Neuroscience 144 (2007) 1075–1086

EFFECT OF μ AND κ OPIOIDS ON INJURY-INDUCED MICROGLIAL ACCUMULATION IN LEECH CNS: INVOLVEMENT OF THE NITRIC OXIDE PATHWAY

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Abstract—Damage to the leech or mammalian CNS increases nitric oxide (NO) production and causes accumulation of phagocytic microglial cells at the injury site. Opioids have been postulated to modulate various parameters of the immune response. Morphine and leech morphine-like substance are shown to release NO and suppress microglial activation. Regarding the known immuno-modulatory effects of selective μ and κ ligands, we have assessed the effect of these agents on accumulation of microglia at the site of injury in leech CNS. Leech nerve cords were dissected, crushed with fine forceps and maintained in different concentrations of opiates in culture medium for 3 h and then fixed and double stained with Hoechst 33258 and monoclonal antibody to endothelial nitric oxide synthase (NOS). Morphine and naloxone ($\geq 10^{-3}$ M) but not selective μ agonist, DAMGO [d-Ala2, N-Me-Phe-Gly5(ol)-enkephalin] and antagonist, CTAP [D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2] inhibited the microglial accumulation. The effect of morphine was abrogated by pre-treatment with naloxone and also non-selective NOS inhibitor, L-NAME [N^{ω}-nitro-L-arginine-methyl-ester; 10^{-3} M] implying an NO-dependent and μ-mediated mechanism. These results are similar to properties of recently found μ-3 receptor in leech, which is sensitive to alkaloids but not peptides. Both selective κ agonist, U50,488 [3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)-benzeneacetamide; $\geq 10^{-3}$ M], and antagonist, nor-binaltorphimine (*nor*-BNI; $\geq 10^{-3}$ M), inhibited the accumulation. The effect of *nor*-BNI was reversed by L-NAME. Immunohistochemistry showed decreased endothelial NOS expression in naloxone and U50,488-treated cords. Since, NO production at the injury site is hypothesized to act as a stop signal for microglias, opioid agents may exert their effect via changing of NO gradient along the cord resulting in disruption of accumulation. These results suggest an immuno-modulatory role for μ and κ opioid receptors on injury-induced microglial accumulation which may be mediated via NO.

Key words: *Hirudo medicinalis*, opioids, nitric oxide, microglia, cell accumulation, nerve injury.

In mammals as in other vertebrates, microglia constitute a physiologically distinct population of immunocompetent cells with mesodermal origin. After insult to the CNS—even minor pathological changes (including trauma, infection and neurodegenerative processes)—these ramified microglia regain an activated phenotype and express antigenic markers specific to the immune system leading eventually to the formation of a glial scar (Kreutzberg, 1996). Migration and accumulation of microglial cells at the lesion are among the earliest reactions following nerve injury, involving several steps, from activation and directed movement to gathering at the lesion. However, the role of microglia in recovery is controversial. They secrete trophic factors that enhance neuronal survival (Barron, 1995; Elkabes et al., 1996) and components of extracellular matrix, such as laminin, that promote growth (von Bernhardi and Muller, 1995). Also, directed migration of microglia may play a beneficial role in the elimination of damaged neurons or invading microorganisms. However, activated microglia can also be injurious to neighboring neurons by producing harmful cytotoxins as free radicals, excitatory amino acids, and inflammatory cytokines that induce neuronal death (Thanos et al., 1993; Barron, 1995; Angelov et al., 1998). Thus, down-regulation of the chemotactic ability of activated microglia may prevent potential neuronal damage in areas of brain injury.

CNS injury triggers a cascade of sequential cellular events, but repair is ordinarily incomplete in mammals (Brecknell and Fawcett, 1996). In contrast, the sequence of cellular events after neuronal injury in the leech CNS leads to successful regeneration of axons, repair of synaptic connections, and restoration of function (Modney et al., 1997). Leech microglia, like leech neurons and large glia, resemble their mammalian counterparts in their morphology, physiology and histochemistry and exhibit macrophage-like activity *in vivo* and in cell cultures. It has been revealed that leech microglia normally lie scattered among axons as an apparently homogeneous population of cells within the nerve cord and collect at the site of lesion within 24 h of nerve injury (Morgese et al., 1983). Experiments in which nerve cords have been isolated in tissue culture medium and then crushed have shown that most, if not all, of the microglia at the lesion migrate there from within the

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Abbreviations: ANOVA, analysis of variance; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; DAMGO, D-Ala2, N-Me-Phe-Gly5(ol)-enkephalin; D-NAME, N^{ω}-nitro-D-arginine-methyl-ester; eNOS, endothelial nitric oxide synthase; KOR, kappa opioid receptor; L-NAME, N^{ω}-nitro-L-arginine-methyl-ester; LPS, lipopolysaccharide; L-15, Leibowitz-15 culture medium supplemented with 2% fetal calf serum, 0.6% glucose, and gentamicin (10 mg/mL); NO, nitric oxide; *nor*-BNI, *nor*-binaltorphimine; NOS, nitric oxide synthase; SPNO, spermine NONOate; U50, 488, 3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)benzeneacetamide.

0306-4522/07$30.00+0.00
doi:10.1016/j.neuroscience.2006.10.036

nervous system. In contrast, there is no apparent shift of cells of the perineural sheath, which are distinctive for their flattened rather than spindle-shaped nuclei. Because the leech nerve cord lies entirely within a blood sinus and can be removed for study without damaging the cord between ganglia, it has been possible in the leech to make clear distinctions between resident microglia and blood macrophages that are a component of lesions in mammalian CNS. Moreover, there is evidently no microglial cell division in the leech in response to injury (McGlade-McCulloh et al., 1989), although in the mammalian brain division adds to the number of microglia at the lesion (Perry and Keane, 1997) and makes cell counts difficult. Distinctive properties of the leech CNS, including its location within a blood vessel and the clear identification of microglia in the living cord, lack of microglial division and great control of variables *ex vivo* at single or identified cell levels, have made it particularly favorable for study of the cells *in situ*.

It has been shown in the leech that immediately after injury, even before microglial accumulate, activity of the constitutive endothelial nitric oxide synthase (eNOS) increases and that some injured microglia, as well as the injured region of the connective glia, express eNOS (Banati et al., 1993; Shafer et al., 1998). Nitric oxide (NO) influences motile cells in both vertebrates and invertebrates via guanylate cyclase activation (Magazine et al., 1996). Microglia distributed throughout the CNS at the time of injury, are one source of nitric oxide synthase (NOS) activity (Shafer et al., 1998). Application of NO donor and NOS inhibitor leads to the hypothesis that the highest concentrations of NO at the crush would stop the migration of cells.

Endogenous opioid peptides play a variety of roles in the CNS from development to immune modulation. These functions are mediated mostly via specific opioid receptors uniquely localized in different brain regions and cells (Sheng et al., 1997). Mechanisms underlying CNS opioid effects may be mediated via immune mediators, such as cytokines, beta-chemokines, and free radicals (i.e. reactive oxygen intermediates and NO) produced by activated glial cells (Taub et al., 1991; Sheng et al., 1997; Chao et al., 1997). Several studies have indicated different neuroprotective or neurotoxic roles for μ and κ opioid agonists and antagonists (Baskin et al., 1994; Caroleo et al., 1994; Sheng et al., 1997). The opioid receptor antagonist, naloxone, has been considered pharmacologically beneficial to endotoxin shock, experimental cerebral ischemia and spinal cord injury (Chang et al., 2000; Liu et al., 2000a). Also, exposure of microglia and neurons to morphine potently induces apoptosis of these brain cells (Hu et al., 2002). The inhibitory effect of morphine on the mobility and phagocytic activity of the invertebrate microglia as well as vertebrate microglia provides additional functional evidence for a possible role of opiate-like compounds on down-regulating immunoregulatory processes, as also observed in the circulating immunocytes (Sonetti et al., 1997). Morphine decreases not only the number of cells emerging form the excised leech ganglia but also the degree of their transformation to the active ameboid forms depending on the cell conformational state. It is important to note that this dose-dependent and naloxone sensitive effect of morphine on microglial cells parallels that on activated immunocytes of the same species (Sonetti et al., 1994).

