Human Neural Precursor Cells Express Functional κ-Opioid Receptors


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ABSTRACT

Neural stem cells (NSCs) play an important role in the developing as well as adult brain. NSCs have been shown to migrate toward sites of injury in the brain and to participate in the process of brain repair. Like NSCs, cultured human neural precursor cells (NPCs) are self-renewing, multipotent cells capable of differentiating into neurons, astrocytes, and oligodendrocytes and of migrating toward chemotactic stimuli. Cellular and environmental factors are important for NPC proliferation and migration. Expression of κ-opioid receptors (KORs) and μ-opioid receptors (MORs) in murine embryonic stem cells and of MORs and δ-opioid receptors in rodent neuronal precursors, as well as hippocampal progenitors has been reported by other investigators. In this study, we demonstrated robust expression of KORs in highly enriched (>90% nestin-positive) human fetal brain-derived NPCs. We found that KOR ligands, dynorphin1–17 and trans-3,4-dichloro-N-methyl-N[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate ([U50,488] but not dynorphin1–17, stimulated proliferation and migration of NPCs in a concentration-dependent manner. NPC proliferation was maximally stimulated at 10−14 M dynorphin1–17 and 10−12 M U50,488. The KOR selective antagonist, nor-binaltorphimine, partially blocked the migratory and proliferative effects of KOR agonists supporting, at least in part, the involvement of a KOR-related mechanism. As has been described for rodent P19 embryonal carcinoma stem cells, retinoic acid treatment markedly suppressed KOR mRNA expression in human NPCs. Taken together, the results of this study suggest that activation of KORs alters functional properties of NPCs/NSCs that are relevant to human brain development and repair.

Self-renewing neural stem cells (NSCs) that are capable of differentiating into neurons, astrocytes, and oligodendrocytes play a crucial role in the developing as well as adult brain. The involvement of NSCs in neurogenesis and brain repair has been the subject of intense scientific interest in recent years given their therapeutic potential in many neurodegenerative diseases and traumatic injuries of the nervous system. The highly complex process of coaxing NSCs to migrate, proliferate, differentiate, and integrate into the neural network of the central nervous system at the right region at the right time awaits elucidation before this potential can be realized. To study various components of this complex process, cell culture models of neural precursor cells (NPCs) have been used, as these cells share with NSCs the ability to proliferate, differentiate, and migrate toward chemotactic stimuli (Ni et al., 2004).

An emerging body of evidence suggests that the endogenous opioid system is involved in neurogenesis. The three major classes of opioid receptors, μ-opioid receptors (MORs), δ-opioid receptors (DORs), and κ-opioid receptors (KORs), have been characterized in the mouse brain (Zhu et al., 1998). Whereas opioid receptor mRNA expression was not detected within the mouse embryo at embryonic days 7.5 and

ABBREVIATIONS: NSC, neural stem cell; NPCs, neural precursor cells; MOR, μ-opioid receptor; DORs, δ-opioid receptor; KOR, κ-opioid receptor; ES, embryonic stem; U50,488, trans-3,4-dichloro-N-methyl-N[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide methanesulfonate; RA, retinoic acid; SDF, stromal cell-derived factor; FITC, fluorescein isothiocyanate; phosphate-buffered saline, phosphate-buffered saline; MTT, 3,3’-3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PE, phycoerythrin; PI, propidium iodide; nor-BNI, nor-binaltorphimine; FITC-AA, FITC-conjugated 2-(3,4-dichlorophenyl)-N-methyl-N[1-(3-aminophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide; RT, reverse transcription; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; HPRT, hypoxanthine phosphoribosyl transferase; Ct, threshold cycle; SNC80, [(1R)-4-((αR,α’R)-29,3R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethylbenzamide; DMEM, Dulbecco’s modified Eagle’s medium.
KOR mRNA and the migratory response to KOR ligands. We also investigated the influence of RA on NPC function. We sought to determine whether these fetal human brain-derived factor (SDF)-1α receptors may modulate early developmental events in neural and non-neural tissues (Zhu et al., 1998). Activation of these opioid receptors by their endogenous ligands may mediate many physiological and behavioral effects. Although MORs and KORs were detected in mouse embryonic stem (ES) cells and in ES cell-derived neural progenitors (Kim et al., 2006), only MOR and DOR mRNAs were detectable in murine neuronal precursors (Hauser et al., 2000) or rat adult hippocampal progenitors (Persson et al., 2003a,b). A recent study of human fetal brain (20–21 weeks old) found distinct anatomical distribution patterns for opioid receptor mRNA, with KOR mRNA being the most abundantly expressed of the three opioid receptor types (Wang et al., 2006). However, localization of KORs specifically to NSCs has not been described.

