μ- and κ-Opioids Induce the Differentiation of Embryonic Stem Cells to Neural Progenitors*

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Growth factors, hormones, and neurotransmitters have been implicated in the regulation of stem cell fate. Since various neural precursors express functional neurotransmitter receptors, which include G protein-coupled receptors, it is anticipated that they are involved in cell fate decisions. We detected μ-opioid receptor (MOR-1) and κ-opioid receptor (KOR-1) expression and immunoreactivity in embryonic stem (ES) cells and in retinoic acid-induced ES cell-derived, nestin-positive, neural progenitors. Moreover, these G protein-coupled receptors are functional, since \([\alpha\text{-Ala}^2,\text{MePhe}^4,\text{Gly-ol}^5]\text{enkephalin, a MOR-selective agonist, and U69,593, a KOR-selective agonist, induce a sustained activation of extracellular signal-regulated kinase (ERK) signaling throughout a 24-h treatment period in undifferentiated, self-renewing ES cells. Both opioids promote limited proliferation of undifferentiated ES cells via the ERK/MAP kinase signaling pathway. Importantly, biochemical and immunofluorescence data suggest that \([\alpha\text{-Ala}^2,\text{MePhe}^4,\text{Gly-ol}^5]\text{enkephalin and U69,593 divert ES cells from self-renewal and coax the cells to differentiate. In retinoic acid-differentiated ES cells, opioid-induced signaling features a biphasic ERK activation profile and an opioid-induced, ERK-independent inhibition of proliferation in these neural progenitors. Collectively, the data suggest that opioids may have opposite effects on ES cell self-renewal and ES cell differentiation and that ERK activation is only required by the latter. Finally, opioid modulation of ERK activity may play an important role in ES cell fate decisions by directing the cells to specific lineages.}

The maintenance of ES cells in an undifferentiated state in vitro is dependent on self-renewing cell division in the presence of leukemia inhibitory factor (LIF), which signals through various receptor complexes (reviewed in Refs. 1–3; see also Refs. 4 and 5). Mouse ES cells can be induced to differentiate into neural cells in the presence of retinoic acid (RA) in vitro (reviewed in Refs. 6 and 7). During this induction, ES cells undergo a series of steps that resemble key stages in the early mouse embryo, supporting the hypothesis that the in vitro pathway represents the normal developmental pathway (8, 9).

Considerable effort has been recently devoted to characterizing intrinsic factors, extrinsic factors, and signaling pathways regulating proliferation and differentiation of stem cells. The ERK/MAP kinase signaling pathway has been implicated in both proliferation and differentiation of many cell types, including stem cells (10). In several studies, ERK activation had a negative influence on self-renewal/proliferation in murine ES cells (4, 11–14). Moreover, LIF-dependent activation of STAT3 was not mediated by ERK activity (15). However, a dual role for the Ras/ERK pathway was proposed for ES cells, affecting both their division and differentiation (16). Ras activation down-regulated levels of Nanog, a protein that is normally expressed in high amounts in self-renewing ES cells (17). A functional role for ERK was proposed in the phosphatidylinositol 3-kinase-dependent regulation of ES cell self-renewal (18). The importance of ERK activation and its duration for cell differentiation has been investigated in various cells (19–22). Some evidence suggests that RA-induced inhibition of LIF signaling in ES cells is ERK-independent (23). However, a specific requirement of the ERK pathway was reported for RA-dependent commitment of murine ES cells (24). Cross-talk may occur by noncanonical actions of RA with the MAP kinase phosphorylation cascade. RA-induced differentiation of ES cells may be achieved by restricting nuclear entry of activated ERK (25). ERK activation is required for regulation of cyclin D1 levels, the increase of which parallels ES cell differentiation (26, 27).

Neurotransmitters as well as growth factors and hormones can influence cell division and differentiation of self-renewing stem cells and neural progenitors (NPs) via ERK in some cases (28, 29). For example, stimulation of the muscarinic acetylcholine receptor, a G protein-coupled receptor, induces DNA synthesis in rat cortical neuroepithelium progenitors via stimulation of phosphatidylinositol 3-kinase and ERK.

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2 The abbreviations used are: LIF, leukemia inhibitory factor; Ab, antibody; DAMGO, \([\alpha\text{-Ala}^2,\text{MePhe}^4,\text{Gly-ol}^5]\text{enkephalin; ES, embryonic stem; ERK, extracellular signal-regulated protein kinase; KOR, κ-opioid receptor; MAP, mitogen-activated protein; ME, 2-mercaptoethanol; MOR, μ-opioid receptor complexes; (reviewed in Refs. 1–3; see also Refs. 4 and 5). Mouse ES cells can be induced to differentiate into neural cells in the presence of retinoic acid (RA) in vitro (reviewed in Refs. 6 and 7). During this induction, ES cells undergo a series of steps that resemble key stages in the early mouse embryo, supporting the hypothesis that the in vitro pathway represents the normal developmental pathway (8, 9).