It has been demonstrated that invertebrates contain opiate substances in their neural tissue. Also, the existence of a third μ-receptor, μ3, has been suggested in immunocytes and in neural tissues of the invertebrates on human monocytes and other human and mammalian tissues (Sonetti et al., 1994; Stefano and Scharrer 1996; Stefano, 1998). The morphine-like receptor localized in leech ganglia had no affinity for any opioid peptides or analogues, but had high affinity for opiate alkaloids (Laurent et al., 2000). This receptor is coupled to cyclase adenylate inhibition as shown by the *in vitro* blockade of cyclic AMP level titration after morphine addition and a quick NO release (Stefano et al., 1993; Stefano and Scharrer 1996). In addition, it has been suggested that a morphine-like substance exists in leech ganglia and its amount significantly increased after a surgical trauma or lipopolysaccharide (LPS) injection. This increase in morphine-like substance supports the idea that this alkaloid-opiate may play a role in nervous, immune and vascular systems (Laurent et al., 2000; Stefano et al., 1993). Kappa opioid receptor (KOR) ligands, on the other hand, may play a neuroprotective and immunomodulatory role against certain CNS insults (Sheng et al., 1997; Hu et al., 1998; Goyagi et al., 2003). KOR activation has been postulated to suppress the humoral immune response and reactive oxygen intermediate production by microglia (Radulovic et al., 1995; Hu et al., 1998). Moreover, KOR agonists such as U50,488 (3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)cyclohexyl)benzeneacetamide) are shown to have neuroprotective effects in animal models of brain ischemic injury and HIV neurotoxicity (Itoh et al., 1993; Chao et al., 1996).

This study was designed to investigate the possible role of various κ and μ opioid agents on the accumulation of microglial cells in the site of injury and the possible involvement of the NO pathway, in the CNS of leech *Hirudo medicinalis*.

EXPERIMENTAL PROCEDURES

Animals and operations

Adult leeches (*Hirudo medicinalis*), weighing 3–5 g at the time of experimentation, were supplied from a breeding colony maintained in the laboratory or from a commercial local supplier (Hejamat Center, Tehran, Iran). Leeches were stored in artificial spring water (0.5 g of solid sea salts/L H_2O) at 10 °C for up to 6 months before use. Ganglia and their associated roots and connectives were dissected (Nicholls and Baylor, 1968). In brief, animals were anesthetized with 10% ethanol–spring water for 20 min. The nervous system of the animal was exposed by a dorsal cut and by removal of the gut. Blood was removed from the exposed area and the sheath covering the connectives and the ganglia of the nervous system was stripped off with fine forceps. The connectives were dissected from the leech and pinned in a dish coated with silicone rubber (Sylgard 184; Dow Corning, Midland, MI, USA) containing leech Ringer (115 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 10 mM Tris-maleate; pH=7.4) (Kuffler and Potter,

1964). Cords were crushed in a standard manner using a pair of fine forceps (Dumont No. 5) ground to a width of 300 μm, and preparations were placed in Liebowitz-15 (L-15, Gibco, Paisley, Scotland) culture medium supplemented with 2% fetal calf serum, 0.6% glucose, and gentamicin (10 mg/mL) (Ready and Nicholls, 1979), referred to here as L-15. Pharmacological reagents were dissolved in L-15. The protocol was approved by the Committee of Ethics of the Faculty of Sciences of Tehran University (357; 8 November 2000). All efforts were made to minimize the number of animals used and their suffering.

Microglial staining and eNOS immunoreactivity

To examine the distribution of eNOS immunoreactivity, crushed cords were immunostained with monoclonal antibodies to eNOS. Microglial cell nuclei were stained with Hoechst 33258 fluorescent dye to see accumulation of cells. In brief, cords were dissected from leeches, crushed in a standard manner and left for 3 h (maximum accumulation at the lesion obtained from previous reports) at room temperature in L-15 with specific reagents. The tissues were then rinsed in PBS and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, at 22 °C for 30 min and then incubated 60 min in blocking solution (2% fetal calf serum, 2% Triton X-100 in PBS; pH 7.5) (Chen et al., 2000; Kumar et al., 2001). Tissue was kept overnight at 4 °C in anti-eNOS monoclonal antibody developed in rabbit (Sigma, Steinheim, Germany), diluted 1:300 in blocking solution. For the secondary antibody, a 1:50 dilution of FITC-conjugated mouse anti-rabbit IgG (gamma chain specific, Sigma) was used. The tissue was mounted in glycerol 85% with Hoechst 33258 dye (Fluka, Steinheim, Germany) (10 mg/mL) and viewed with an Olympus fluorescence microscope (Olympus, Tokyo, Japan) using appropriate filters. Several through-focus images were taken using DP-70 Olympus camera.

Determining the effects of different μ opioid receptor ligands on injury-induced microglial accumulation and eNOS immunoreactivity

In order to investigate the effects of various μ opioid receptor ligands on the accumulation of microglia at the injury site, crushed cords were incubated in different concentrations of non-selective μ opioid receptor agonist and antagonist, morphine and naloxone (10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} M and 5×10^{-2} M; Temad, Tehran, Iran), as well as selective μ agonist, DAMGO [D-Ala2, N-Me-Phe-Gly5(ol)-enkephalin; 10^{-5}-10^{-2} M; Sigma] and selective μ antagonist CTAP [D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; 10^{-5}-10^{-2} M; Sigma], respectively for 3 h, fixed 30 min in 4% paraformaldehyde and stained as described above (n=6–7 for each group). Since morphine and naloxone showed significant effects on the level of microglial accumulation, the involvement of NO was assessed in this regard using a non selective NOS inhibitor, L-NAME (N$^{\omega}$-nitro-L-arginine-methyl-ester, Sigma). Thus, crushed cords were pre-treated with L-NAME (10^{-3} M), for 30 min and then incubated in morphine (10^{-2}) in L-15 for 3 h. The concentration of L-NAME was obtained from previous studies (Chen et al., 2000). In addition, to assess the involvement of μ receptor in the effect of morphine, one group was pre-treated with naloxone (10^{-2} M) for 30 min and then incubated in morphine (10^{-2} M; n=6–7 for each group).

Effect of selective κ receptor agonists and antagonists on microglial accumulation and involvement of NO

Accumulation was assessed in cords treated with selective κ agonist U50,488 (Sigma) and antagonist *nor*-binaltorphimine (*nor*-BNI; Sigma) in different concentrations (10^{-5}-10^{-2} M), as described above. Moreover, the effect of pre-treatment of cords with L-NAME (10^{-3} M) in *nor*-BNI treated groups was assessed (n=6–7 for each group).

Statistical analysis

To quantify peak accumulation for each group, the sampling technique was used with stained tissues, referred to here as a "line count" (Chen et al., 2000; Duan et al., 2003). A hairline in the offline through-focus images obtained from experiments was positioned perpendicular to the longitudinal axis of the crush, and the number of nuclei within the sheath touching or intersecting the line at the crush was then counted in a double-blind manner. To avoid counting the same cell twice, each time the line was moved 5 μm, the length of a nucleus, toward the center of the crush and counts repeated. Counts were made for both connectives at the anterior and posterior margins of the crush in which accumulation was highest, three series each and the counts were summed. This method measures accumulation at the highest concentration of cells. All results are presented as mean±S.E.M. Data were assessed by one-way analysis of variance (ANOVA). If a significant F value was obtained, post hoc analyses (Tukey-Kramer's multiple comparison tests) were performed to determine the effects of various treatments on the accumulation of microglia at the site of injury. P-values less than 0.05 were considered as significant. Calculations were performed using the SPSS statistical package (version 11.5).

RESULTS

Microglial accumulation and eNOS immunoreactivity are highest at the site of crush

Nerve cords were double-stained with a monoclonal antibody for eNOS and with the fluorescent nuclear dye Hoechst 33258 (Chen et al., 2000). As illustrated in Fig. 1A, eNOS immunoreactivity was not seen in uncrushed connectives (Shafer et al., 1998); also, microglial cells were evenly distributed throughout the connectives (Morgese et al., 1983) (Fig. 1B). Fig. 1D shows that cords fixed 3 h after the crush show eNOS immunoreactivity at the lesion (Chen et al., 2000; Shafer et al., 1998), while uncrushed control cords do not exhibit eNOS activity (Fig. 1C).

