such as methadone and fentanyl, also shift to the right in the absence of the spinophilin gene.

(D) In contrast to morphine, spinophilin knockout mice are more sensitive to the analgesic effects of clonidine.

(E) Wild-type mice develop tolerance to the analgesic effects of morphine after four daily injections (20 mg/kg, i.p.), while the spinophilin knockout mice develop tolerance to morphine much earlier and show no analgesic response by the second exposure to the drug.

(F) Neurabin (NB) knockout mice develop tolerance to morphine at the same rate as their wild-type controls. Responses are expressed as % maximal possible effect (MPE = [Latency-baseline]/[cutoff latency]). Data are expressed as means ± SEM, *$p < 0.05$ for genotype over treatment, two-way ANOVA followed by Bonferroni test.

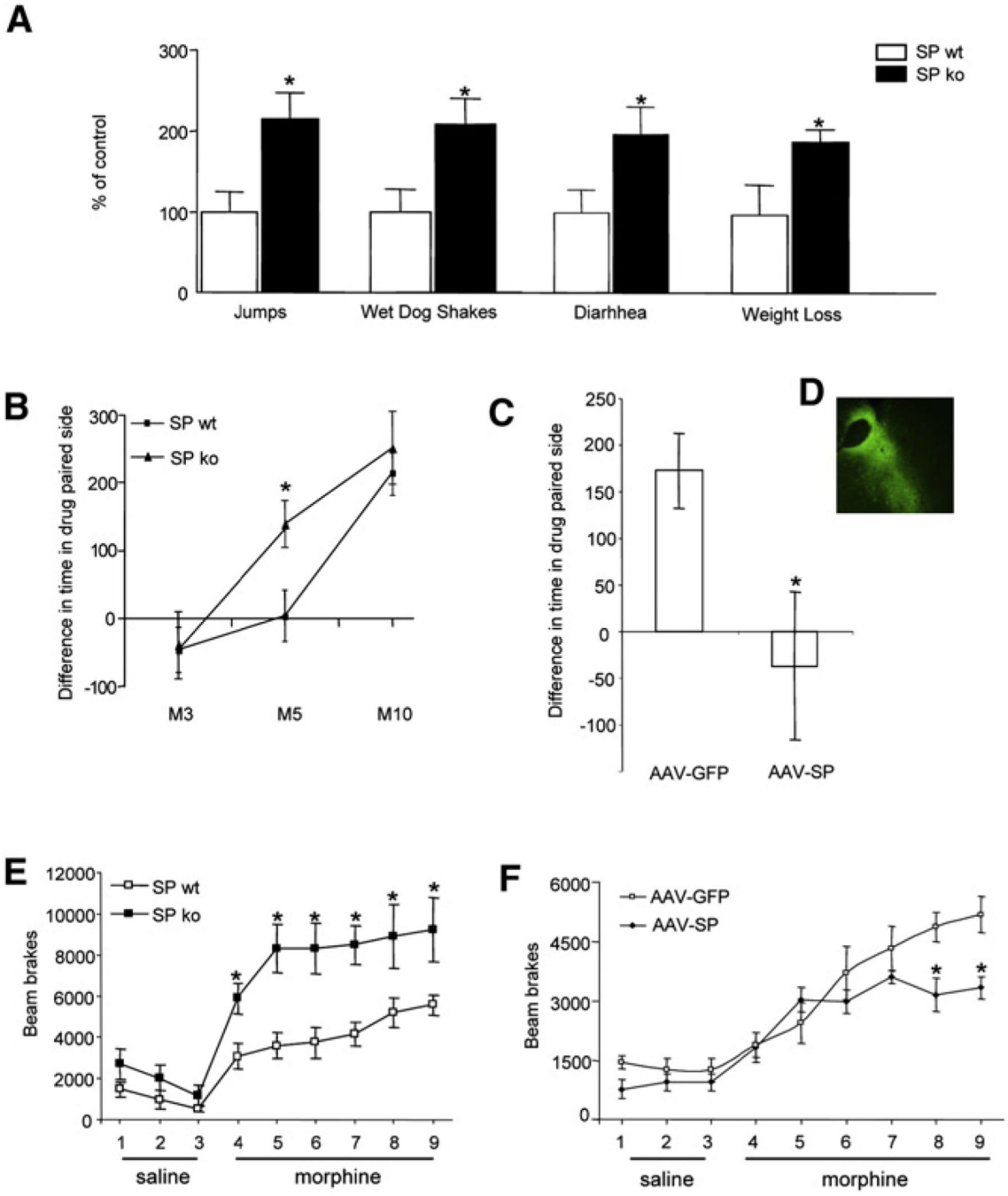

Figure 2. Spinophilin Knockout Mice Exhibit Enhanced Morphine Reward, Physical Dependence, and Locomotor Activation

(A) Spinophilin (SP) knockout (KO) mice show a much higher degree of morphine dependence, as signs of naloxone (1 mg/kg, s.c.) precipitated opiate withdrawal are twice as intense in KO mice compared to their wild-type littermates (SP WT, n = 8–9 per group).

(B) Spinophilin knockout mice are also more sensitive to the rewarding effects of morphine in the place preference test (n = 10–12 per group).

(C) On the other hand, overexpression of spinophilin in the nucleus accumbens of C57/Bl6 mice using AAV-spinophilin decreases sensitivity to the rewarding actions of morphine (5 mg/kg, s.c.) in this paradigm compared to AAV-GFP injected control mice (n = 6–8 per

group.) Data are expressed as means ± SEM, *p < 0.05 for genotype over treatment, two-way ANOVA followed by Bonferroni test.