The endogenous KOR peptide dynorphin has been shown to exhibit neuroprotective and excitotoxic properties (Hauser et al., 2005) and is involved in pain and seizures due to viral infection of the brain (Solbrig and Koob, 2004; Solbrig et al., 2006). Dynorphins have also been found to modulate glial DNA synthesis during brain ontogeny (Gorodinsky et al., 1995). It has been suggested that dynorphin plays a role in maintaining the balance of the endogenous opioid system (Lee, 1995) and that responses induced by dynorphin may be mediated by both opioid and nonopioid systems (Yarygin et al., 1998). The synthetic KOR agonist U50,488 has been reported to modulate cocaine-induced behavior in animals (Glick et al., 1995; Heidbreder et al., 1995; Shippenberg et al., 1996; Negus, 2004) and to have a neuroprotective effect (Hiramatsu et al., 2004). U50,488 was also shown to exhibit dichotomous neurotrophic effects similar to those of dynorphin (Gorodinsky et al., 1995) on [3H]thymidine incorporation, which were associated with differences in the developmental stages (7- to 21-day) of fetal rat brain cell aggregates (Barg et al., 1993).

Retinoic acid (RA), which is commonly used to differentiate stem cells, is a vitamin A derivative that is essential in embryo growth and development (Jacobs et al., 2006; McCaffery et al., 2006). An imbalance of RA can disrupt neural and non-neural tissues (Zhu et al., 1998). Activation of opioid receptors may modulate early developmental events in neural and non-neural tissues (Zhu et al., 1998). The synthetic KOR agonist U50,488 has been reported to modulate cocaine-induced behavior in animals (Glick et al., 1995; Heidbreder et al., 1995; Shippenberg et al., 1996; Negus, 2004) and to have a neuroprotective effect (Hiramatsu et al., 2004). U50,488 was also shown to exhibit dichotomous neurotrophic effects similar to those of dynorphin (Gorodinsky et al., 1995) on [3H]thymidine incorporation, which were associated with differences in the developmental stages (7- to 21-day) of fetal rat brain cell aggregates (Barg et al., 1993).

Materials and Methods

Reagents. All reagents were purchased from the sources indicated: Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium, gentamicin, and biotin-conjugated anti-FITC IgG (Invitrogen, Carlsbad, CA); phosphate-buffered saline (PBS), glucose, glutamine, poly-D-lysine, penicillin/streptomycin, trypsin, 3,3′,3′′-[4,5-dimethyl-2-thiazoylyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypan blue, ExtrAvidin-R-phycocerythin (PE), anti-glial fibrillary protein (an astrocyte marker) antibody, sodium dodecyl sulfate, and RA (Sigma-Aldrich, St. Louis, MO); propidium iodide (PI) (Millipore Corporation, Billerica, MA); human fibroblast growth factor-basic, human epidermal growth factor, N2 plus supplement, CXCL12/SDF-1α, anti-human nestin, and anti-human Tuj (a neuronal marker) antibodies (R&D systems, Minneapolis, MN); dynorphin1–17 and dynorphin2–17 (American Peptide Co., Inc., Sunnyvale, CA); and nor-binaltorphimine (nor-BNI) (Tocris Cookson, Ellisville, MO). U50,488 was a gift from Upjohn (now Pfizer, New York, NY), and anti-KOR antibody was generously provided by Dr. R. P. Elde, University of Minnesota, Minneapolis, MN. The KOR antisemur was produced in rabbits immunized with a conjugate of the C terminus of the rat KOR1 sequence (366–380), DPASMDVGGNPKP (Arvidsson et al., 1995; Shuster et al., 1999). The KOR antisemur specificity was verified by blockade of staining with cognate peptide concentrations of 1 to 100 nM, whereas shorter synthetic peptides (residues 366–373, 369–376, and 374–380) were unable to block at 1 mM (Arvidsson et al., 1995).