Alkaloid μ ligands attenuate the injury-induced microglial accumulation

Fig. 2 shows the curve for effect of different concentrations of morphine and naloxone on injury-induced microglial accumulation. One-way ANOVA revealed that both morphine and naloxone exerted significant effects ($F_{(6,36)}$= 9.628, P<0.001 and $F_{(6,35)}$=6.885, P<0.001, respectively). Further analysis indicated that morphine (10^{-3}, 10^{-2} and 5×10^{-2} M) decreased microglial accumulation in comparison with control L-15 group (P=0.014, P<0.001 and P=0.001, respectively). In addition, further analysis showed that naloxone at concentrations equal to or greater than 10^{-3} M significantly decreased the accumulation of microglial cells. However, neither DAMGO nor CTAP, μ selective peptides, showed a significant effect on cell accumulation ($F_{(4,25)}$=0.490, P=0.743 and $F_{(4,25)}$=0.353, P=0.84, respectively) (Fig. 3). Fig. 4 shows the accumulation of cells in cords treated with L-15, morphine, DAMGO and CTAP. Fig. 5 shows the microglial accumu-

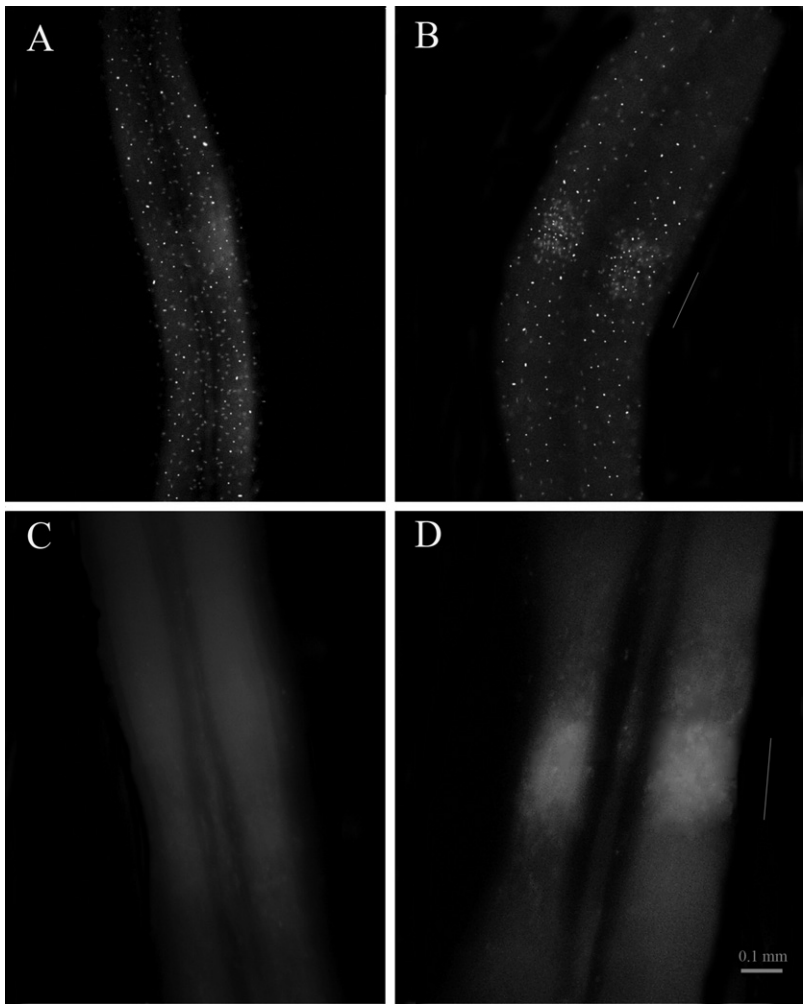

Fig. 1. Appearance of eNOS immunoreactivity and microglial cell accumulation at sites of CNS damage. The connectives connecting ganglia are paired with the bundled axons of each connective surrounded by a cellular sheath. Adult leech nerve cords were crushed and stained with a monoclonal antibody against human eNOS and with the fluorescent nuclear dye Hoechst 33258. The approximate longitudinal extent of the crushes is indicated by white lines. In uncrushed cords, microglial cells are distributed throughout the connectives (A) and eNOS immunoreactivity is absent (C). In crushed cords, fixed 3 h after crushing, microglial cells are accumulated at the injury site (B) and eNOS immunoreactivity is predominant at the crush site (D).

lation and eNOS immunoreactivity in a naloxone treated cord. Altogether, these results indicate that only alkaloid ligands but not peptides are able to reduce the accumulation of microglial cells.

Effect of morphine on microglial accumulation is both naloxone sensitive and NO-mediated

It has been hypothesized that injury-induced NO acts as a stop signal for microglial migration. Thus, the highest concentrations of NO at the crush may stop the migration of cells. Incubation of crushed cords in L-NAME (1 mM) reduces NO levels and microglial cell in the absence of NO, continue to migrate and do not accumulate at the lesion (Chen et al., 2000). Three groups of crushed cords were incubated in L-NAME 1 mM), D-NAME (N^{ω}-nitro-D-arginine-methyl-ester, 1 mM) and L-15. Consistent with previous results, there was a significant difference between microglial cell accumulation at the lesion site (one-way ANOVA, $F_{(2,15)}=18.182$, $P<0.001$) in injured tissue treated with L-NAME (1 mM) compared with tissue treated with D-NAME ($P<0.001$) and L-15 ($P<0.001$) (figure not shown). Moreover, morphine is known to stimulate NO release in leech neural tissue (Laurent et al., 2000). If microglia aggregate at a lesion as a result of locally increased NO, then morphine by producing a general increase in NO might disrupt the injury-induced NO gradient and interfere with accumulation. This experiment was conducted to assess the involvement of NO in the effect of morphine on microglial accumulation. Fig. 6 shows the effect of pre-treatments with L-NAME and naloxone on morphine modulation of microglial accumulation. L-NAME (1 mM) exerted a significant effect on morphine-induced attenuation of microglial accumulation ($F_{(2,15)}=8.149$, $P=0.004$) and completely blocked the effect of morphine ($P=0.012$ and $P=0.958$ compared with morphine and L-15 control group, respectively). Pre-treatment of crushed cords with naloxone (10^{-2} M) for 30 min showed a significant interaction ($F_{(2,15)}=$

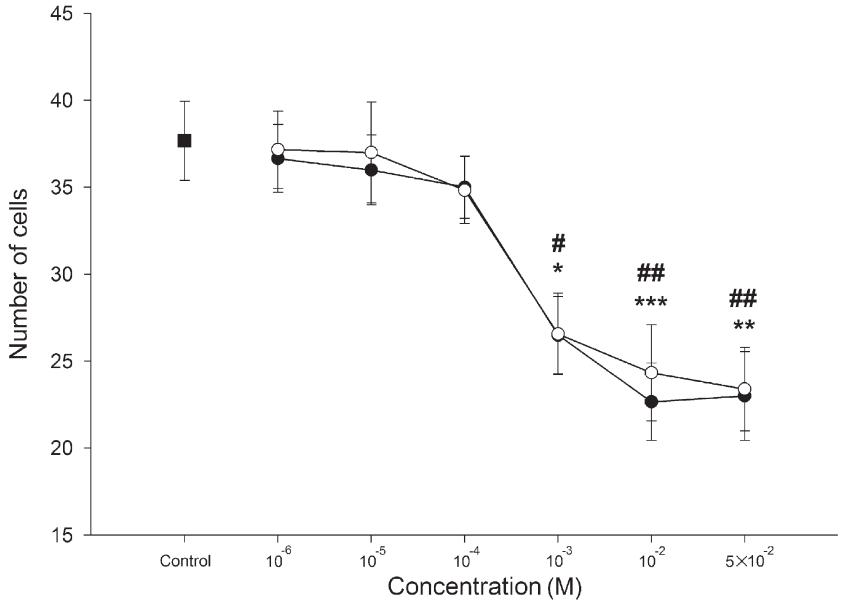

Fig. 2. Alkaloid μ opioid ligands attenuated the accumulation of microglial cells at the injury site. Leech nerve cords were crushed and incubated in different concentrations of morphine and naloxone in L-15 for 3 h, then fixed and stained with fluorescent Hoechst 33258 dye. For each sample the number of microglial cells was counted (see Experimental Procedures). The curves show the effect of different concentrations of morphine (●) and naloxone (○) on accumulation of microglial cells. The data are shown as means of cell counts±S.E.M. * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ for morphine-treated groups different from control group (■). $^{\#}P<0.05$, and $^{\#\#}P<0.01$ for naloxone-treated groups different from control group (Tukey-Kramer's multiple comparison tests).