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(30). The activated CB1 receptor inhibits neuronal progenitor differentiation via attenuation of ERK signaling in E17 cortical cultures and in adult dentate gyrus (31). This endocannabinoid system was also found to promote astroglial differentiation by acting on neural progenitor cells (32). Glutamate activates N-methyl D-aspartate (NMDA) receptors and promotes proliferation of E15 precursors derived from the germinal zone of the ventral telencephalon in vivo and in vitro (33). Haloperidol acting via dopamine D2 receptors increases the number of NPs, neurons, and glia in the adult rat brain (34). G protein βγ subunits of heterotrimeric G proteins are required for proper mitotic-spindle orientation and asymmetric cell fate decisions of cerebral cortical progenitors (35). These studies were performed with brain-derived stem cells or NPs, but little is known about the consequences of G protein-coupled receptor-ERK cross-talk in blastoctyst-derived ES cells or their NPs.

Since the plasticity of uncommitted stem cells has opened new perspectives in tissue regeneration, recent research has been directed to understanding the signaling mechanisms that control proliferation and/or differentiation of ES cells. Here, we detected μ- and κ-ORs in ES cells and in ES cell-derived NPs. More importantly, we found that both opioids induced ES cell differentiation via ERK and an ERK-independent attenuation of proliferation in RA-driven NPs.

EXPERIMENTAL PROCEDURES

Mouse ES Cells and Their Growth Conditions—D3 (ATCC) and PRX-129/S6 # 7 ES cells (Primogenix, Inc.) were used. PRX-129/S6 # 7 ES cells were isolated from the inner cell mass of a day 3.5 129/S6/SvEv mouse blastocyst. Cells have a normal male karyotype and are specific pathogen-free. They were injected into blastocysts and produced chimeras at a high rate (first injection: 10/15 pups, no perinatal death). PRX-129/S6 # 7 cells were grown on mouse embryonic fibroblasts in the presence of LIF, passage 10 cells were propagated 2–3 times on gelatin-coated flasks, and these cells are currently used in the laboratory. Quality control and characterization of the D3 ES cells are documented by their depositor (36). D3 ES cells were propagated on irradiated STO cell feeders (ATCC) for several passages before they were transferred to gelatin-coated flasks.

D3 and PRX-129/S6 # 7 ES cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, LIF (100 ng/ml), and 0.1 mM 2-mercaptoethanol (ME) on gelatin-coated dishes. These growth conditions are known to prevent differentiation via ERK and an ERK-independent attenuation of vanadate, 1% Nonidet-40, 1 mM phenylmethylsulfonyl fluoride, 20 mM HEPES, 10 mM EGTA, 40 mM β-glycerophosphate, 2.5 mM MgCl2, 2 mM sodium vanadate, 1% Nonidet-40, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin) and spun at 14,000 × g, and protein concentration of the supernatants was determined. Cell lysates (10–20 μg of protein/lane) were separated by 10% SDS-PAGE. Proteins were blotted on Immobilon membranes. Nonspecific sites were blocked with 5% milk in Tris-buffered saline plus 0.2% Tween 20. Blots were incubated with monoclonal phospho-ERK Ab (1:2000; Cell Signaling) for at least 15 h at 4 °C, followed by incubation with a horseradish peroxidase-conjugated IgG (1:2000; Sigma) for 1 h at room temperature. Bands were visualized with an ECL chemiluminescence detection system (GE Healthcare) and exposure to Classic Blue sensitive x-ray film. Band intensities were determined by densitometry with an Eastman Kodak Co. DC120 digital camera, Kodak ds 1D version 3.0.2 (Scientific Imaging Systems), and NIH Image ImageJ version 1.32 software. ERK stimulation in opioid-treated cells was expressed as -fold change over basal levels in control cells.

OR and Nestin Immunofluorescence Microscopy—ES cells or ES cell-derived NPs were grown in glass chamber slides (Nunc). The cells were fixed in 4% PA for 20 min at room temperature and permeabilized in 0.1% Triton/PBS for 5 min. Cells were then incubated in PBS containing 0.5% bovine serum albumin and 0.1% Tween 20 for 30 min to reduce nonspecific binding, followed by overnight incubation at 4 °C with the following rabbit polyclonal OR Abs: MOR-1 raised against C terminus (1:2500; Neuronetics); MOR-1 raised against N terminus (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); KOR-1 (1:50; Santa Cruz Biotechnology). In some cases, a mouse monoclonal nestin Ab (1:1000; Chemicon) was added together with the OR Abs. After washing, Alexa Fluor 594-conjugated (red) and/or Alexa Fluor 488-conjugated (green) secondary Abs (1:1000) were applied for 1 h at room temperature. DAPI (1:200) was added together with the secondary Abs. The slides were treated with anti-fade reagent (Molecular Probes, Inc., Eugene, OR)
and examined for immunofluorescence with NIKON-OP-TIPHOT-2 or OLYMPUS AH3 microscopes with simultaneous recording of dual fluorescence label images.