NPC Cultures. NPC cultures were prepared from 7- to 9-week-old human fetal brain tissues, as described previously (Ni et al., 2004). In brief, human fetal brain tissues obtained under a protocol approved by the Human Subjects Research Committee of the University of Minnesota were mechanically dissociated, resuspended in DMEM/F-12 media (containing 5 mM glucose and glutamine, N2 plus supplement, penicillin and streptomycin, and 20 ng/ml human fibroblast growth factor-basic/20 ng/ml human epidermal growth factor) and plated onto poly-D-lysine-coated 10-cm tissue culture Petri dishes. This stage is considered as passage 0. When cell cultures reached 50 to 60% confluence with clones of cells, they were subcultured by trypsin (0.0125%) at a density of 2 × 10⁴ cells/cm². The KOR antagonist nor-BNI (10 μM) was added to the upper chambers of a 96-well chemotaxis device (Neuro Probe Inc., Gaithersburg, MD) (3 × 10⁵ cells/well) separated from the lower chambers with an 8-μm pore size of polycyvinylpyrrolidone-free polycarbonate filter. The lower chambers were filled with KOR ligands, dynorphin1–17, and U50,488 or dynorphin2–17, at the indicated concentrations. After 8 h of incubation, NPCs that had migrated from upper chambers into lower chambers were quantified by Diff-Quik staining (Dade Diagnostics, Aguaada, Puerto Rico). To determine whether the migration toward KOR ligands was a KOR-mediated mechanism, NPCs were treated with the KOR antagonist nor-BNI for 30 min before the migration assay. Likewise, to determine whether RA altered migration, NPCs were treated with RA for 30 min before migration toward dynorphin1–17 and U50,488 was measured.

NPC Proliferation Assay. NPC cultures were either untreated or pretreated with nor-BNI for 30 min before treatment with the indicated concentrations of dynorphin1–17 or U50,488 at 1 day after plating followed by culture medium replacement (containing either medium or nor-BNI ± dynorphin1–17 or U50,488) every other day for 7 days. [3H]thymidine was added to NPC cultures on day 7 for 24 h followed by washing with media and lyssin with 2 N NaOH. Cell lysates were collected into vials containing scintillation cocktail and counted for γH radioactivity in a scintillation counter (Beckman Coulter, Fullerton, CA).

Cell Viability Assay. To determine the effect of RA on NPC viability, a MTT assay, which provides quantitative assessment of mitochondrial integrity, was used. After designated treatment time periods of NPCCs with RA, MTT (final concentration of 1 mg/ml) was added to cell cultures for 4 h followed by addition of lysin buffer [20%
SDS (w/v) in 50% N,N-dimethylformamide, pH 4.7, adjusted with 2.5% acetic acid and 1 N HCl (32:1) for 16 h. Cell lysate and absorbance was read at 600 nm (Molecular Devices, Sunnyvale, CA) to reflect uptake of MTT by live cells. Alternatively, NPCs treated with RA (10^{-7} M) for 72 h were stained with either trypan blue or PI to distinguish live versus dead cells.

**Immunocytochemical Staining.** To detect KOR expression, NPC cultures grown on poly-d-lysine-coated plastic chamber slides were fixed with 4% paraformaldehyde for 20 min followed by washing with PBS and incubation with 10% normal donkey serum in PBS for 1 h at room temperature. Primary anti-KOR antibody was added, and the mixture was incubated overnight at 4°C. After washing, secondary antibody (FITC or rhodamine conjugate) was added for 1 h at room temperature followed by viewing under a fluorescent microscope.