9.074, $P=0.003$). Naloxone (10^{-2} M) completely reversed the effect of morphine on microglial accumulation ($P=0.012$ and $P=0.818$ compared with morphine and L-15 control group, respectively) and caused accumulation similar to L-15 control group. Therefore, these findings suggest a naloxone sensitive and NO-mediated action for the effect of morphine on injury-induced microglial accumulation.

KOR ligands reduce the microglial accumulation via NO pathway

Both U50,488 and *nor*-BNI, selective κ agonist and antagonist, respectively, showed significant effects on microglial accumulation ($F_{(3,20)}=5.429$, $P=0.006$ and $F_{(3,20)}=9.185$, $P=0.001$, respectively). U50,488 and *nor*-BNI (10^{-3}, 10^{-2}

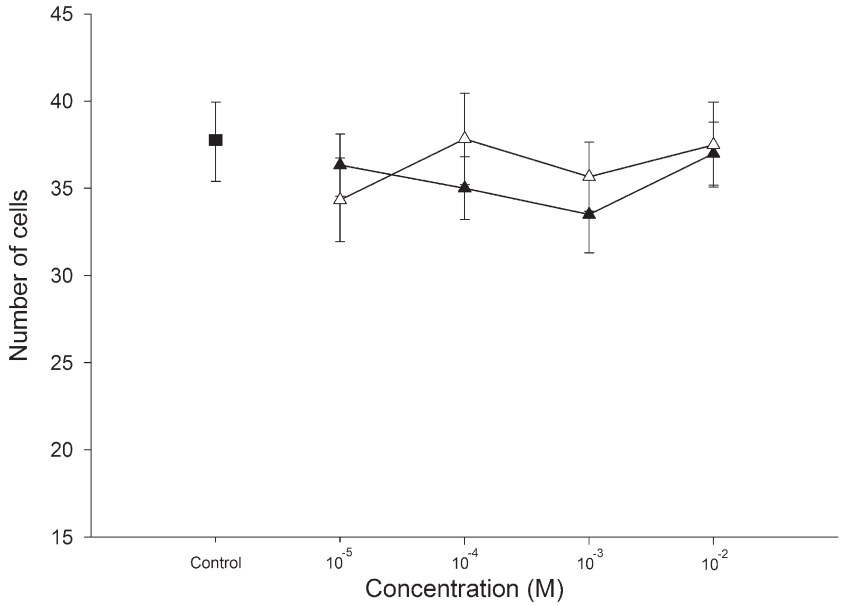

Fig. 3. The effect of different concentrations of selective μ agonist, DAMGO (▲), and selective μ antagonist, CTAP (Δ), on accumulation of microglial cells at the site of injury. The data are shown as means of cell counts±S.E.M. Selective peptide-treated cords showed accumulation similar to control cords (■) and no significant effect was seen.

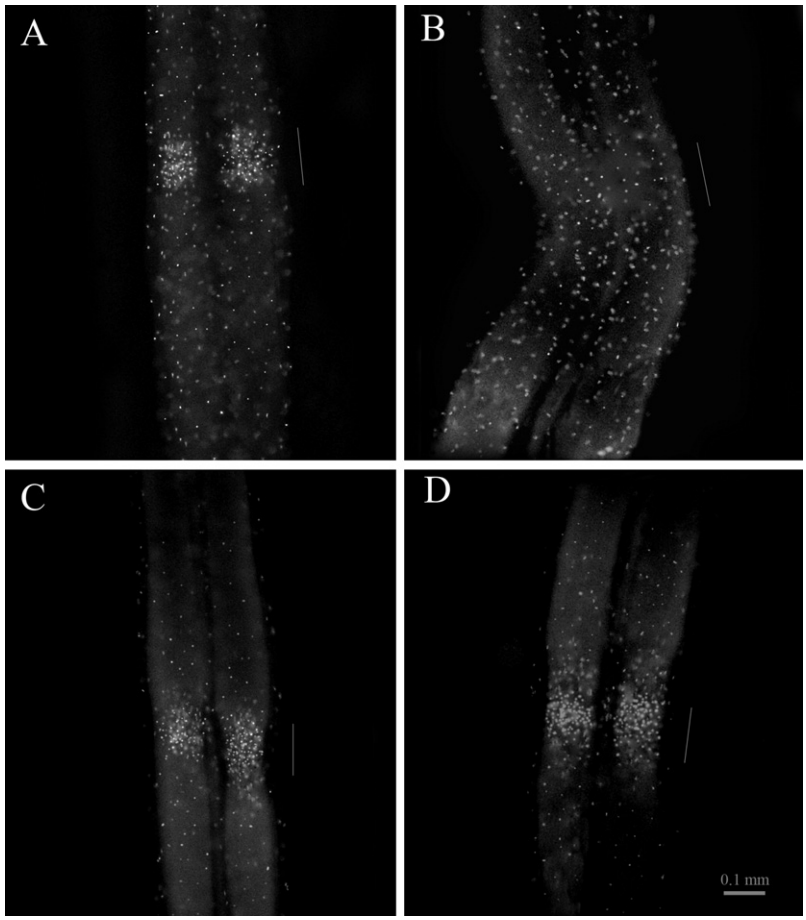

Fig. 4. Morphine but not selective μ peptides reduces the accumulation of microglial cells at the site of injury. Leech nerve cords were crushed and incubated in different μ opioid agents in L-15 for 3 h, then fixed and stained with fluorescent Hoechst 33258 dye. Microglial cells accumulated at the lesion site (vertical line) in L-15-treated control group (A). No accumulation was seen in cords treated with morphine (10^{-2} M) (B). Accumulation similar to control cords was seen in DAMGO- (10^{-2} M) and CTAP- (10^{-2} M) treated cords, respectively (C, D).

M) reduced the microglial accumulation at injury site in comparison with their control group ($P=0.008$ and $P<0.001$, respectively) (Fig. 7). Fig. 8 illustrates microglial accumulation and eNOS immunoreactivity in κ ligand-treated cords. The effect of pre-treatment of *nor*-BNI treated cords with L-NAME (10^{-3} M) is shown in Fig. 9,

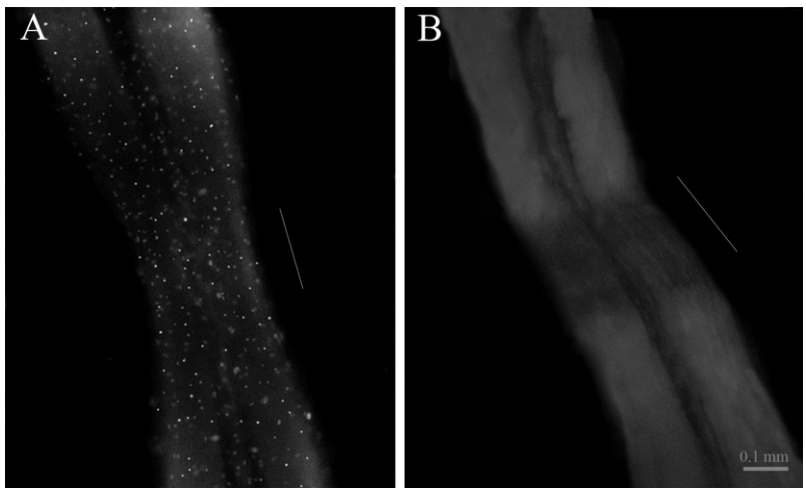

Fig. 5. The effect of naloxone on accumulation and eNOS immunoreactivity is shown in cords stained with Hoechst 33258 (A) and monoclonal antibody to eNOS (B). Naloxone (10^{-3} M) blocks the accumulation of microglial cells and expression of eNOS at the injury site.

