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GLIAL-RESTRICTED PRECURSORS: PATTERNS OF EXPRESSION OF OPIOID RECEPTORS AND RELATIONSHIP TO HUMAN IMMUNODEFICIENCY VIRUS-1 Tat AND MORPHINE SUSCEPTIBILITY *IN VITRO*

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Abstract—Recent evidence suggests that human immunodeficiency virus (HIV)-induced pathogenesis is exacerbated by opioid abuse and that the synergistic toxicity may result from direct actions of opioids in immature glia or glial precursors. To assess whether opioids and HIV proteins are directly toxic to glial-restricted precursors (GRPs), we isolated neural stem cells from the incipient spinal cord of embryonic day 10.5 ICR mice. GRPs were characterized immunocytochemically and by reverse transcriptase–polymerase chain reaction (RT-PCR). At 1 day *in vitro* (DIV), GRPs failed to express μ opioid receptors (MOR or MOP) or κ-opioid receptors (KOR or KOP); however, at 5 DIV, most GRPs expressed MOR and KOR. The effects of morphine (500 nM) and/or Tat (100 nM) on GRP viability were assessed in GRPs at 5 DIV by examining the apoptotic effector caspase-3 and cell viability (ethidium monoazide exclusion) at 96 h following continuous exposure. Tat or morphine alone or in combination caused significant increases in GRP cell death at 96 h, but not at 24 h, following exposure. Although morphine or Tat caused increases in caspase-3 activity at 4 h, this was not accompanied with increased cleaved caspase-3 immunoreactive or ethidium monoazide-positive dying cells at 24 h. The results indicate that prolonged morphine or Tat exposure is intrinsically toxic to isolated GRPs and/or their progeny *in vitro*. Moreover, MOR and KOR are widely expressed by Sox2 and/or Nkx2.2-positive GRPs *in vitro* and the pattern of receptor expression appears to be developmentally regulated. The temporal requirement for prolonged morphine and HIV-1 Tat exposure to evoke toxicity in glia may coincide with the attainment of a particular stage of maturation and/or the development of particular apoptotic effector pathways and may be unique to spinal cord GRPs. Should similar patterns occur *in vivo* then we predict that immature astroglia and oligodendroglia may be preferentially vulnerable to HIV-1 infection or chronic opiate exposure. **© 2007 IBRO. Published by Elsevier Ltd. All rights reserved.**

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Abbreviations: AMC, Ac-DEVD-7-amino-4-methylcoumarin; BSA, bovine serum albumin; DIV, days *in vitro*; DMEM, Dulbecco's modified Eagle's medium; DSHB, Developmental Studies Hybridoma Bank; EGF, epidermal growth factor; EMA, ethidium monoazide; FGF-2, fibroblast growth factor-2; β-FNA, β-funaltrexamine; GRP, glial-restricted precursor; HIV, human immunodeficiency virus; O-2A, oligodendrocyte-type 2 astrocyte; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptase–polymerase chain reaction.

Key words: human immunodeficiency virus, opiate drug abuse, glial progenitors, neural stem cells, gliogenesis, cell death.

Injection drug abuse is a major cause of the spread of human immunodeficiency virus (HIV)/AIDS. Heroin, morphine and other opioid drugs of abuse not only promote HIV infection and the progression of AIDS (Nath et al., 2002; Steele et al., 2003), but also appear to intrinsically exacerbate the frequency and severity of HIV encephalitis (HIVE) in the CNS (Bell et al., 1998; Gurwell et al., 2001; Anthony et al., 2005). Despite the prevalence of HIV infection among drug abusers and importance of the opioid system in the pathogenesis of HIV, it is uncertain how opioid abuse augments the neuropathology of HIV (Nath et al., 2000; Nath, 2002; Hauser et al., 2005).

Developing neural cells can express μ (MOR or MOP), δ (DOR or DOP), and/or κ (KOR or KOP) opioid receptors, which can be important in regulating cell proliferation, differentiation, and survival (Hauser and Mangoura, 1998). The particular effects seen for each receptor type are contextual and differ among cell types and at different stages of development. Immature neurons (Hauser et al., 2000; Eisch and Harburg, 2006; Narita et al., 2006a), astrocytes (Stiene-Martin and Hauser, 1990; Eriksson et al., 1990, 1991; Stiene-Martin and Hauser, 1991; Hauser et al., 1996; Stiene-Martin et al., 1998; Belcheva et al., 2005), oligodendrocytes (Knapp et al., 1998; Stiene-Martin et al., 2001), and their precursors (Persson et al., 2003a,b, 2006; Kim et al., 2006) can respond uniquely to opioids. For example, MOR receptor activation can inhibit proliferation in immature astroglia, while activation of the same receptor type in immature oligodendroglia increases proliferation (Knapp et al., 1998).