**Fluorescence-Activated Cell Sorting of KOR Expression on NPCs.** Trypsinized NPCs (10^6 cells) treated for 30 min with 30 μM FITC-conjugated 2-(3,4-dichlorophenyl)-N-methyl-N-[1-(3-aminophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide (FITC-AA), a FITC-conjugated KOR ligand (a generous gift from Dr. J. M. Bidlack, University of Rochester, Rochester, NY) (Ignatowski and Bidlack, 1998) with or without nor-BNI pretreatment (300 μM for 20 min) were washed and resuspended in biotin-conjugated anti-FITC IgG. After washing, NPCs were resuspended in PE, and after washing again, NPCs were subjected to fluorescence-activated cell sorting to determine the percentage of KOR expression. NPCs with the same treatment were viewed under fluorescent microscope.

**Real-Time qPCR.** Brain tissue, NPC, or RA-treated NPC cDNA with an RNase Mini Kit (QIAGEN, Valencia, CA) was DNase treated (Ambion, Austin, TX) followed by cDNA synthesis with SuperScript II (Invitrogen). KOR cDNA of brain tissues and NPCs was amplified in a final reaction volume of 50 μl containing 5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0, and 1% Triton X-100), 3 μl of 25 mM MgCl2, 1 μl of 10 mM dNTP mixture, 2 U of Taq DNA polymerase (Promega, Madison, WI), 1 μl each of 25 μM sense and anti-sense primers, 2 μl of cDNA, and water. The amplification cycles were set at 94°C for 60 s for 1 cycle, 94°C for 30 s, 65°C for 30 s, and 72°C for 60 s for 40 cycles followed by 72°C for 10 min for 1 cycle. The amplified PCR product was electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light to verify the DNA fragment size (246 base pairs for KOR).

**Statistical Analysis.** Data are expressed as means ± S.E.M. For comparison of means of multiple groups, analysis of variance was used, followed by Fisher’s protected least significant difference test.

**Results**

**KOR Expression in Brain Tissues and NPCs.** All seven human fetal brain tissues (8- to 16-week-old specimens) and all nine NPC cultures (passages 1 and 2) isolated from 7- to 9-week-old specimens examined were found to express KOR mRNA using RT-PCR (Fig. 1, A and B). The regions of the brain tissues obtained varied from cortex, midbrain, or whole brain depending on the specimen collected. For each specimen, a portion of brain tissue was used for total RNA extraction. When analyzed by RT-qPCR, after normalization to the housekeeping gene (∆Ct = KOR Ct – HPRT Ct, expression level = 2^{-ΔCt}), KOR mRNA expression levels varied among brain tissue specimens (from 5.17 to 49.52) and NPC cultures (from 3.1 to 17.27). Regardless, KOR mRNA was constitutively expressed in all of the brain specimens and in all NPC cultures derived from fetal brain tissues. Immunocytochemical staining of cultured NPCs demonstrated the expression of KOR colocalized mostly with nestin-positive cells (Fig. 1C). The NPC cultures were ≥90% nestin-positive, and KOR expression was estimated to be approximately 30 to 40% positive in these NPC cultures. NPCs labeled with FITC-AA (a KOR ligand) followed by biotinylated anti-FITC IgG and ExtrAvidin-R-PE incubation demonstrated KOR expression (Fig. 1Dc) and blockade by nor-BNI pretreatment (Fig. 1Dd). Minimal background (Fig. 1Da) (biotin-conjugated anti-FITC IgG alone) or nonspecific fluorescence (Fig. 1Db) (biotin-conjugated anti-FITC IgG + PE) was observed. The same treatment of NPCs subjected to flow cytometry analysis also demonstrated KOR expression and blockade by nor-BNI pretreatment (Fig. 1E).