**Real Time Quantitative Reverse Transcriptase (qRT)-PCR**—Total RNA from ES cells and RA-induced NPs was isolated using the Qiagen RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. cDNA was generated from 1 μg of total RNA using the High Capacity cDNA archive kit from Applied Biosystems (Foster City, CA) with random primers as described by the manufacturer. qRT-PCR was performed with SYBR green chemistry on an Applied Biosystems 7500 instrument. The primers were designed using Primer Express (Applied Biosystems) and supplied by Integrated DNA Technologies (Coralville, IA). Primer sequences for MOR-1 were as follows: forward, 5'-GCCACGTTCCATCAGGTAG-3' and reverse, 5'-GCCAAGGGTTCATCAGGTAG-3'. Primer sequences for KOR-1 were as follows: forward, 5'-AGAGAGAAGCGGCAAGCA-3' and reverse, 5'-GCCAAGGGTTCATCAGGTAG-3'. The cDNA templates for qRT-PCR were diluted 1:10, and the 50 μl SYBR-green reaction consisted of 1X SYBR-green Master Mix (Applied Biosystems), 300 nM forward and reverse primers, and 5 μl of diluted cDNA. Four replicates of each qRT-PCR reaction were run on 96-well plates. The amplification efficiencies of MOR-1 and KOR-1 were consistent with those of the endogenous control, glyceraldehyde-3-phosphate dehydrogenase. Relative quantification measurements were made as described (43) by using the comparative C_T method (ΔΔC_T method) in which the gene C_T values are normalized by subtracting glyceraldehyde-3-phosphate dehydrogenase C_T.

**Immunoblotting with MOR-1- and KOR-1-specific Abs**—For detection of receptor protein levels in ES cells or ES cell-derived NPs, immunoblotting with MOR-1- and KOR-1-specific Abs was adopted. For this purpose, cells were lysed in a modified radioimmune precipitation buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM Na_3VO_4, 1 mM NaF), and samples containing 20–50 μg of protein were loaded on 10% SDS gels. Receptor band(s) were detected using two rabbit polyclonal MOR-1 Abs: C terminus MOR-1 Ab (1:200; Neuromics) and N terminus MOR-1 Ab (1:200; Santa Cruz Biotechnology). KOR-1 immunoreactivity was detected with a rabbit polyclonal KOR-1-specific Ab (1:50; Santa Cruz Biotechnology). Horse-radish peroxidase-linked IgGs were applied as secondary Abs. Receptor band(s) were detected using two rabbit polyclonal MOR-1 Abs: C terminus MOR-1 Ab (1:200; Neuromics) and N terminus MOR-1 Ab (1:200; Santa Cruz Biotechnology). Horse-radish peroxidase-linked IgGs were applied as secondary Abs. Bands were visualized by chemiluminescence detection as described for ERK.

**SOX-1 Immunostaining and Counting**—D3 ES cells were grown in chamber slides, in serum/LIF/ME-containing media, and were treated with 1 μM DAMGO or U69,593 for 24 h. In some cases, cells were pretreated for 1 h either with MOR antagonist (CTAP, 1 μM) or KOR antagonist (nor-BNI, 1 μM) before the addition of opioid agonists for 24 h. Control and opioid-treated ES cells were washed with PBS and fixed with 2% PA for 10 min at room temperature. Cells were then permeabilized with 0.4% Triton/PBS for 5 min and incubated in PBS containing 10% fetal calf serum and 0.4% Triton X-100 for 30 min to reduce nonspecific binding, followed by incubation with chicken polyclonal SOX-1 Ab (1:500; Chemicon) overnight at 4 °C. After washing, Alexa Fluor 594 (red)-conjugated anti-chicken secondary Ab (1:1000; Molecular Probes) was applied for 1 h at room temperature. Cells were counterstained with DAPI (1:200) to visualize nuclei for cell counts. Chamber slides were treated with anti-fade reagent (Molecular Probes) and examined for immunofluorescence as described above. NIH Image J version 1.32 software was used to count cells. The percentage of SOX-1-positive cells was estimated from the ratio between the total number of cells (DAPI-stained) and the number of SOX-1-stained cells. Cells from 3–5 fields/slide were counted, and 3–4 slides/treatment group were used for the counting. The total number of counted cells was 500–800/treatment group.

**Cell Proliferation Assays**—For these studies, equal numbers of cells were seeded per well in 12-well dishes. Cell numbers were estimated by serial dilutions and counting cells (volume of 10 μl) in several visual fields using a bright line hemacytometer and light microscopy. Basal levels or opioid-induced changes in DNA synthesis were assessed by measuring the rate of [methyl-3H]thymidine incorporation into cells. Cells were grown in media with or without opioids for 24 h. In some experiments, the MEK-selective inhibitor U0126 (1 μM) was present for 28 h, whereas opioid agonists were added for 24 h, and [methyl-3H]thymidine (0.02 μCi/ml) was present for the last 4 h. [methyl-3H]Thymidine incorporation was measured as described in our previous studies with some modifications (44). Briefly, cells were washed with PBS, followed by incubation with 5% trichloroacetic acid at 4 °C for 30 min. Then cells were collected in 2% NaHCO_3/0.1 N NaOH, and [methyl-3H]thymidine incorporation was determined by liquid scintillation counting.