(D) Low-power (10×) magnification of immunofluorescence for GFP, showing the AAV-GFP vector targeting the nucleus accumbens.
(E) Spinophilin ko mice display greater locomotor responses to acute and repeated morphine administration than their wild-type littermates (n = 5 per group).
(F) On the contrary, overexpression of spinophilin in the nucleus accumbens of C57/B16 mice via infection with AAV-Spinophilin does not affect the locomotor activating effects of acute morphine but prevents the development of locomotor sensitization (n = 5 per group). Data are expressed as means ± SEM, *p < 0.05 for genotype versus treatment, two-way ANOVA followed by Bonferroni post hoc test.

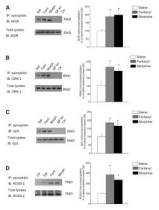

Figure 3. MOR Coimmunoprecipitates with Spinophilin

(A) Mice were treated with morphine (15 mg/kg) or fentanyl (0.125 mg/kg) for 30 min. Striatal extracts were immunoprecipitated (IP) with an anti-spinophilin antibody, and the immunoprecipitate was immunoblotted (IB) for MOR. MOR was immunoprecipitated with spinophilin under basal conditions, and this interaction was strengthened following acute fentanyl or morphine treatment. (B) Activation of MOR similarly increases association of spinophilin with GRK2 (B), Gβ5 (C), and RGS9-2 (D). Mice were treated with fentanyl or morphine as in (A), and striatal extracts were immunoprecipitated for spinophilin and immunoblotted for GRK2, Gβ5, or RGS9-2, respectively. For all experiments, data are expressed as means ± SEM, n = 3 per treatment group, Sal = saline treatment, Fent = fentanyl treatment, Morph = morphine treatment, SP KO = striata from morphine treated spinophilin KO mice, Ctr (Control) = striata from morphine-treated mice immunoprecipitated with an anti-Flag antiserum.