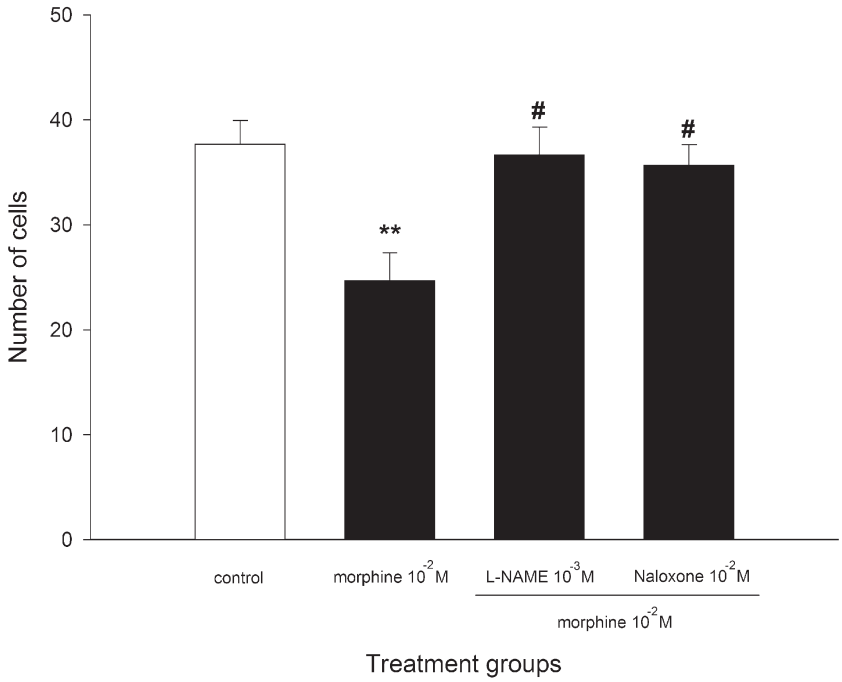

Fig. 6. Effect of morphine on microglial accumulation is both naloxone sensitive and NO-mediated. Inhibition of NO synthesis by L-NAME, a non-selective NOS inhibitor, reversed the effect of morphine on microglial cell accumulation at the injury site. Pre-treatment of cords with μ antagonist, naloxone, showed the same effect. The data are shown as means of cell counts±S.E.M. ** $P<0.01$ in comparison with L-15-treated control group. # $P<0.05$ in comparison with morphine-treated cords (Tukey-Kramer's multiple comparison tests).

where it exerted a significant effect on microglial accumulation ($F_{(2,15)}=9.207$, $P=0.002$). L-NAME completely reversed the effect of κ selective antagonist on microglial accumulation and caused accumulation similar to L-15-treated control group ($P=0.07$ compared with *nor*-BNI and $P=0.972$ compared with control group).

DISCUSSION

The leech CNS has been favorable for the study of nerve repair and synapse regeneration, which occurs successfully at the level of individual, identified neurons. Leech microglia are uniformly distributed at rest (Morgese et al.,

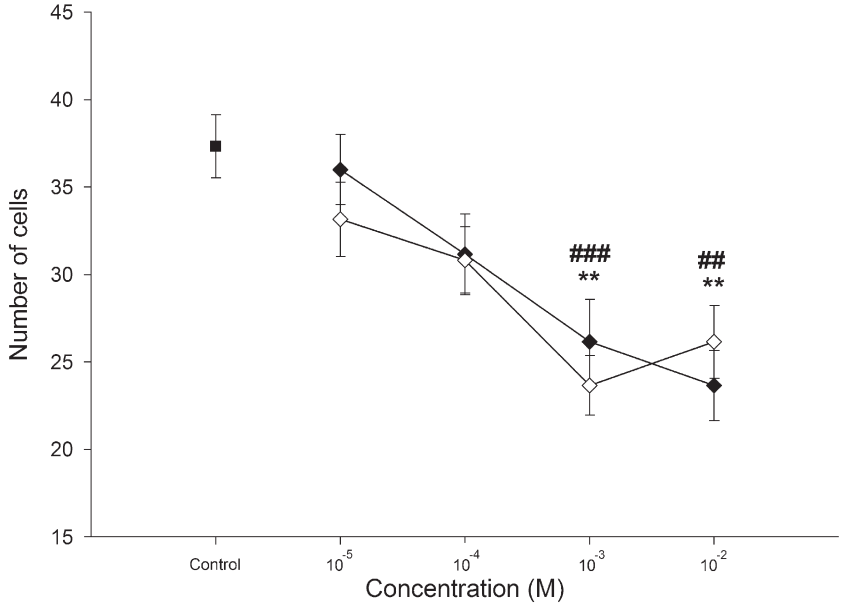

Fig. 7. Kappa ligands attenuate the accumulation of microglial cells at the injury site. The curve shows the effect of different concentrations of U50,488 (♦) and *nor*-BNI (◇) on accumulation of microglial cells. The data are shown as means of cell counts±S.E.M. ** $P<0.01$ for U50,488-treated groups different from control (■). ## $P<0.01$ and ### $P<0.001$ for *nor*-BNI-treated groups different from control (Tukey-Kramer's multiple comparison tests).

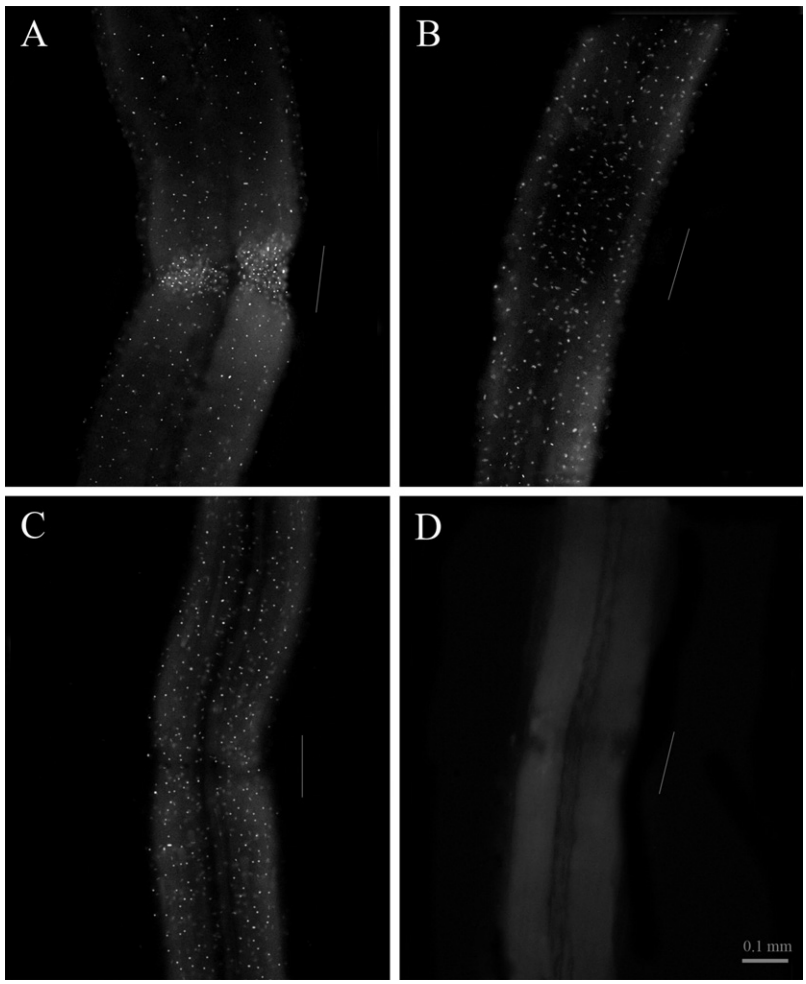

Fig. 8. Kappa ligands reduce the accumulation of microglial cells at the lesion site. Cords are stained with monoclonal antibody to eNOS and Hoechst 33258 fluorescent dye. Microglial cells accumulated at the lesion (vertical line) in L-15-treated control groups (A); however, no accumulation was seen in cords treated with *nor*-BNI (10^{-2} M) (B). In addition, U50,488 (10^{-2} M) blocked the microglial accumulation (C) and eNOS immunoreactivity (D) in crushed cords.