**BrdUrd Labeling of Cells**—D3 ES cells or RA-induced NPs were grown until they reached about 50% confluence. They were then treated with either 1 μM DAMGO or U69,593 for 24 h. In some cases, cells were pretreated with inhibitors or opioid antagonists as described in the figure legends. At the end of the treatment, medium was replaced with BrdUrd labeling medium (10 μM; BrdUrd detection kit I; Molecular Probes), and cells were incubated for 20–30 min. After several washes, cultures were fixed with ethanol fixative for 20 min at −20 °C. For nestin co-labeling, cells were incubated together with a monoclonal BrdUrd Ab (1:10; Roche Applied Science kit) and polyclonal nestin Ab (1:1000; Covance) at 37 °C for 30 min. For Sox-1 co-labeling, cells were first incubated with a monoclonal BrdUrd Ab (1:10; Roche Applied Science kit) at 37 °C for 30 min and then incubated in PBS containing 10% fetal calf serum and 0.4% Triton X-100 for 30 min to reduce nonspecific binding, followed by counterstaining with a chicken Sox-1 polyclonal Ab (1:500; Chemicon) overnight at 4 °C. After washing, cells were treated with fluorescein-conjugated anti-mouse IgG (green, 1:10; Roche Applied Science kit) together with Alexa Fluor 594 (red)-conjugated anti-chicken or anti-rabbit secondary Abs (1:1000; Molecular Probes) for 1 h at room temperature. Slides were examined for immunofluorescence as described above.

**RESULTS**

**Characterization of ES Cells and ES Cell-derived NPs**—The state of differentiation of D3 or PRX ES cells and RA-induced...
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ES cell-derived NPs was characterized by immunocytochemistry and immunoblotting. For this purpose, D3 and PRX ES cells were maintained under serum/LIF/ME-induced self-renewal conditions, or they were differentiated in the presence of RA. For the immunocytochemistry experiments, cultures were fixed with 4% PA for 20 min at room temperature. Cells were then permeabilized in 0.1% Triton/PBS for 5 min and incubated in PBS containing 0.5% bovine serum albumin and 0.1% Tween 20 for 30 min to reduce nonspecific binding, followed by incubation with mouse monoclonal nestin Ab (1:10000) overnight at 4 °C. After washing, Alexa Fluor 594 (red)-conjugated anti-mouse secondary Ab (1:10000) was applied for 1 h at room temperature. DAPI (1:200) was added together with the secondary Ab. Representative images show DAPI/nestin staining in short term (2–5 days) RA-differentiated cells (dD3 and dPRX). Only DAPI staining is present on the double DAPI/nestin images from undifferentiated cells (D3 and PRX). Magnification was ×20–40. B, immunoblot analysis. D3 ES cells were maintained in serum/LIF/ME-containing media, or they were differentiated with 1 μM RA (short term differentiated D3 ES cells) and grown for additional 2–3 days before use. Cell lysates (40 μg of protein) were run on 10% SDS-PAGE, and immunoblotting was performed with an Oct4 Ab (lane 1), immortalized rat astrocytes stably transfected with MOR-1 (lane 2), rat brain membrane (lane 3), late passage immortalized rat astrocytes (negative control) (lane 4), dD3 ES cells (lane 5), for N terminus MOR-1 Ab, D3 ES cells (lane 6), and dD3 ES cells (lanes 7); for KOR Ab, D3 ES cells (lane 1), late passage immortalized rat astrocytes (negative control) (lane 2), and dD3 ES cells (lanes 3).

FIGURE 2. Immunoblot analysis of MOR-1 and KOR-1 in D3 ES cells and in NPs. D3 ES cells were grown in serum/LIF/ME-containing media (undifferentiated cells: D3 ES cells), or they were differentiated with 1 μM RA (short term differentiation: dD3 ES cells). Cell lysates (20–50 μg of protein) were run on 10% SDS-PAGE, and immunoblotting was carried out with MOR-1 (C terminus, 1:2000; Neuromics), MOR-1 (N terminus, 1:2000; Santa Cruz Biotechnology) or KOR-1 (1:50; Santa Cruz Biotechnology) Abs. Shown are representative immunoblots from 4–8 experiments: for C terminus MOR-1 Ab, D3 ES cells (lane 1), immortalized rat astrocytes stably transfected with MOR-1 (lane 2), rat brain membrane (lane 3), late passage immortalized rat astrocytes (negative control) (lane 4), dD3 ES cells (lane 5), for N terminus MOR-1 Ab, D3 ES cells (lane 6), and dD3 ES cells (lane 7); for KOR Ab, D3 ES cells (lane 1), late passage immortalized rat astrocytes (negative control) (lane 2), and dD3 ES cells (lanes 3).

