Human Neural Precursor Cells Express Functional Kappa Opioid Receptors


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Running title: Functional Properties of KORs in NPCs

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Text Pages: 15
Tables: 0
Figures: 7
References: 32
Abstract: 240
Introduction: 704
Discussion: 887

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Abbreviations: NSCs, neural stem cells; NPCs, neural precursor cells; KORs, kappa opioid receptors; MORs, mu opioid receptors; DORs, delta opioid receptors; U50,488, trans-3,4-dichloro-N-methyl-N[2-(1-pyrrolidinyl)cyclohexyl]benzeneaceamide methanesulfonate; SDF-1α, stromal cell-derived factor-1α; MTT, 3,3’-3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; nor-BNI, nor-binaltorphimine; FITC-
AA, FITC-conjugated 2-(3, 4-dichlorophenyl)-N-methyl-N-[1-(3-aminophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide; SNC80, [(+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide].
Abstract

Neural stem cells (NSCs) play an important role in the developing as well as adult brain. NSCs have been shown to migrate towards 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 towards chemotactic stimuli. Cellular and environmental factors are important for NPC proliferation and migration. Expression of kappa opioid receptors (KORs) and mu opioid receptors (MORs) in murine embryonic stem cells and of MORs and delta 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 U50,488, but not dynorphin2-17, 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, norbinaltorphimine (nor-BNI), 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.
Introduction

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 (CNS) 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, mu opioid receptors (MORs), delta opioid receptors (DORs) and kappa opioid receptors (KORs), have been characterized in the mouse brain (Zhu et al., 1998). While opioid receptor mRNA expression was not detected within the mouse embryo at E7.5 and E8.5 days, subsequent spatial and temporal expression pattern of MOR, KOR and DOR mRNA at distinct ages suggests that opioid 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 mRNA were
detectable in murine neuronal precursors (Hauser et al., 2000) or rat adult hippocampal progenitors (Persson et al., 2003a; Persson et al., 2003b). A recent study of human fetal brain (20 to 21-week-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 non-opioid systems (Yarygin et al., 1998). The synthetic KOR agonist trans-3,4-dichloro-N-methyl-N[2-(1-pyrolidinyl)cyclohexyl]benzeneaceamide methanesulfonate (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 dynorphin (Gorodinsky et al., 1995) on $^3$H-thymidine incorporation which were associated with differences in the developmental stages (7-day or 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 patterning and differentiation (low RA concentrations) or induce abnormal development of cerebellum and hindbrain nuclei (high RA concentrations) (McCaffery et al., 2003). RA also has been shown to regulate many genes including those of opioid receptors (Beczkowska et al., 1996; Bi et al., 2001; Royal et al., 2005).

We have previously described the culture of human NPC that express both the stem cell marker nestin (≥90%) and a functional CXCR4 chemokine receptor, as demonstrated by their migration toward the CXCR4 ligand stromal cell-derived factor (SDF)-1α (Ni et al., 2004). In the present study, we sought to determine whether these fetal human brain tissue-derived NPCs express KORs, and if so, what influence KOR ligands (dynorphin and U50,488) would have on their function. We also investigated the influence of RA on NPC KOR mRNA and migratory response to KOR ligands.
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-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypan blue, extravidin-R-phycoerythrin (PE), anti-glial fibrillary protein (GFAP; an astrocyte marker) antibody, sodium dodecyl sulfate (SDS) and RA (Sigma, St. Louis, MO); propidium iodide (PI, Millipore, Billerica, MA); human fibroblast growth factor-basic (hFGFb), human epidermal growth factor (hEGF), 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., Sunnyvale, CA); nor-binaltorphimine (nor-BNI; Tocris, 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 antiserum was produced in rabbit immunized with a conjugate of the C terminus of the rat KOR1 sequence (366-380, DPASMRDVGGMNKPV) (Arvidsson et al., 1995; Shuster et al., 1999). The KOR antiserum specificity was verified by blockade of staining with cognate peptide concentrations of 1 to 100 nM while 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 previously described (Ni et al., 2004). Briefly, 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/F12 media (containing 8 mM glucose and glutamine, N2 plus supplement, penicillin and streptomycin, and 20 ng/ml hFGFb/20 ng/ml hEGF) and plated onto poly-D-lysine coated 10-cm tissue culture petri dishes. This stage is considered as passage 0. When cell cultures reached 50-60% confluence with clones of cells, they were subcultured by trypsin (0.0125%) at a density of 2x10^5 cells per 10-cm culture petri dish or 2x10^3 cells per well of 24-well plates and considered as passage 1. Medium was replaced every other day. NPC cultures at passages 1-3 were used throughout the study.