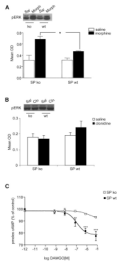

Figure 4. Spinophilin Modulates Opiate Regulation of ERK Phosphorylation and Adenylyl Cyclase Activity

Spinophilin knockout (KO) mice show greater morphine induction of ERK phosphorylation in striatum after morphine administration compared to wild-type littermates (WT). Immunoblot analysis of phosphoERK levels in striatum of KO and WT mice 20 min after an injection of saline (Sal) or 10 mg/kg morphine (Morph) (A) or after injection of saline or 0.4 mg/kg clonidine (Clon) (B). n = 4–5 per group, data are expressed as means ± SEM, *$p < 0.05$ for genotype versus treatment, two-way ANOVA followed by Bonferroni post hoc test. (C) Striatal membranes (~10 μg protein/10 μl) from spinophilin KO and WT mice were treated with different concentrations of DAMGO (0–10 μM) for 15 min at 30°C, and the amount of cAMP formed was determined as described in Experimental Procedures. Results are the means ± SEM of five individual animals in triplicate. **$p < 0.001$, ***$p < 0.0005$, t test.

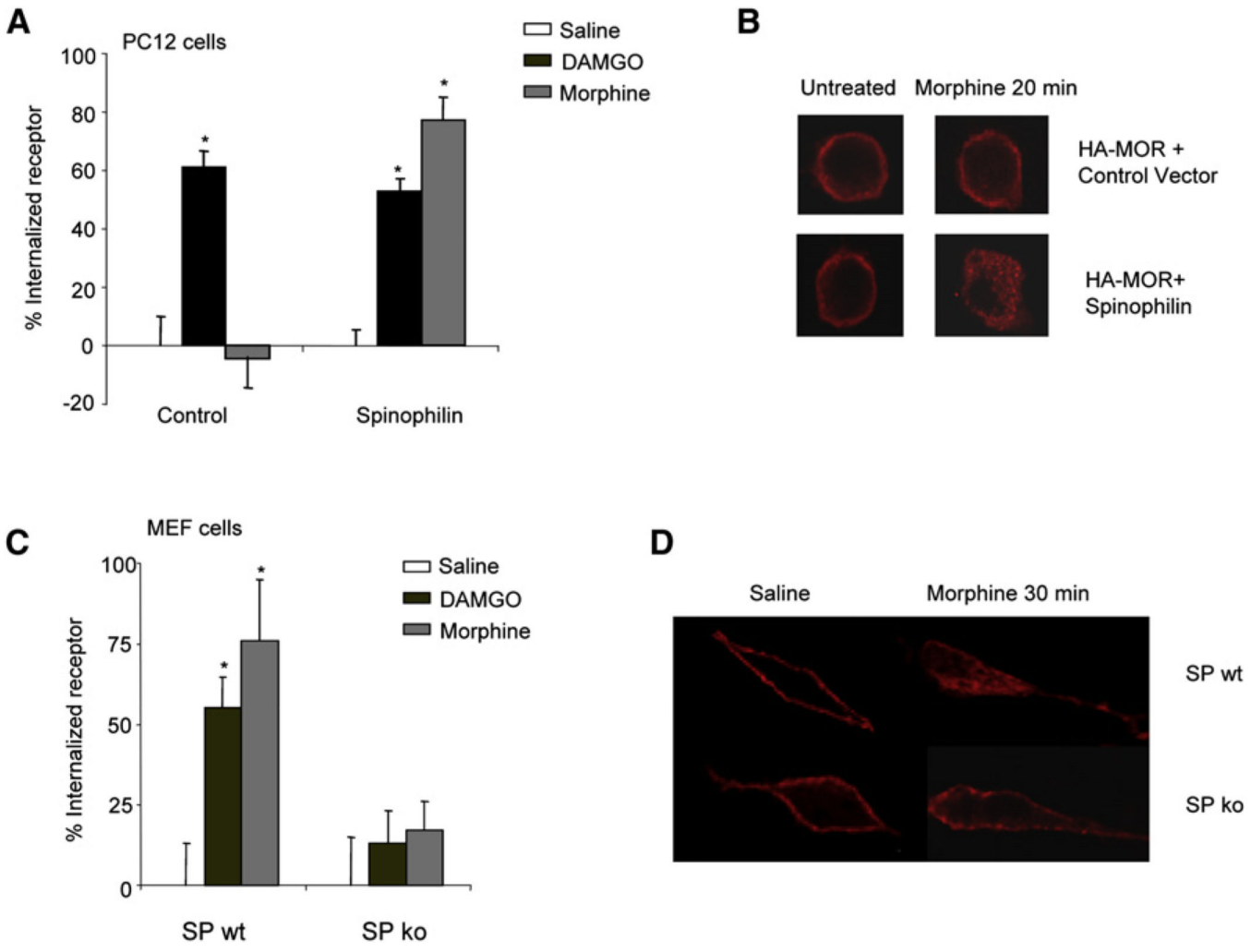

Figure 5. Regulation of MOR Internalization by Spinophilin