of cell lysates, and immunofluorescence microscopy of cells at different stages of development. In qRT-PCR experiments, we found that in comparison with ES cells, NPs have 1.37- and 2-fold increases in MOR-1 and KOR-1 gene expression, respectively (n = 3). As shown in Fig. 2, immunoblotting performed with polyclonal MOR-1 (C terminus; Neuromics)-, MOR-1 (N terminus; Santa Cruz Biotechnology)-, and KOR-1 (Santa Cruz Biotechnology)-specific Abs shows the presence of major 50 and 55 kDa bands that correspond to the expected molecular masses for MOR-1 and KOR-1, respectively. In addition to the 50 kDa band, the N terminus MOR-1 Ab reveals the existence of higher molecular mass bands (Fig. 2, lanes 6 and 7), which were suggested to correspond to the glycosylated form(s) of the receptor by several groups (45–47). Both MOR-1 and KOR-1 bands were detected in cell lysates, obtained from D3 ES cells maintained under self-renewal conditions (serum/LIF/ME). RA treatment does not appear to induce significant changes in MOR-1 or KOR-1 gene expression or protein levels (Fig. 2) in D3 NPs. Brain homogenate was used as a positive control, and lysates from late passage immortalized rat astrocytes were used as negative controls, because these cells lose detectable amounts of ORs with passaging (42).

Immunofluorescence microscopic analysis further supports the occurrence of MOR-1 and KOR-1 in D3 ES cells and NPs (Fig. 3). Once again, the undifferentiated state of D3 ES cells is confirmed by the absence of nestin (green)-stained cells (D3; Fig. 3, E and G). In contrast, D3 ES cell-derived NPs (dD3) show heavy nestin staining (Fig. 3, F and H). The presence of MOR-1 (red) immunoreactive cells was confirmed by applying the two polyclonal MOR-1 Abs that were used for the immunoblotting experiments (Fig. 2). It appears that the N terminus MOR-1 Ab detects the receptor mainly on the cell surface of ES cells (Fig. 3B) and NPs (Fig. 3D). Fig. 3, E and F, shows MOR-1 immunostaining using the C terminus MOR-1 Ab. KOR-1 immunoreactive cells were detected in self-renewing D3 ES cells (Fig. 3G) and in NPs (Fig. 3H).
Thereupon, cells were treated with the growth in media devoid of serum/LIF/ME for at least 24 h. Immunoblotting experiments with a phospho-ERK Ab that recognized KOR-induced ERK activation was measured by performing proliferation and differentiation, we sought to determine the functionality of these receptors. MOR- or MOR antagonist (CTAP) and KOR antagonist (nor-BNI, 1 μM) before the addition of opioid agonists for 5 min. ERK phosphorylation was measured by immunoblotting with a phospho-ERK Ab, directed against phosho-Thr202/Tyr204.

Although serum induces high basal levels of phosphorylated ERK in many cell types, ERK basal levels were low in ES cells grown in serum-containing media (Fig. 5A). The low basal levels of ERK phosphorylation in these cells support the notion that ERK activity may not be required for self-renewal of ES cells as recently reported (see Introduction).

**Opioids Induce Limited Proliferation of Undifferentiated ES Cells via the ERK Pathway**—To correlate the ERK data with cell proliferation, we studied opioid regulation of ES cell growth. In undifferentiated D3 ES cells, the findings are that DAMGO, U69,593, and morphine all induced limited proliferation. Specifically, DAMGO and morphine caused a 40% rise in proliferation over basal levels, whereas U69,593 caused a 30% increase (Fig. 6). The mitogen, epidermal growth factor (10 ng/ml, M), or U69,593 (0.1 μM) for 5 min. In some cases, cells were pretreated for 1 h either with MOR antagonist (CTAP, 1 μM) or KOR antagonist (nor-BNI, 1 μM) before the addition of opioid agonists for 5 min. ERK phosphorylation was measured by immunoblotting with a phospho-ERK Ab, directed against phosho-Thr202/Tyr204.

**FIGURE 3.** Immunofluorescence microscopic detection of MOR-1 and KOR-1 in undifferentiated D3 ES cells and in RA-induced NPs. D3 ES cells were maintained in serum/LIF/ME-induced self-renewal conditions (undifferentiated cells, D3). Upon treatment with 1 μM RA, they undergo differentiation (dD3). Cells were fixed and permeabilized as in Fig. 1. Treatments were followed by overnight incubation with polyclonal MOR-1 (C terminus, 1:2500 (A–D); N terminus, 1:50 (E and F)) or KOR (1:50) Abs at 4 °C. Mouse monoclonal nestin Ab (1:1000) was added to E–H slides. After washing, Alexa Fluor 594 (red) or Alexa Fluor 488 (green)-conjugated secondary Abs (1:1000) were applied for 1 h at room temperature. In some cases, DAPI (1:200) was added together with the secondary Ab (A–D). Immunofluorescence in cells was examined as described under “Experimental Procedures.” Appropriate controls, such as omission of primary Ab, have been run to confirm the specificity of the Abs (A and C). Representative images show MOR-1 (B and E) or KOR-1 (G) (red) staining in undifferentiated (D3) cells or in short term differentiated ES cells (dD3) (D). Double images of MOR-1/nestin (F) or KOR-1/nestin (H) staining in short term differentiated ES cells (dD3) are also presented. Magnification was ×20–40.