**NPC migration assay** Untreated or treated NPCs were added to upper chambers of a 96-well chemotaxis device (Neuro Probe Inc., Gaithersburg, MD) (3x10^5 cells/well) separated from the lower chambers with an 8 µm-pore size of polyvinylpyrrolidone-free polycarbonate filter. The lower chambers were filled with KOR ligands, dynorphin1-17 and U50,488, or dynorphin2-17 at 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, Aguada, PR). To determine if the migration towards KOR ligands was a KOR-mediated mechanism, NPCs were treated with KOR antagonist nor-BNI for 30 min prior to the migration assay. Similarly, to determine if RA altered migration, NPCs were treated with RA for 30 min prior to measuring migration toward dynorphin1-17 and U50,488.

**NPC proliferation assay** NPC cultures were either untreated or pretreated with nor-BNI for 30 min prior to treatment with indicated concentrations of dynorphin1-17 or U50,488 at one 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. ^3^H-thymidine was
added to NPC cultures on day 7 for 24 h followed by washing with media and lysis with 2 N NaOH. Cell lysates were collected into vials containing scintillation cocktail and counted for \(^3\)H 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 NPCs with RA, MTT (final concentration of 1 mg/ml) was added to cell cultures for 4 h followed by addition of lysis buffer (20% SDS [w/v] in 50% N,N-dimethyl formamide, pH 4.7, adjusted with 2.5% acetic acid and 1 N HCl [32:1]) for 16 h. Cell lysate was collected 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 vs. 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 (RT). Primary anti-KOR antibody was added and incubated overnight at 4°C. After washing, secondary antibody [fluorescein (FITC)- or rhodamine-conjugate] was added for 1 h at RT followed by viewing under fluorescent microscope.

**FACS of KOR expression on NPCs** Trypsinized NPCs (10\(^6\) cells) treated for 30 min with FITC-conjugated 2-(3, 4-dichlorophenyl)-N-methyl-N-[1-(3-aminophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide (30 µM) (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. Following washing NPCs were resuspended in PE. After washing, NPCs were subjected to FACS to determine the percentage of KOR expression. NPCs with the same treatment were viewed under fluorescent microscope.

**Reverse transcription-polymerase chain reaction (RT-PCR)** Total RNA isolated from brain tissues, NPCs or RA-treated NPCs with RNaseasy® 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 10x PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0], 1% Triton X-100), 3 µl of 25 mM MgCl₂, 1 µl of 10 mM dNTP mixture, 2U of Taq DNA polymerase (Promega, Madison, WI), 1 µl of each of 25 µM sense and anti-sense primers, 2 µl of cDNA, and water. The amplification cycles were set at 94°C for 60s for 1 cycle, 94°C for 30s, 65°C for 30s and 72°C for 60s for 40 cycles followed by 72°C for 10 min for 1 cycle. The amplified PCR product was electrophorezed on 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet light to verify the DNA fragment size (246 bp for KOR).

**Real-time quantitative PCR (qPCR)** Brain tissue, NPC or RA-treated NPC cDNA was diluted 1:10 and 2 µl of diluted cDNA was used in SYBR® premix Ex Taq™ (perfect real time) quantitative PCR (qPCR) (Takara, Fisher Sci., Chicago, IL). The qPCR primer sets for housekeeping gene and KOR were: sense 5’-
GACCTGCTGGATTACATCAAAGCAGT-3’ and antisense 5’-
CTTTGGATTATACTGCTGAGCAAG-3’ for hypoxanthine
phosphoribosyltransferase (HPRT); sense 5’-GTCTGCTGACTCCATTCCATATT-3’ and antisense 5’-ATCCTGAAGTGATTTCGGACTCTGC-3’ for KOR. Results of qPCR threshold cycle (Ct) of KOR were normalized to the Ct of each housekeeping genes and expressed as fold change relative to control (as $2^{-\Delta\Delta Ct}$) or as expression level ($2^{-\Delta Ct}$).