**Effects of KOR Ligands on NPC Migration.** Given the finding that all NPC cultures tested contained cells that expressed KOR mRNA, we next investigated whether these receptors were functionally active by studying the migration of NPCs toward KOR ligands. In these studies, NPCs were found to migrate toward both the endogenous ligand dynorphin1-17 and the synthetic ligand U50,488 in a concentration-dependent manner but not toward dynorphin2-17 (Fig. 2), which does not bind to KOR because of the absence of the first amino acid, tyrosine. Furthermore, treatment of NPCs with the KOR-selective antagonist nor-BNI (10^{-6} M) significantly blocked this migration toward dynorphin1-17 (10^{-8} M: 61% inhibition; 10^{-6} M: 51% inhibition) and U50,488 (10^{-8} M: 42% inhibition; 10^{-6} M: 41% inhibition) (Fig. 3), demonstrating that a KOR-mediated mechanism is involved in the migratory effect of KOR ligands on NPCs.

**Effects of KOR Ligands on NPC Proliferation.** To assess the effect of KOR ligands on the proliferation of NPCs, cells were treated for 7 days with either dynorphin1-17 or U50,488 followed by an assessment of proliferation through quantifying [3H]thymidine uptake. Both KOR ligands stimulated NPC proliferation in a concentration-dependent manner (Fig. 4). Whereas dynorphin-mediated stimulation of proliferation was maximal at 10^{-14} M (Fig. 4A), the stimulatory effect of U50,488 appeared to be bell-shaped and was maximal at 10^{-10} M (Fig. 4B), and at these concentrations, dynorphin2-17 did not stimulate NPC proliferation (data not shown).

Pretreatment of NPCs with nor-BNI for 30 min before dynorphin1-17 and U50,488 treatment for 7 days partially blocked the stimulatory effect of the KOR agonists on proliferation (26% inhibition of dynorphin1-17; 20% inhibition of U50,488) (Fig. 5, A and B). Nor-BNI alone did not significantly affect NPC proliferation (Fig. 5, A and B).

**Regulation of KOR mRNA Expression by RA.** As RA has been shown to regulate KOR mRNA expression in rodent P19 embryonal carcinoma stem cells, we were interested in determining whether a similar effect was seen in human NPCs. NPCs that were treated with RA showed a markedly reduced expression of KOR mRNA, an effect that was a
time-dependent manner (Fig. 6). After 48 h of treatment, KOR mRNA expression was inhibited 23-fold, and by 72 h, 31-fold inhibition was observed. RA (10^{-7} M) treatment for 72 h was not associated with cellular toxicity assessed by MTT assay, trypan blue, or PI staining, and it inhibited (60%) NPC proliferation and minimally inhibited nestin mRNA expression (2–4-fold) (data not shown).
NPCs with RA (10^{-7} M) for 30 min also significantly inhibited their migratory response toward dynorphin1–17 and U50,488 (Fig. 7).

Discussion

In this study, we have demonstrated for the first time that human NPCs constitutively express functional KORs. The expression of KOR in NPCs was mostly colocalized with nestin, a neural precursor/stem cell marker, and KOR expression was estimated to be present in 30 to 40% NPCs. That these KORs are functionally active was demonstrated by the stimulatory effects of KOR ligands on two salient properties of NSCs/NPCs, i.e., cell migration and proliferation. The finding that NPCs migrate toward dynorphin1–17 and U50,488 but not toward dynorphin2–17 suggested that a KOR-mediated mechanism is involved, which was supported by significant inhibition (41–61%) of migratory activity toward KOR agonists by the KOR selective antagonist nor-BNI. Likewise, dynorphin2–17 did not stimulate NPC proliferation, but the blockade of dynorphin1–17 and U50,488-enhanced NPC proliferation by nor-BNI was less robust (20–26%). Although nor-BNI significantly blocked NPC migration toward dynorphin1–17 and U50,488, as well as the proliferative activity of these agonists, the blockade by nor-BNI was only partial, suggesting that a non-KOR-mediated mechanism may also be operative in these effects of KOR ligands on NPCs.