**FIGURE 4.** Opioid stimulation of ERK phosphorylation in ES cells. D3 ES cells grown in serum/LIF/ME-free media were treated with vehicle, DAMGO (0.1 μM), or U69,593 (0.1 μM) for 5 min. In some cases, cells were pretreated for 1 h either with MOR antagonist (CTAP, 1 μM) or KOR antagonist (nor-BNI, 1 μM) before the addition of opioid agonists for 5 min. ERK phosphorylation was measured by immunoblotting with a phospho-ERK Ab, directed against phosho-Thr202/Tyr204.

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FIGURE 5. Time course of opioid stimulation of ERK phosphorylation in undifferentiated ES cells. D3 ES cells grown in serum/LIF/ME-containing media were treated at different time points with opioids (1 μM). A, representative gels of ERK phosphorylation. B and C, curves of quantified ERK phosphorylation. n = 3–12. *, significantly greater than controls, p < 0.05; **, significantly greater than controls, p < 0.01.

FIGURE 6. Opioids induce limited proliferation in undifferentiated D3 ES cells. Proliferation was measured by [methyl-3H]thymidine incorporation into ES cells. D3 ES cells grown in serum/LIF/ME-containing media were treated with either vehicle (Con), morphine (0.5 μM), DAMGO (1 μM), or U69,593 (1 μM) for 24 h, and 0.02 μCi/ml [methyl-3H]thymidine was added for the last 4 h. In some experiments, the MEK-selective inhibitor U0126 (1 μM) was present for 28 h, whereas opioid agonists were added for 24 h, and [methyl-3H]thymidine was present for the last 4 h. n = 3–5. **, p < 0.01.

transcription factors that are expressed in ectodermal cells upon acquisition of neural progenitor identity (49, 50). By counting cells (≥1000 cells/treatment group), we estimated that only a few of the control cells (4 ± 1.1%, n = 8) were Sox-1–positive. Exposure of undifferentiated ES cells to 1 μM MOR (Fig. 7B) or KOR (Fig. 7C) opioids for 24 h initiated the appearance of about 7–8-fold more Sox-1–positive cells than in control cells (Fig. 7, D and E). The corresponding, selective MOR (CTAP) and KOR (nor-BNI) antagonists blocked agonist-induced appearance of Sox-1–positive cells, suggesting that this is an OR-mediated process. CTAP (10 ± 2.6%, n = 4) and nor-BNI (2 ± 0.5%, n = 4), alone had no significant effect on the number of Sox-1–labeled cells.

Opioid Regulation of ERK Phosphorylation in RA-induced ES Cell-derived NPs—To investigate opioid modulation of ERK activity upon RA-induced differentiation of ES cells, RA-induced embryoid bodies were dissociated and plated in serum containing Dulbecco’s modified Eagle’s medium for an additional 2–5 days (short term differentiation) or for 5–12 days (long term differentiation). After growing in serum-deprived media for 24 h, these cells were treated with opioids for various times, and ERK phosphorylation was measured by immunoblotting (Fig. 8). The results support the following conclusions: 1) basal levels of ERK phosphorylation in long term differentiated cells were significantly higher (2.2 ± 0.2, n = 9, p < 0.05) than basal levels of ERK activation in short term differentiated cells (NPs), suggesting that terminal differentiation of these cells may require activated ERK; 2) MOR and KOR agonists elicit similar effects on ERK activation in short and long term differentiated cells (Figs. 8 and 9).

More detailed time course experiments with DAMGO and U69,593 were also conducted (Fig. 9). These data further confirm that the two opioids induce a similar biphasic ERK activation profile with peaks occurring at 2–15 min and at about 2–6 h in short and long term differentiated ES cells (Fig. 9, A–D). Thus, the opioid-induced sustained ERK activation seen in
undifferentiated ES cells changes in differentiated cells to a biphasic ERK activation profile.

Opioids Inhibit NP Proliferation—To evaluate opioid effects on RA-induced NP proliferation, we performed a quantitative analysis of Sox-1/BrdUrd double-labeled, control, and opioid-treated NPs (Fig. 10). Since Sox-1 is a selective marker of proliferating NPs, we counted the BrdUrd-labeled cells among the Sox-1-positive cells and thus estimated the changes in NP proliferation upon treatment with opioids. The data presented in Fig. 10 indicate that 1 μM DAMGO or U69,593 decreased proliferation of NPs by 50–60% in 24 h. The inhibitory effect of both opioid agonists was reversed by the corresponding MOR (CTAP) and KOR (nor-BNI) antagonists, indicating that DAMGO and U69,593 were acting via their respective receptors. Interestingly, nor-BNI alone induced a 27% increase (59 ± 4.7%, n = 4, p = 0.0149) in cell proliferation over basal levels. This finding may be due to the possibility that NPs secrete endogenous κ-opioid peptides that may have inhibitory effects on basal levels of cell division. Upon the addition of KOR antagonist, the effects of both endogenous and exogenous KOR ligands were suppressed, resulting in an increase in proliferation over basal levels. In contrast, in the presence of CTAP alone, NP proliferation was similar to basal levels (34 ± 5.4%, n = 4, p = 0.168).