Statistical analysis  Data are expressed as mean ± SEM. For comparison of means of multiple groups, analysis of variance (ANOVA) was used, followed by Fisher’s PLSD 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 (passage 1 and 2) isolated from 7- to 9-week old specimens examined were found to express KOR mRNA using RT-PCR (Fig. 1A 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 housekeeping gene ($\Delta C_t = KOR \text{ Ct} - \text{HPRT Ct}$, expression level $= 2^{-\Delta C_t}$) 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 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 $\geq 90\%$ nestin-positive, and KOR expression was estimated to be approximately 30-40% 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 towards both the endogenous ligand dynorphin$_{1-17}$ and the synthetic ligand U50,488 in a concentration-dependent manner, but not towards dynorphin$_{2-17}$ (Fig. 2), which does not bind to KOR due to 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 towards dynorphin$_{1-17}$ ($10^{-8}$ M: 61% inhibition, $10^{-6}$ M: 51% inhibition) and U50,488 ($10^{-8}$ M: 42% inhibition, $10^{-6}$ M: 41% inhibition) (Fig. 3), demonstrating 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 dynorphin$_{1-17}$ or U50,488 followed by an assessment of proliferation through quantifying $^3$H-thymidine uptake. Both KOR ligands stimulated NPC proliferation in a concentration-dependent manner (Fig. 4). While dynorphin-mediated stimulation of proliferation was maximal at $10^{-14}$ M (Fig. 4A), U50,488’s stimulatory effect appeared to be bell-shaped and was maximal at $10^{-10}$ M (Fig. 4B). And at these concentrations, dynorphin$_{2-17}$ did not stimulate NPC proliferation (data not shown).

Pretreatment of NPCs with nor-BNI for 30 min prior to dynorphin$_{1-17}$ and U50,488 treatment for 7 days partially blocked the stimulatory effect of the KOR agonists on proliferation (26% inhibition of dynorphin$_{1-17}$; 20% inhibition of U50,488) (Fig. 5A and B). Nor-BNI alone did not significantly affect NPC proliferation (Fig. 5A 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 to
determine whether a similar effect is seen in human NPCs. NPCs that were treated with RA showed a markedly reduced expression of KOR mRNA, an effect that was 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). Treatment of NPCs with RA (10^{-7} M) for 30 min also significantly inhibited their migratory response towards dynorphin1-17 and U50,488 (Fig. 7).
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 30-40% of 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 U50,448, but not toward dynorphin2-17, suggested a KOR-mediated mechanism is involved which was supported by significant inhibition (41% to 61%) of migratory activity towards KOR agonists by the KOR selective antagonist nor-BNI. Similarly, dynorphin2-17 did not stimulate NPC proliferation, but the blockade of dynorphin1-17- and U50,448-enhanced NPC proliferation by nor-BNI was less robust (20% to 26%). Although nor-BNI significantly blocked NPC migration towards 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; Persson et al., 2003b). However, it has been reported that the MOR gene is silent while 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 different methodologies for in vitro cell culture.

Thus far, it has been reported that the DOR agonist SNC80 \[+(+)-4-[(\alpha R)-\alpha-(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide\] promoted neural differentiation in a multipotent mouse stem cell line, while the MOR agonist DAMGO and the KOR agonist U50,488 had no effect on differentiation (Narita et al., 2006). On the other hand, DAMGO 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 \(\beta\)-endorphin and agonists for MOR/DOR stimulated proliferation (Persson et al., 2003a). In mouse cerebellar granule neuron precursors, MOR activation by morphine and DOR (\(\delta_2\)) 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 CNS have been extensively studied, while 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 depletion 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 a negative feedback on NPC migration towards KOR ligands awaits further investigation. Currently, we can only speculate that RA treatment could desensitize rather than downregulate KOR since the inhibitory effect of RA on NPC migration occurred in such a short period of time (30 min treatment). Also, as RA is a vitamin A derivative these findings could have pathobiological significance regarding 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 have 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.
We are grateful to Dr. Fred Kravitz for his invaluable input in this study.
References


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Legends for figures

Figure 1. Expression of KOR in fetal brain tissues and NPCs. Total RNA extracted from (A) seven brain tissue specimens (8- to 16-week-old, lanes 1-7) and (B) nine NPC cultures (lanes 1-9) derived from 7- to 9-week-old brain tissue specimens was DNase treated and reverse transcribed to cDNA followed by PCR to detect KOR expression. (C) NPCs were immunostained for (a) nestin (green), (b) KOR (red) and (c) DAPI (blue) and three images were merged in (d). (D) NPCs labeled with (a) biotin-conjugated anti-FITC IgG (as background), (b) biotin-conjugated anti-FITC IgG + PE (as nonspecific fluorescence), (c) FITC-AA (30 µM, 30 min), biotin-conjugated anti-FITC IgG and PE, or (d) FITC-AA (30 µM, 30 min) after pretreatment with nor-BNI (300 µM for 20 min) followed by biotin-conjugated anti-FITC IgG and PE were viewed under fluorescence microscopy. (E) NPCs with the same treatment as in (D) were analyzed by flow cytometry.

Figure 2. Migration of NPCs toward KOR ligands. NPCs (10^6 cells/well) were loaded onto chemotaxis upper chambers separated by a membrane (8 µm) from the bottom chambers filled with medium control, KOR ligands (dynorphin1-17 and U50,488) or dynorphin2-17 at indicated concentrations for 8 h. Membranes were stained with Diff-Quik to assess migration as cell counts per 5 fields for each well. Data presented are mean ± SEM of triplicates of 3 separate experiments using NPCs from different brain tissue specimens. *p<0.05 and **p<0.01 vs. medium control (C).