The expression levels of KOR mRNA varied among brain specimens and the NPCs derived from brain tissues when analyzed by RT-qPCR. Such variation could be attributed to many factors, including the region and age of the brain tissues, genetic differences, or exposure of the fetus to nutritional elements or drugs used by the pregnant mother. For example, maternal cannabis and alcohol exposure was found to impair expression of opioid-related genes (Wang et al., 2006). Although MOR and KOR were detected in murine ES cells (Kim et al., 2006), only MOR and DOR mRNA were detectable in either murine neuronal precursors (Hauser et al., 2000) or rat adult hippocampal progenitors (Persson et al., 2003a,b). However, it has been reported that the MOR gene is silent, whereas DOR and KOR genes are expressed in murine stem cells (Wei and Loh, 2002). This discrepancy...
among reports may be related to the age of the specimens or to different brain regions examined, as well as to different methodologies for in vitro cell culture.

Thus far, it has been reported that the DOR agonist SNC80 promoted neural differentiation in a multipotent mouse stem cell line, whereas the MOR agonist [d-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin and the KOR agonist U50,488 had no effect on differentiation (Narita et al., 2006). On the other hand, [d-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin and the KOR agonist U69,593 were found to induce differentiation of mouse ES cells (Kim et al., 2006). In rat adult hippocampal progenitors MOR and DOR antagonists decreased proliferation (Persson et al., 2003b), but β-endorphin and agonists for MOR/DOR stimulated proliferation (Persson et al., 2003a). In mouse cerebellar granule neuron precursors, MOR activation by morphine and DOR (δ₂) activation by deltorphin inhibited proliferation and differentiation, respectively (Hauser et al., 2000). Furthermore, chronic administration of morphine decreased neurogenesis in adult rat hippocampus (Eisch et al., 2000). Clearly, results from these rodent studies were different from our current study of human NPCs regarding KOR expression and the effects of KOR agonists on human NPCs. These conflicting results could reflect animal species differences or different methodologies for in vitro studies versus in vivo studies.

The important roles of RA in regulating neural development and controlling growth and differentiation of the developing central nervous system have been extensively studied, whereas the influence of RA in the adult brain on long-term potentiation and neurogenesis of the hippocampus has received much less attention (McCaffery et al., 2006). It has been reported that RA is critical in early-stage development of adult neurogenesis as deletion of RA significantly reduced neuronal differentiation, resulted in decreased expression of immature neuronal markers, and reduced cell survival (Jacobs et al., 2006). However, excessive concentrations of RA resulted in abnormal development of cerebellar and hindbrain nuclei in human embryos exposed to RA (McCaffery et al., 2003). Consistent with studies of rodent P19 embryonal carcinoma stem cell cultures (Bi et al., 2001; Park et al., 2005), we also found that treatment of human NPCs with RA resulted in profound down-regulation of KOR mRNA expression. Remarkably, treatment of these cells for only 30 min with RA also had a marked inhibitory effect on their migration toward KOR ligands. Whether or not RA treatment triggered certain signaling pathway(s) leading to negative feedback on NPC migration toward KOR ligands awaits further investigation. Currently, we can only speculate that RA treatment could desensitize rather than down-regulate KOR because the inhibitory effect of RA on NPC migration occurred in such a short period of time (30-min treatment). Furthermore, as RA is a vitamin A derivative, these findings could have pathobiological significance for the well-established deleterious effects of vitamin A excess on fetal brain development.

With opioid receptor knockout mice being used extensively in studies to delineate the role of opioid receptors in many experimental models, the network of opioid receptors and their endogenous ligands in brain development has become widely recognized. Activation of opioid receptors and their downstream signaling pathways has been found to modulate many cellular functions. The current finding that human...
NPCs express KORs may contribute to an understanding of the involvement of this class of opioid receptors in development of the human fetal brain as well as their potential role in the adult brain in memory formation and in directing NSCs to sites of brain injury.

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