1983), and are rapidly activated and migrate directly toward a nerve injury, stopping at the lesion and accumulating there (McGlade-McCulloh et al., 1989), a process that appears to be regulated by NO (Chen et al., 2000). NOS activity at the lesion is one of the earliest events after crushing the leech nerve cord (Shafer et al., 1998). A delayed appearance of NO has been reported in injured and diseased tissues of the mammalian CNS (Blottner et al., 1995). For example, after peripheral axotomy, NOS mRNA (Verge et al., 1992) and protein (Vizzard et al., 1995) increase in some neuronal cell populations and NOS inhibition is shown to enhance peripheral nerve regeneration (Zochodne et al., 1997). Possible sources of NO may be macrophages that migrate to lesions and express the inducible isoform of NOS (iNOS), or neurons that express the Ca^{2+}/calmodulin-dependent eNOS and neuronal (nNOS) isoforms of NOS (Paakkari and Lindsberg, 1995). It has been shown that application of the NO donor spermine NONOate (SPNO) to the entire injured leech cord stops the cells in place, effectively preventing them from migrating to the crush, resulting in reduced microglial accumulation at the lesion site. It is hypothesized that application of NO donor all along the cord, eliminates the NO gradient between injury site and other parts of connectives and leads to prevention of cell migration. Also, incubation of crushed cords in the NOS inhibitor, L-NAME, reduces NO levels and microglial cell accumulation but does not affect the migration rate or population of microglia moving, supporting the hypothesis that injury-induced NO may serve as a stop signal for migrating microglia and disturbing the injury-induced NO gradient along the cord, could alter microglial migration (Chen at al., 2000). It implies that NO concentration changes may be enough to alter the microglial accumulation and these two agents by different and opposite mechanisms both prevent cell migration.

Morphine can induce NO production in macrophages, human endothelial cells and cells from diverse animals (Magazine et al., 1996). Moreover, morphine is shown to induce NO release from leech glial cells (Laurent et al., 2000). It is shown that both morphine and sodium nitroprusside, a substance that liberates NO, can cause previously activated and ameboid immunocytes and microglial

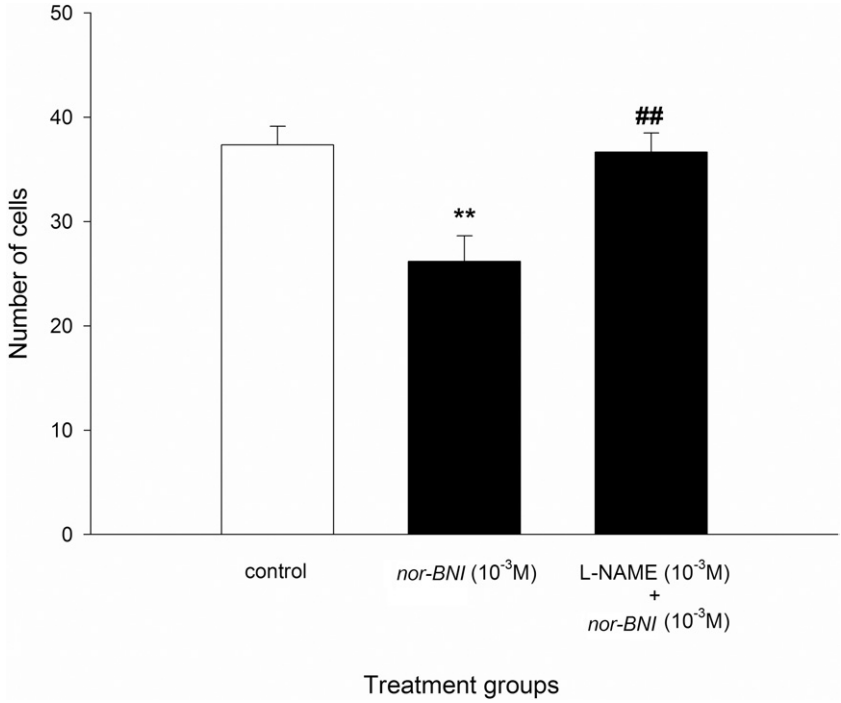

Fig. 9. The effect of κ antagonist, *nor*-BNI, is mediated via NO. L-NAME (10^{-3}M) inhibited the effect of *nor*-BNI (10^{-3}M) on microglial accumulation. The data are shown as means of cell counts±S.E.M. ** $P<0.01$ in comparison with L-15-treated control group. ## $P<0.01$ in comparison with cords treated with *nor*-BNI (Tukey-Kramer's multiple comparison tests).

as well as human endothelial cells to become round (Stefano et al., 1993; Magazine et al., 1996). Morphine diminishes the microglial egress from the excised leech ganglia and in resting microglia the opiate alkaloid causes the cells to sprout short spikes and elongate existing filopodia; however, they maintain a rounded shape. It is shown that the effects of morphine addition to the microglia cultures depend on the cell conformational state: in microglia in the resting form the opiate alkaloid caused the cells to sprout short spikes and elongate existing filopodia; however, they maintained a rounded shape. Furthermore, these cells have a clear tendency to aggregate. In contrast, a different and more impressive response is observed after microglial exposure to morphine with the cells in transitional ameboid conformation: microglia retire from the lamellipodia and become round as they lose intercellular and substrate adherence (Sonetti et al., 1997). The NO release inhibitor significantly inhibits morphine-induced conformational changes in these cell types, suggesting that the effect of morphine on cell conformation may be mediated, at least in part, by NO (Magazine et al., 1996). On the basis of these observations, one may surmise that the cell rounding previously attributed to morphine's direct action on these cells may actually be due to the action of morphine-induced NO release or be a combined action of both compounds. The present paper demonstrates that morphine attenuates microglial accumulation at the site of injury. This effect is inhibited by L-NAME and is also naloxone sensitive implying a possible involvement of NO and opioid receptor. One explanation could be that morphine, like SPNO, induces NO release all along the cord, altering the NO gradient and therefore inhibiting microglial migration to the site of injury. These observations are also consistent with the above-mentioned effects of morphine on microglial conformational changes that are inhibited by L-NAME and suggested to be mediated via NO pathway (Magazine et al., 1996). Concentrations required to see the effects in this ex vivo model are higher than microglial cell culture studies but are relevant to other studies using nerve cord or tissue samples (Chen et al., 2000; Duan et al., 2003).

Recently, it has been demonstrated that invertebrates contain opiate substances in their neural tissue (Laurent et al., 2000). Also, the existence of a third μ-receptor, μ-3, has been suggested in immunocytes and in neural tissues of the invertebrates, on human monocytes, and other human and mammalian tissues (Sonetti et al., 1994). The morphine-like receptor localized in leech ganglia has no affinity for any opioid peptides or analogues, but has high affinity for opiate alkaloids. This receptor appears to be coupled to NO release, which is associated with the induced conformational rounding in invertebrates and in human beings caused by morphine (Sonetti et al., 1997). In this study, opioid alkaloids (morphine and naloxone) but not selective opioid peptides (DAMGO and CTAP) show the effect that is consistent with the finding and properties of μ-3 receptor. Our data do not directly show the involvement of μ-3 receptor in this regard and further studies investigating the exact properties and involvement of this receptor in nerve-injury sequential events are required. The similarity between the effect of morphine and naloxone on microglial accumulation needs to be explained by more detailed investigations, but it could be attributed to

the effect of these agents on NO concentration along the cord as seen in NO donor and L-NAME-treated cords. Incubation of crushed cords in the NOS inhibitor, L-NAME, and also naloxone reduces NOS activity and NO production at the lesion leading to disturbance of cell accumulation. In previous experiments, L-NAME (10^{-3} M) is shown to completely inhibit microglial accumulation (Chen et al., 2000). In the present study, naloxone reduces eNOS immunoreactivity and exerts the same effect. However, these suggestions are based on observed correlations and warrant further studies using molecular markers to evaluate the migration machinery. Moreover, it is shown that a surgical trauma or LPS injection provokes a large increase in the level of morphine-like substance within 24–48 h in ganglia of leech (Laurent et al., 2000). Although, we do not know about the exact role of released endogenous opioid ligand in sequential events after nerve lesion, blockade of microglial accumulation at the injury-site by naloxone may suggest the involvement of this ligand.