The involvement of the ERK signaling pathway in opioid regulation of NP proliferation was evaluated by treatment of the cells with the MEK inhibitor, U0126, for 1 h before opioid agonist exposure (Fig. 10). U0126 alone did not significantly affect basal levels of NP proliferation (44 ± 3.2%, n = 4, p = 0.832 versus controls of 43 ± 4.1%, n = 6). Interestingly, administration of U0126 in the presence of DAMGO did not reverse the inhibitory effect induced by the MOR agonist alone, suggesting
that MOR regulation of NP proliferation is independent of the ERK signaling pathway. Furthermore, the inhibitory effect of U69,593 was only partially reversed upon blockade of the ERK signaling by U0126.

**DISCUSSION**

The analysis of our data reveals several important findings. 1) MOR-1 and KOR-1 gene expression and immunoreactivity was detected in undifferentiated ES cells and in ES cell-derived NPs. This is the first evidence of the occurrence of MOR-1 and KOR-1 in blastocyst-derived ES cells. 2) MOR-1 and KOR-1 that occur in ES cells and NPs show functionality as established by the detection of opioid-induced temporal regulation of ERK/MAP kinase signaling. A sustained activation of ERK was characteristic for the opioid-treated undifferentiated ES cells, whereas the RA-induced NPs showed a biphasic profile of opioid-induced ERK activity. 3) The ERK signaling pathway mediated μ- and κ-opioid-induced limited proliferation in undifferentiated D3 ES cells, although in the absence of opioids, these cells may not require ERK activation for their self-renewal. More importantly, opioids induced ES cell asymmetric division to generate nestin/Sox1-positive NPs and reduced the self-renewal of ES cells. Thus, a novel finding here is that opioid-induced sustained activation of ERK may play a role in the initial differentiation of ES cells. 4) Opioids attenuated proliferation of the population of NPs that were generated upon differentiation of ES cells with RA. This inhibitory effect is ERK-independent for DAMGO and partially for U69,593 and correlated with MOR and KOR agonist induction of a biphasic rather than a sustained ERK activation profile in NPs.

In prior studies, KOR was found in the P19 embryonic carcinoma cell line, which contains pluripotent stem cells (51–53). Although these cells are similar to ES cells derived from 4–5-day-old mouse embryos, they are transformed cells and therefore capable of overexpressing genes not expressed in their parent cell. RA promoted expression of the MOR gene, whereas the KOR gene was first suppressed and then reactivated in P19...
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FIGURE 10. Opioids inhibit proliferation of ES cell-derived, RA-differentiated NPs. A, Sox-1 and BrdUrd immunocytochemistry. D3 ES cell-derived, RA-induced NPs were grown in chamber slides in Dulbecco’s modified Eagle’s medium containing fetal calf serum for 2 days. Cells were serum-starved for 24 h and treated with vehicle, DAMGO (1 μM) or U69,593 (1 μM), for an additional 24 h. In some cases, cells were pretreated for 1 h either with MOR antagonist (CTAP, 1 μM), KOR antagonist (nor-BNI, 1 μM), or the MEK-selective inhibitor U0126 (1 μM), followed by opioid agonist treatment. Cells were then incubated with BrdUrd and co-labeled with monoclonal BrdUrd and chicken Sox-1 polyclonal Abs as described under “Experimental Procedures.” Representative double BrdUrd (green)/Sox-1 (red) images are shown in control and opioid-treated cells. Magnification was ×20. B, quantification of BrdUrd/Sox-1 counting data. NIH ImageJ version 1.32 software was used to count cells. Cells from >5 fields/well were counted (>1000 cells/treatment group). The total number of NPs was estimated by counting Sox-1-stained cells. BrdUrd-positive cells were expressed as a percentage of the total number of Sox-1-labeled NPs. *p < 0.0004; #, significantly greater than agonist alone (p ≤ 0.0281).

cells (54–56). KOR was also found on the cell surface and nuclear fractions of GTR1 ES cells (57–59). In addition, MOR and δ-opioid receptor, but not KOR, are expressed in adult hippocampal progenitors, and opioid peptides, such as β-endorphin, can regulate proliferation of these progenitor cells via ERK (60). Reduced ERK signaling via MOR decreases proliferation of these progenitor cells, increases the number of in vitro generated neurons, and reduces the number of astrocytes and oligodendrocytes. Finally, opiates were found to inhibit neurogenesis in the adult rat hippocampus (61).