We previously found that μ opioid receptor (MOR or MOP) activation exacerbated HIV-1 Tat cytotoxicity in oligodendrocyte-type 2 astrocyte (O-2A) progenitors (Khurdayan et al., 2004). The interactive toxicity was highly selective for O-2A progenitors and was not evident in more differentiated type 2 astrocytes and oligodendrocytes. To determine whether opiates and HIV-1 proteins are intrinsically toxic to glial precursors, we examined the effects of morphine, a prototypic opioid drug of abuse and preferential MOR agonist, on glial-restricted precursors (GRPs) isolated from neural stem cells from embryonic mouse spinal cord.

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Moreover, because morphine can have subtle actions via κ opioid receptors (KOR or KOP), and because MOR and KOR are expressed by developing neural cells (Hauser and Mangoura, 1998), and can independently affect cellular response/responsiveness to HIV-1 (Peterson et al., 1993, 1999, 2001; Chao et al., 1996, 2001), the patterns of expression of MOR and KOR were assessed in detail. GRPs were characterized by nestin, Sox2, A2B5 and Nkx2.2 immunoreactivity. Our findings indicate that opioid receptors are widely expressed by Sox2-positive and Nkx2.2-positive GRPs. In addition, both Tat and morphine alone are intrinsically lethal to GRPs, while in combination they display no additional interactive toxicity. This differs from the situation with A2B5-positive, bipotential O-2A progenitors, in which toxicity was seen only with combined Tat and morphine exposure (Khurdayan et al., 2004), suggesting a developmentally or regionally specific response of glial progenitors to HIV and/or opioid toxicity.

EXPERIMENTAL PROCEDURES

Cell culture

Pregnant ICR mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA). All experimental protocols conformed to local Institutional Animal Care and Use Committee (IACUC) and national (PHS) guidelines on the care and ethical use of animals. Experiments were designed and conducted to minimize the number of mice used and their discomfort. On gestational day 10.5±0.5 (E10.5), mice were killed with ether vapor and the embryos removed aseptically and kept on ice in cell culture media. Spinal cords were isolated from each embryo and pooled together such that the neural cells derived from the spinal cords of an entire litter (eight to 12 embryos) constituted a single statistical observation. As reported previously (Kalyani et al., 1997), cells were dissociated by incubating spinal cords in 0.05% trypsin–EDTA solution (15 min) on ice, followed by incubation in cell culture medium containing 1% bovine serum albumin (BSA; Roche Diagnostics, Indianapolis, IN, USA) for 15 min and then by triturating 18-times through a fire-polished, glass Pasteur pipette. GRPs were isolated by immunopanning in dishes precoated with A2B5 antibodies prepared from an A2B5-expressing hybridoma cell line (clone 105[HB-29]; ATCC, Manassas, VA, USA) for 1 h at room temperature at pH 7.0 (Mayer-Proschel et al., 1997; Mujtaba et al., 1999; Cao et al., 2005). Non-adherent cells were removed by rinsing with Dulbecco's phosphate-buffered saline (PBS) and discarded. A2B5-adherent cells were gently removed, using a cell scraper, before plating onto poly-L-ornithine (15 μg/ml; Sigma, St. Louis, MO, USA) and laminin (20 μg/ml; GIBCO-Invitrogen Corporation, Gaithersburg, MD, USA) coated cell culture plates (24 or 48 wells). Approximately 95% of cells isolated by panning were A2B5 immunoreactive. Cultures containing less than 95% A2B5 positive cells were discarded. GRPs were cultured for 1 or 5 days *in vitro* (DIV) at 35.5 °C in 5% CO_2/95% air and high humidity in Dulbecco's modified Eagle's medium/F12 (DMEM/F-12) containing N-2 supplement, recombinant murine fibroblast growth factor-2 (FGF-2, also known as basic FGF) (20 ng/ml; PeproTech Inc., Rocky Hill, NJ, USA) and platelet-derived growth factor-AA (PDGF-AA) (10 ng/ml; PeproTech Inc.) antibiotics (penicillin and streptomycin).