Consistently, several evidences support the neuro-immuno-modulatory effect of opioids (Sheng et al., 1997). Naloxone is shown to protect neurons via reduction of reactive oxygen species production by microglia (Chang et al., 2000; Liu et al., 2000a). It also reduces LPS induced neurotoxicity (Liu et al., 2000b) and microglia-induced degeneration of dopaminergic substantia nigra neurons (Lu et al., 2000). As mentioned before, morphine stimulates NO release and induces conformational changes in human monocytes, granulocytes, and endothelial cells and in invertebrate immunocytes and microglia via NO (Magazine et al., 1996; Liu et al., 1996; Stefano, 1998). Morphine alters human microglial cell production and chemotaxis toward the activated complement component C_{5a} and beta-chemokine, RANTES (Hu et al., 2000). It also induces chemotaxis and brain-derived neurotrophic factor expression in microglia (Takayama and Ueda, 2005) and apoptosis of human microglia and neurons (Hu et al., 2002).

KOR are known modulators of brain activity (Tortella and DeCoster, 1994). In recent years, increased attention has been paid to the immunosuppressive effects of κ opioid ligands on macrophages (Carr et al., 1989) as well as on lymphocytes (Taub et al., 1991; Caroleo et al., 1994). These studies have suggested the existence of KORs on these immune cells. They suppress the humoral immune response both *in vivo* and *in vitro* (Radulovic et al., 1995). It has been postulated that activation of KOR (by U50,488) has an immuno-modulatory effect on the production of reactive oxygen intermediates by cytokine-primed microglial cells in culture. Pre-treatment of microglial cell cultures with an equal concentration of the selective KOR antagonist, *nor*-BNI, completely blocks the inhibitory effect of U50,488 implying the role of KOR in superoxide generation (Hu et al., 1998). Moreover, the inhibitory effect of KOR ligand on microglial expression of HIV-1-related neurotoxicity (Chao et al., 1996, 2000; Gekker et al., 2004) is well documented. In addition, they have neuroprotective properties in animal models of ischemic brain injury (Itoh et al., 1993; Widmayer et al., 1994). U50,488 has been shown to have superior neuroprotective activity against ischemic injury when compared with dynorphin (Baskin et al., 1994). Various effects of KOR are linked to the NO pathway (Park et al., 2002). Brain ischemic neuroprotection of KOR agonists is supposed to be related to the reduced neuronal NO production (Goyagi et al., 2003; Zeynalov et al., 2006; Chen et al., 2005). In this report, we have shown that KOR ligands reduce the accumulation of microglia at the site of injury. In light of the above data, we have investigated the effect of L-NAME on microglial accumulation alteration by *nor*-BNI. L-NAME has abrogated the effect of *nor*-BNI, implying that NO release may be the mechanism involved in this effect. In contrast with finding of μ opioid receptor, the existence of KOR is not yet investigated in leech CNS. However, the demonstration of 62% homology between human brain KOR and mu opioid receptor suggests a potential for functional overlap. In addition an endogenous opioid ligand might interact with more than one type of opioid receptors (Pogozheva et al., 2005).

CONCLUSION

In conclusion, the present paper is the first report of opioid modulation of microglial accumulation at the site of injury in leech CNS. Both κ ligands and μ alkaloid ligands showed the effect which was possibly mediated via NO. Effects of these agents on CNS regeneration and repair in leech and other animals warrant further investigations. Although the clinical significance of the findings in the present study is unknown, the therapeutic potential for the use of alkaloid μ antagonist, naloxone and κ antagonist *nor*-BNI in brain injury should be considered.

Acknowledgments—The authors are greatly indebted to Dr. K. J. Muller for his help and advice throughout the work. We express our thanks to Drs. F. F. De-miguel, A. R. Dehpour and M. H. Ghahremani for their helpful suggestions and Mr. H. Akbari for expert technical assistance. This study was supported by a grant from the Vice-Chancellor for Research of Tehran University of Medical Sciences to S.N.O. and N.Y.F.A.

REFERENCES

Angelov DN, Walther M, Streppel M, Guntinas-Lichius O, Neiss WF, Probstmeier R, Pesheva P (1998) Tenascin-R is antiadhesive for activated microglia that induce downregulation of the protein after peripheral nerve injury: a new role in neuronal protection. J Neurosci 18:6218–6229.

Banati RB, Gehrmann J, Schubert P, Kreutzberg GW (1993) Cytotoxicity of microglia. Glia 7:111–118.

Barron KD (1995) The microglial cell. A historical review. J Neurol Sci 134:57–68.

Baskin DS, Widmayer MA, Browning JL, Heizer ML, Schmidt WK (1994) Evaluation of delayed treatment of focal cerebral ischemia with three selective kappa-opioid agonists in cats. Stroke 25: 2047–2053.

Blottner D, Grozdanovic Z, Gossrau R (1995) Histochemistry of nitric oxide synthase in the nervous system. Histochem J 27:785–811.

Brecknell JE, Fawcett JW (1996) Axonal regeneration. Biol Rev Camb Philos Soc 71:227–255.

Caroleo MC, Arbitrio M, Melchiorri D, Nistico G (1994) A reappraisal of the role of the various opioid receptor subtypes in cell-mediated immunity. Neuroimmunomodulation 1:141–147.

Carr DJ, DeCosta BR, Kim CH, Jacobson AE, Guarcello V, Rice KC, Blalock JE (1989) Opioid receptors on cells of the immune system: evidence for delta- and kappa-classes. J Endocrinol 122:161–168.

Chang RC, Rota C, Glover RE, Mason RP, Hong JS (2000) A novel effect of an opioid receptor antagonist, naloxone, on the production of reactive oxygen species by microglia: a study by electron paramagnetic resonance spectroscopy. Brain Res 854:224–229.

Chao CC, Gekker G, Hu S, Sheng WS, Shark KB, Bu DF, Archer S, Bidlack JM, Peterson PK (1996) Kappa opioid receptors in human microglia downregulate human immunodeficiency virus-1 expression. Proc Natl Acad Sci U S A 93:8051–8056.

Chao CC, Hu S, Shark KB, Sheng WS, Gekker G, Peterson PK (1997) Activation of mu opioid receptors inhibits microglial cell chemotaxis. J Pharmacol Exp Ther 281:998–1004.

Chao CC, Hu S, Gekker G, Lokensgard JR, Heyes MP, Peterson PK (2000) U50,488 protection against HIV-1-related neurotoxicity: involvement of quinolinic acid suppression. Neuropharmacology 39:150–160.

Chen A, Kumar SM, Sahley CL, Muller KJ (2000) Nitric oxide influences injury-induced microglial migration and accumulation in the leech CNS. J Neurosci 20:1036–1043.

Chen CH, Toung TJ, Hurn PD, Koehler RC, Bhardwaj A (2005) Ischemic neuroprotection with selective kappa-opioid receptor agonist is gender specific. Stroke 36:1557–1561.

Duan Y, Haugabook SJ, Sahley CL, Muller KJ (2003) Methylene blue blocks cGMP production and disrupts directed migration of microglia to nerve lesions in the leech CNS. J Neurobiol 57:183–192.

Elkabes S, DiCicco-Bloom EM, Black IB (1996) Brain microglia/macrophages express neurotrophins that selectively regulate microglial proliferation and function. J Neurosci 16:2508–2521.

Gekker G, Hu S, Wentland MP, Bidlack JM, Lokensgard JR, Peterson PK (2004) Kappa-opioid receptor ligands inhibit cocaine-induced HIV-1 expression in microglial cells. J Pharmacol Exp Ther 309:600–606.