We have characterized the mechanism of ERK activation by ORs and demonstrated that opioids either enhanced or inhibited DNA synthesis in several types of primary cultures and cell culture model systems (40–42, 44, 62, 63). In most of these cases, opioids inhibited stimulated proliferation as seen here (Fig. 10). It was important to determine how opioids regulate the ERK signaling pathways in ES cells and in ES cell-derived NPs. As discussed in the Introduction, several studies suggest that ERK activation may not be required for ES cell self-renewal (4, 11–14). Our thymidine incorporation results further support this hypothesis. For example, the finding that MEK inhibitor U0126 alone enhanced basal levels of ES cell proliferation by about 40% supports the notion that undifferentiated ES cells can proliferate when ERK signaling is blocked and suggest that ERK activity may not be necessary for ES cell self-renewal. Moreover, our U0126 data on basal levels of ERK suggest that activation of this kinase may have inhibitory effects on ES cell self-renewal. In addition, under self-renewal conditions (serum/LIF/ME), ES cells maintain low basal ERK activity, data that further support the lack of requirement for ERK activity by these cells (Fig. 5). In contrast, the results also suggest that opioid regulation of ES cell asymmetric cell division in undifferentiated ES cells is ERK-dependent. Therefore, we propose that in undifferentiated ES cells, two processes take place. There is an ongoing ERK-independent, symmetric cell division leading possibly to ES cell self-renewal and an opioid-induced ERK-dependent, asymmetric cell division leading to ES cell differentiation (Fig. 11). Thus, both μ- and κ-opioids promote ES asymmetric cell division at a slightly more rapid rate than that of serum/ME/LIF-induced self-renewal of ES cells.

The above findings raise the following questions. If ERK signaling is not the regulator of ES cell division/self-renewal, then what is the signaling pathway that modulates this process? It was proposed that mouse ES cells utilize phosphatidylinositol 3-kinase to progress through the G1 phase and to avoid differentiation (18, 27). What is the mechanism of opioid induction of NP appearance? It has been suggested that Sox proteins might contribute to the transcripational activation of the MOR gene and that MOR could mediate some Sox-regulated developmental processes (64).

ERK signal duration impacts different cellular responses in many cells and may produce different outcomes in the same cells, such as proliferation, differentiation, or apoptosis (10, 26, 44, 65–68). In some cells, sustained but not transient activation of ERK is required to initiate proliferation (41, 65, 69, 70). Sustained ERK activity appears to be required by many cells to pass the G1 restriction point and to enter S phase, in which

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FIGURE 11. Hypothetical model of ES cell division upon opioid treatment. A, the symmetrical ES cell division is ERK-independent in the absence of opioids. B, opioids initiate asymmetrical cell division in ES cells. ES cell self-renewal is ERK-independent but may be reduced upon sustained activation of ERK. The appearance of NPs is due to an ERK-dependent cell division.

cellular DNA is replicated (65, 71). In contrast, other studies have shown that Ras/Raf mediated a transient epidermal growth factor-induced ERK activation that leads to proliferation, whereas Rap/Raf-mediated sustained activation of ERK is required for PC12 cell differentiation (22, 72). Our data suggest that the opioid-induced sustained activation of ERK triggers ES asymmetric cell division, and the “newly” formed cells appear to be ES cell-derived NPs.

The important role of the ERK signaling pathway in neural differentiation has been well established. Whereas some reports indicate that ERK signaling inhibits differentiation of some types of cells (73, 74), other studies support the idea that the ERK signaling may be a positive regulator of this process (75). A recent paper discusses the existence of a biphasic regulation of ERK activity during myogenic differentiation and suggests that the signaling pathway(s) may play a dual role in this multistep process, wherein the late phase is responsible for the formation of postmitotic myotubes (76). A similar study shows that an early stimulation and late inhibition of ERK activity by insulin-like growth factor mediates the switch in insulin-like growth factor action from inhibition to stimulation of skeletal muscle cell differentiation (77). Finally, it was found that the early stage of neuronal differentiation of mouse ES cell line P19 triggered by aggregation of the cells and RA treatment is accompanied by biphasic activation of ERK signaling: a transient phase and a second, sustained ERK activation that is maintained until the appearance of neural phenotype (78).

Here, we propose that opioid-induced sustained activation of ERK may be a required step for the development of ES cell-derived NPs, and opioid-induced biphasic ERK phosphorylation may play a supporting role in the increased differentiation rate during the generation and maturation of NPs. This hypothesis is based on the following findings. As seen in Fig. 7, basal levels of ERK activity are higher in long term differentiated ES cells than in short term differentiated cells (NPs). In addition, μ- and κ-opioids induce a moderate, biphasic activation of ERK signaling in short and long term differentiated ES cells. More importantly, we found that the biphasic stimulation of ERK phosphorylation by opioids accompanies the opioid-induced inhibition of proliferation in NPs. Although MOR regulation of NP proliferation was independent of the ERK signaling pathway, the inhibitory effect of U69,593 was only partially reversed upon blockade of the ERK signaling by U0126. This finding may be explained by the involvement of dual signaling pathways in KOR modulation of NP proliferation.

In conclusion, our studies suggest that MOR-1, KOR-1, and their exogenous ligands are able to modulate ES cell proliferation and differentiation. Thus, opioids and opioid-induced ERK signaling may play an important role in ES cell fate decisions by directing the cells to specific lineages.

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