HIV-1 Tat protein

Recombinant Tat_{1-72} from HIV_{BRU} was prepared as described previously (Ma and Nath, 1997) with slight modifications (Gurwell et al., 2001). A deletion mutant variant of Tat_{1-72} ($Tat_{\Delta 31-61}$) lacking the core and basic neurotoxic domains (Nath et al., 1996) or immunoneutralized Tat_{1-72} was used as a control as previously described (Gurwell et al., 2001; Khurdayan et al., 2004).

Experimental treatments

GRPs were treated with morphine sulfate (500 nM), Tat_{1-72}, immunoneutralized Tat_{1-72}, or mutant $Tat_{\Delta 31-61}$ (100 nM). Some cultures were treated with the MOR antagonist β-funaltrexamine (β-FNA; Research Biochemical International, Natick, MA, USA) (1.5 μM).

PCR

Opioid receptor mRNA was assessed by reverse transcriptase–polymerase chain reaction (RT-PCR). GRPs were grown for 1 or 5 DIV and total RNA isolated from GRPs using GenElute™ Mammalian total RNA kit (Sigma). The first strand of cDNA was synthesized using 0.2 μg total RNA and random decamers. A PCR cycle consisted of 30 s at 94 °C, 30 s at 55 °C and 50 s at 68 °C for 35 cycles using platinum *Taq*DNA polymerase (GIBCO-Invitrogen Corporation). Negative controls included all the reagents essential for amplification with the exception of template DNA. 15S ribosomal control primers (5′-TTCCGCAAGTTCACCTACC-3′ and 5′-CGGGCCATGCTTTACG-3′) (Ambion, Austin, TX, USA) were included as an internal standard. The sense primer used for MOR started from proximal initiation sites (P_{m1}: 5′-CTCAGAGAGTGGCGCTTTGGGGATGC-3′) and the antisense primer was 5′-CGCTAAGGCGTCTGCCAGAGCAAG-3′. KOR primers consisted of 5′-ATCAGCGATCTGGAGCT-3′ and 5′-GCAAGGAGCATTCAATGAC-3′ (Wei et al., 2000). The final PCR products were analyzed on 2% agarose gels. The identities of the amplified PCR products were confirmed by sequencing (Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL, USA).

Immunocytochemistry

The presence of opioid receptors on GRPs was characterized immunocytochemically. After washing two times, GRPs grown for 1 or 5 DIV were fixed using 3% Zamboni's solution for 30 min, then permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. Immunodetection was performed by incubating with 1% donkey serum in PBS with rabbit anti-MOR-1 antisera (1:1000 dilution; AB1580, Chemicon International, Temecula, CA, USA), rabbit anti-δ (DOR-1 or OPRD1; AB1560 N-terminus; Chemicon) or rabbit anti-κ (KOR-1 or OPRK1; H-70; Santa Cruz Biotechnology, Santa Cruz, CA, USA) opioid receptor antisera (both at 1:500 dilutions) overnight at 4 °C; general procedures as previously described (Hauser et al., 2000). The above anti MOR-1 opioid receptor antibodies were generated against a synthetic peptide (CTNHQLENLEAETAPLP) corresponding to C-terminus of the cloned μ receptor (Chen et al., 1996). This synthetic peptide contains the amino acid sequence (NHQLENLEAETAPLP) originally used to generate anti-MOR-1 antibodies (Arvidsson et al., 1995) (with an N-terminal cysteine for conjugation), which we have extensively employed to characterize MOR expression in developing glia (Hauser et al., 1996; Stiene-Martin et al., 2001). Anti-opioid receptor primary antibodies were detected using donkey anti rabbit secondary antibodies conjugated to Cy3 (Invitrogen–Molecular Probes, Eugene, OR, USA) (1:250 dilutions) for 1 h at room temperature in the dark. Controls lacking primary antibodies were used to assess antibody specificity and MOR and KOR expression was independently confirmed by RT-PCR.

Cells were incubated overnight at 4 °C in goat anti-Sox2 or Nkx2.2 polyclonal antibodies (both at 1:100 dilutions; Santa Cruz Biotechnology). Sox2 or Nkx2.2 primary antibodies were detected using secondary antibodies conjugated to Cy2 or Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR, USA) (1:250 dilution) by

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