Figure 3. Blockade of KOR agonist migratory activity by KOR antagonist. NPCs were untreated or treated with nor-BNI (10^-6 M) for 30 min prior to being loaded onto
chemotaxis upper chamber to assess migration towards medium (C), dynorphin1-17 or U50,488 (10^{-8} M and 10^{-6} M) in lower chambers for 8 h. Membranes were stained with Diff-Quik to assess migration as cell counts per 5 fields for each well. Data presented are mean ± SEM of triplicates of 2 (for dynorphin1-17) to 3 (for U50,488) separate experiments using NPCs derived from different brain tissue specimens. †† p<0.01 vs. medium control; * p<0.05 and ** p<0.01 vs. respective treatment without nor-BNI.

Figure 4. Effects of dynorphin1-17 and U50,488 on NPC proliferation. After plating NPCs onto poly-D-lysine-coated 24-well plates (2x10^4 cells/well) for 24 h, (A) dynorphin1-17 or (B) U50,488 at indicated concentrations was added at day 1 and at each media change (every other day) of NPC cultures. After treatment for 7 days, ³H-thymidine was added to NPC cultures for 16 h followed by washing and cell lysate collection into scintillation cocktail for radioactivity counting. Data presented as percent of untreated control are mean ± SEM of triplicates of 4 separate experiments using NPCs from different brain tissue specimens. ** p<0.01 vs. untreated control.

Figure 5. Blockade of KOR agonist proliferative activity by KOR antagonist. After plating NPCs onto poly-D-lysine-coated 24-well plates (2x10^4 cells/well) for 24 h, NPCs were either untreated (control) or pretreated with nor-BNI (10^{-8} M) for 30 min prior to (A) dynorphin1-17 (10^{-13} M) or (B) U50,488 (10^{-11} M) treatment at day 1. At each media change (every other day), fresh media containing either drug alone or in combination were added to NPC cultures. After treatment for 7 days, ³H-thymidine was added to NPC cultures for 16 h followed by washing and cell lysate collection into scintillation cocktail for radioactivity counting. Data presented as percent of untreated control are mean ±
SEM of triplicates of 4 separate experiments using NPCs from different brain tissue specimens. *p<0.05 vs. dynorphin1-17 alone.

Figure 6. Inhibition of KOR mRNA expression in NPCs by RA. Total RNA extracted from NPCs treated with RA (10^{-7} M) for 24, 48 and 72 h was DNase treated, reverse transcribed to cDNA and subjected to RT qPCR for KOR mRNA detection. Levels of KOR mRNA expression were normalized to housekeeping gene HPRT and expressed as fold change relative to Control. Data presented are mean of 3 separate experiments using NPCs from different brain tissue specimens.

Figure 7. Inhibition of NPC migration by RA. NPCs were untreated or treated with RA (10^{-7} M) for 30 min prior to being loaded onto chemotaxis upper chamber to assess migration towards medium (C), dynorphin_{1-17} or U50,488 (10^{-12} to 10^{-6} M) in lower chambers for 8 h. Membranes were stained with DiffQuick to assess migration as cell counts per 5 fields for each well. Data presented are mean ± SEM of triplicates of 3 separate experiments using NPCs from different brain tissue specimens. †p<0.05 and ††p<0.01 vs. medium control; *p<0.05 and **p<0.01 vs. respective treatment without RA.
Figure 2

[Graph showing migration rates for different concentrations of Dynorphin 1-17, U50,488, and Dynorphin 2-17. The x-axis represents Log [M], and the y-axis represents migration (cell count per 5 fields). Significant differences are indicated with * and ** symbols.]
Figure 3

![Bar graph showing migration (cell counts per 5 fields) for different conditions with Dynorphin1-17 and U50,488 at various log concentrations.](image-url)
Figure 5

A

NPC proliferation
($\text{[^3]H-Thymidine uptake as \% of control}$)

Dynorphin$_{1-17}$ ($10^{-13}$ M)

Dynorphin$_{1-17}$ ($10^{-15}$ M)

nor-BNI

B

NPC proliferation
($\text{[^3]H-Thymidine uptake as \% of control}$)

U50,488 ($10^{-11}$ M)

U50,488 ($10^{-11}$ M)

nor-BNI
Figure 6

![Graph showing KOR mRNA expression (fold change relative to Control) over different treatment hours (24, 48, 72).](image-url)
Figure 7

A

Dynorphin_{1-17}  RA (10^{-7} M)

**Migration (cell count per 5 fields)**

B

U50,488  RA (10^{-7} M)

Log [M]  Log [M]