(A) Graph represents percent internalization 30 min following DAMGO (5 μM) or morphine (10 μM) treatment in PC12 cells transfected with control vector or spinophilin construct. Internalization was quantified using ELISA. Data are expressed as means ± SEM, n = 4–5 per group, *p < 0.05 between treatments for each group, ANOVA followed by PLSD test.

(B) PC12 cells transiently transfected with control vector and HA-MOR or with spinophilin plasmid and HA-MOR were treated with saline or morphine (10 μM) for 30 min. MOR cellular localization was determined immunofluorescence to the HA tag.

(C) Graph represents percent internalization 30 min following DAMGO or morphine treatment (as in [A] and [B]) in mouse embryonic fibroblasts (MEF) from spinophilin (SP) knockout (KO) and wild-type (WT) 14-day-old embryos infected with an HSV-HA-MOR vector. Internalization was quantified using ELISA. Data are expressed as means ± SEM, n = 4 per group, *p < 0.05 between treatments for each group, ANOVA followed by PLSD test.

(D) MEF from SP WT and KO embryos were infected with an HSV-HA-MOR vector and 24 hr later were treated with saline or morphine (10 μM) for 30 min. MOR cellular localization was detected using immunofluorescence. For immunofluorescence studies, each set of experiments was repeated three times, and each treatment was performed in duplicate.