Goyagi T, Toung TJ, Kirsch JR, Traystman RJ, Koehler RC, Hurn PD, Bhardwaj A (2003) Neuroprotective kappa-opioid receptor agonist BRL 52537 attenuates ischemia-evoked nitric oxide production in vivo in rats. Stroke 34:1533–1538.

Hu S, Peterson PK, Chao CC (1998) Kappa-opioid modulation of human microglial cell superoxide anion generation. Biochem Pharmacol 56:285–288.

Hu S, Chao CC, Hegg CC, Thayer S, Peterson PK (2000) Morphine inhibits human microglial cell production of, and migration towards, RANTES. J Psychopharmacol 14:238–243.

Hu S, Sheng WS, Lokensgard JR, Peterson PK (2002) Morphine induces apoptosis of human microglia and neurons. Neuropharmacology 42:829–836.

Itoh J, Ukai M, Kameyama T (1993) U-50,488H, a κ-opioid receptor agonist, markedly prevents memory dysfunctions induced by transient cerebral ischemia in mice. Brain Res 619:223–228.

Kuffler SW, Potter DD (1964) Glia in the leech central nervous system: physiological properties and neuron-glia relationship. J Neurophysiol 27:290–320.

Kumar SM, Porterfield DM, Muller KJ, Smith PJ, Sahley CL (2001) Nerve injury induces a rapid efflux of nitric oxide (NO) detected with a novel NO microsensor. J Neurosci 21:215–220.

Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. Trends Neurosci 19:312–318.

Laurent V, Salzet B, Verger-Bocquet M, Bernet F, Salzet M (2000) Morphine-like substance in leech ganglia, evidence and immune modulation. Eur J Biochem 267:2354–2361.

Liu B, Du L, Hong JS (2000a) Naloxone protects rat dopaminergic neurons against inflammatory damage through inhibition of microglia activation and superoxide generation. J Pharmacol Exp Ther 293: 607–617.

Liu B, Du L, Kong LY, Hudson PM, Wilson BC, Chang RC, Abel HH, Hong JS (2000b) Reduction by naloxone of lipopolysaccharide-induced neurotoxicity in mouse cortical neuron-glia co-cultures. Neuroscience 97:749–756.

Liu Y, Shenouda D, Bilfinger TV, Stefano ML, Magazine HI, Stefano GB (1996) Morphine stimulates nitric oxide release from invertebrate microglia. Brain Res 722:125–131.

Lu X, Bing G, Hagg T (2000) Naloxone prevents microglia-induced degeneration of dopaminergic substantia nigra neurons in adult rats. Neuroscience 97:285–291.

Magazine HI, Liu Y, Bilfinger TV, Fricchione GL, Stefano GB (1996) Morphine-induced conformational changes in human monocytes, granulocytes, and endothelial cells and in invertebrate immunocytes and microglia are mediated by nitric oxide. J Immunol 156:4845–4850.

McGlade-McCulloh E, Morrissey AM, Norona F, Muller KJ (1989) Individual microglia move rapidly and directly to nerve lesions in the leech central nervous system. Proc Natl Acad Sci U S A 86:1093–1097.

Modney BK, Sahley CL, Muller KJ (1997) Regeneration of a central synapse restores non-associative learning. J Neurosci 17:6478–6482.

Morgese VJ, Elliott EJ, Muller KJ (1983) Microglial movement to sites of nerve lesion in the leech CNS. Brain Res 272:166–170.

Nicholls JG, Baylor DA (1968) Specific modalities and receptive fields of sensory neurons in the CNS of the leech. J Neurophysiol 31:740–756.

Paakkari I, Lindsberg P (1995) Nitric oxide in the central nervous system. Ann Med 27:369–377.

Park SW, Li J, Loh HH, Wei LN (2002) A novel signaling pathway of nitric oxide on transcriptional regulation of mouse kappa opioid receptor gene. J Neurosci 22:7941–7947.

Perry GW, Keane RW (1997) Modulation of microglial form and immune function by factors released from goldfish optic nerves. Int J Neurosci 91:133–146.

Pogozheva ID, Przydzial MJ, Mosberg HI (2005) Homology modelling of opioid receptor-ligand complexes using experimental constraints. AAPS J 7:E434–E448.

Radulovic J, Miljevic C, Djergovic D, Vujic V, Antic J, von Horsten S, Jankovic BD (1995) Opioid receptor-mediated suppression of humoral immune response in vivo and in vitro: involvement of kappa opioid receptors. J Neuroimmunol 57:55–62.

Ready DF, Nicholls J (1979) Identified neurones isolated from leech CNS make selective connections in culture. Nature 281:67–69.

Shafer OT, Chen A, Kumar SM, Muller KJ, Sahley CL (1998) Injury-induced expression of endothelial nitric oxide synthase by glial and microglial cells in the leech central nervous system within minutes after injury. Proc R Soc Lond B Biol Sci 265:2171–2175.

Sheng WS, Hu S, Gekker G, Zhu S, Peterson PK, Chao CC (1997) Immunomodulatory role of opioids in the central nervous system. Arch Immunol Ther Exp (Warsz) 45:359–366.

Sonetti D, Ottaviani E, Bianchi F, Rodriguez M, Stefano ML, Scharrer B, Stefano GB (1994) Microglia in invertebrate ganglia. Proc Natl Acad Sci U S A 91:9180–9184.

Sonetti D, Ottaviani E, Stefano GB (1997) Opiate signaling regulates microglia activities in the invertebrate nervous system. Gen Pharmacol 29:39–47.

Stefano GB (1998) Autoimmunovascular regulation: morphine and anandamide and ancondamide stimulated nitric oxide release. J Neuroimmunol 83:70–76.

Stefano GB, Digenis A, Spector S, Leung MK, Bilfinger TV, Makman MH, Scharrer B, Abumrad NN (1993) Opiate-like substances in an invertebrate, a novel opiate receptor on invertebrate and human immunocytes, and a role in immunosuppression. Proc Natl Acad Sci U S A 90:11099–11103.

Stefano GB, Scharrer B (1996) The presence of the m3 opiate receptor in invertebrate neural tissues. Comp Biochem Physiol 113: 369–373.

Taub DD, Eisenstein TK, Geller EB, Adler MW, Rogers TJ (1991) Immunomodulatory activity of mu- and kappa-selective opioid agonists. Proc Natl Acad Sci U S A 88:360–364.

Takayama N, Ueda H (2005) Morphine-induced chemotaxis and brain-derived neurotrophic factor expression in microglia. J Neurosci 12:430–435.

Thanos S, Mey J, Wild M (1993) Treatment of the adult retina with microglia-suppressing factors retards axotomy-induced neuronal degradation and enhances axonal regeneration *in vivo* and *in vitro*. J Neurosci 13:455–466.

Tortella FC, DeCoster MA (1994) Kappa opioids: therapeutic considerations in epilepsy and CNS injury. Clin Neuropharmacol 17:403–416.

Verge VM, Xu Z, Xu XJ, Wiesenfeld-Hallin Z, Hokfelt T (1992) Marked increase in nitric oxide synthase mRNA in rat dorsal root ganglia after peripheral axotomy: in situ hybridization and functional studies. Proc Natl Acad Sci U S A 89:11617–11621.

Vizzard MA, Erdman SL, de Groat WC (1995) Increased expression of neuronal nitric oxide synthase (NOS) in visceral neurons after nerve injury. J Neurosci 15:4033–4045.

von Bernhardi R, Muller KJ (1995) Repair of the central nervous system: lessons from lesions in leeches. J Neurobiol 27:353–366.

Widmayer MA, Turner TD, Browning JL, Baskin DS (1994) U50,488 reduces the severity of tissue damage in a rabbit model of focal cerebral ischemia. Mol Chem Neuropathol 22:211–223.

Zeynalov E, Nemoto M, Hurn PD, Koehler RC, Bhardwaj A (2006) Neuroprotective effect of selective kappa opioid receptor agonist is gender specific and linked to reduced neuronal nitric oxide. J Cereb Blood Flow Metab 26:414–420.

Zochodne DW, Misra M, Cheng C, Sun H (1997) Inhibition of nitric oxide synthase enhances peripheral nerve regeneration in mice. Neurosci Lett 228:71–74.

(Accepted 26 October 2006)
(Available online 12 December 2006)