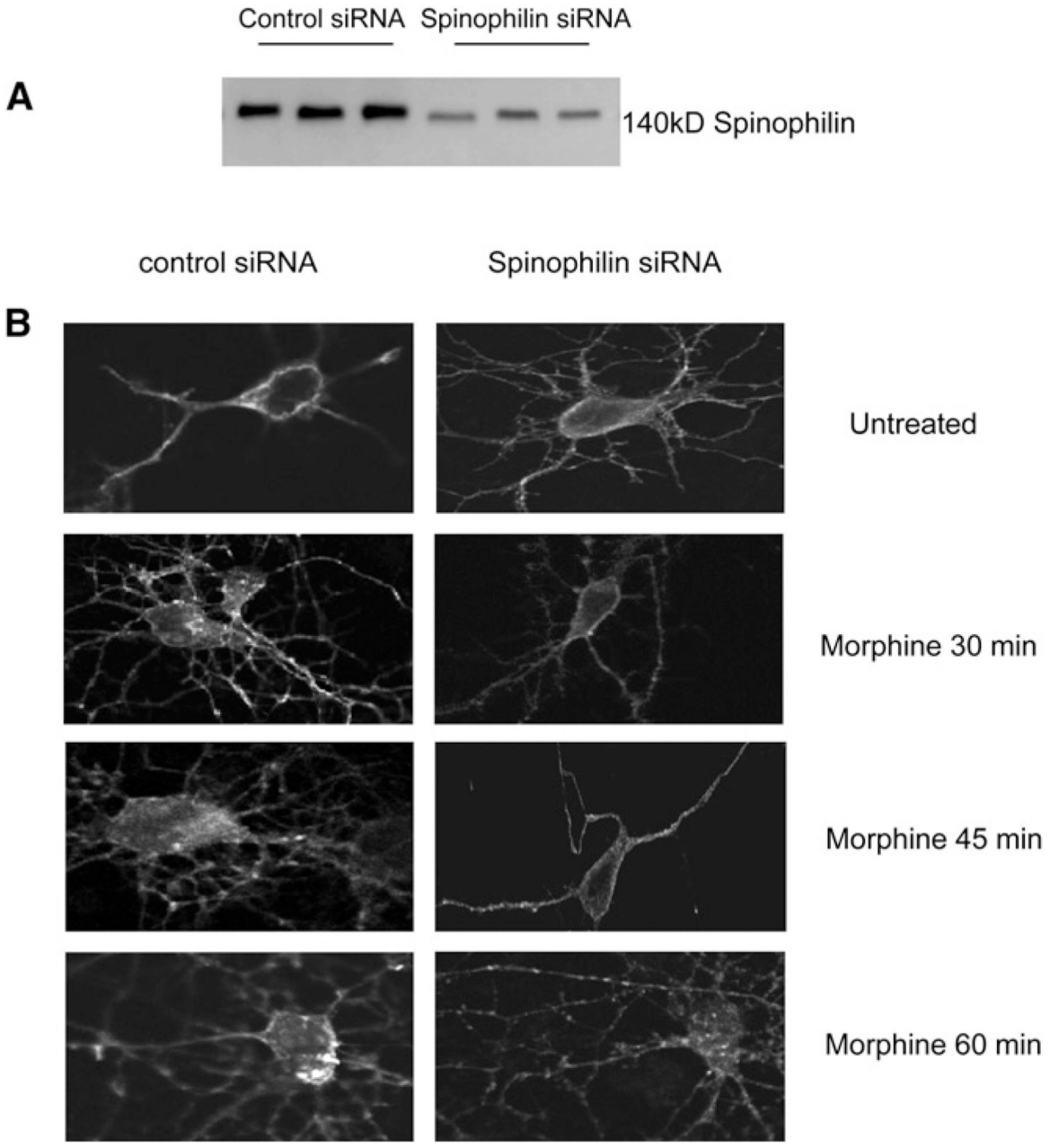

Figure 6. Knockout of Spinophilin Delays MOR Internalization

(A) Blot shows spinophilin levels in cultured striatal neurons infected with control or spinophilin siRNA.

(B) Knockdown of spinophilin expression using siRNA prevents MOR endocytosis in cultured striatal neurons. Neurons were transfected with siRNA for spinophilin or with control siRNA and 48 hr later were infected with HSV-HA-MOR. The next day, cells were treated with morphine (10 μM) for 30, 45, or 60 min, and MOR internalization was observed using immunofluoresence. While MOR is internalized within 30 min in striatal neurons transfected with control siRNA, in neurons transfected with spinophilin siRNA, MOR was internalized at 60 min.

Table 1

Acute Opiate Administration Regulates Spinophilin Levels in Nucleus Accumbens

	Acute Morphine	Acute Methadone	Acute Fentanyl	Acute Clonidine
Spinophilin levels (% control)	62 ± 8.9*	80.8 ± 3.3*	73 ± 9.5*	109 ± 17
Neurabin levels (% control)	106 ± 2.8	115 ± 9.4	111 ± 5.6	

Decreased levels of spinophilin (but not of neurabin) in the nucleus accumbens of C57Bl/6 mice 2 hr after acute morphine (20 mg/kg), methadone (20 mg/kg), or fentanyl (0.250 mg/kg) administration. In contrast, clonidine administration had no significant effect on spinophilin levels in this brain region. Data represent % change from saline control and are expressed as means ± SEM, n = 4–5 per group,

* $p < 0.05$ from control by t test.