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Opioid Antagonist Modulation of DNA Synthesis in Mouse Tongue Epithelium Is Circadian Dependent

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ZAGON, I. S., Y. WU AND P.J. McLAUGHLIN. *Opioid antagonist modulation of DNA synthesis in mouse tongue epithelium is circadian dependent.* PHARMACOL BIOCHEM BEHAV 48(3) 709–714, 1994.—In addition to neuromodulation, endogenous opioids also serve as growth factors. To investigate the possible involvement of opioid peptides in the renewal of epithelium, mice were given systemic injections of the opioid antagonist, naltrexone (NTX) (10 mg/kg). Disruption of opioid-receptor interaction by NTX resulted in an elevation (43–150% from control levels) of the number of basal epithelial cells undergoing DNA synthesis in the tip, dorsal, and ventral surfaces of the tongue. This stimulatory effect lasted up to 8 h following drug exposure, and was recorded at 1700 h but not 0700 h. These results are the first indication that endogenous opioids function in cellular renewal processes, and do so in a circadian rhythm-dependent manner.

Opioids Growth factors Tongue Epithelium Naltrexone Circadian rhythm Mouse Growth DNA synthesis

REGULATION of cell proliferation by an endogenous opioid system has been documented in the developing nervous system (including the retina) [see (23) for review] and heart (11), glial cells in culture (16), neural and nonneural cancers [see (18,21) for reviews], as well as bacteria (22). Recent evidence suggests that an opioid peptide also modulates wound healing of mammalian corneal epithelium (13). [Met^5]-Enkephalin, a pentapeptide derived from proenkephalin A, has been identified as a principal opioid peptide involved in growth (19,20) and has been termed opioid growth factor (OGF). This peptide is an inhibitory agent that modulates cell replication, differentiation, cellular and tissue organization, and cell survival. Application of potent opioid antagonists, such as naltrexone, disrupts opioid-receptor interaction and produces growth stimulation, indicating that OGF is a tonically active peptide in cellular events. OGF is known to operate in a receptor-mediated fashion (19,20). Pharmacological and biochemical studies have determined that an opioid receptor, zeta (ζ), associated with the nucleus, is involved in OGF action (17).

Cell replication not only occurs during development and in wound healing, but plays a role in the continuous renewal of epithelial tissues such as skin, gastrointestinal tract, and tongue throughout life [e.g., (6)]. The present studies were designed to examine the question of whether endogenous opioid peptides function as growth factors in adult, mammalian epithelial tissues. The tongue was chosen as a model system for exploration of this thesis, given its accessible location, the presence of relatively few appendages (e.g., hair), and the possibility of examining structurally distinct regions undergoing cell renewal (5,10). Because DNA synthesis (and cell division) oscillates in a circadian manner in the tongue (8,9,12), this study also presented the opportunity to address how opioid peptide regulation of cellular replication interfaces with circadian dependence. The strategy of using an opioid antagonist (i.e., naltrexone) disruption of opioid-receptor interaction to examine the repercussions on DNA synthesis in the lingual basal epithelium was employed.

METHOD

Animals

Adult C57/BL6 male mice (Charles River Laboratories, Wilmington, MA) were used in this study. All animals were acclimated for at least 8 weeks prior to study. Animals were

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housed in solid-bottom metal cages (four animals/cage) in an environment of 21 ± 0.5°C with a relative humidity of 50 ± 10%. The room had a complete exchange of air 15–18 times per hour and a 12 L : 12 D cycle (light from 0700 to 1900) with no twilight; water and Purina 5010 Rodent Chow were continuously available. Mice were divided into two groups and injected IP with 10 mg/kg naltrexone (Sigma) or an equal volume of sterile water (vehicle). This dosage of naltrexone is 1.75% of the LD_{50} in adult mice (2). Previous studies (18) have demonstrated that a dosage of 10 mg/kg naltrexone is capable of blocking morphine-induced antinociception for up to 24 h. Single, acute injections were given 2 h prior to sacrifice at 0700 or 1700 h. Some animals were given acute injections 4, 6, or 8 h prior to sacrifice at 1700 h. Mice were anesthetized with sodium pentobarbital and killed by intracardiac perfusion with 10% neutral buffered formalin.

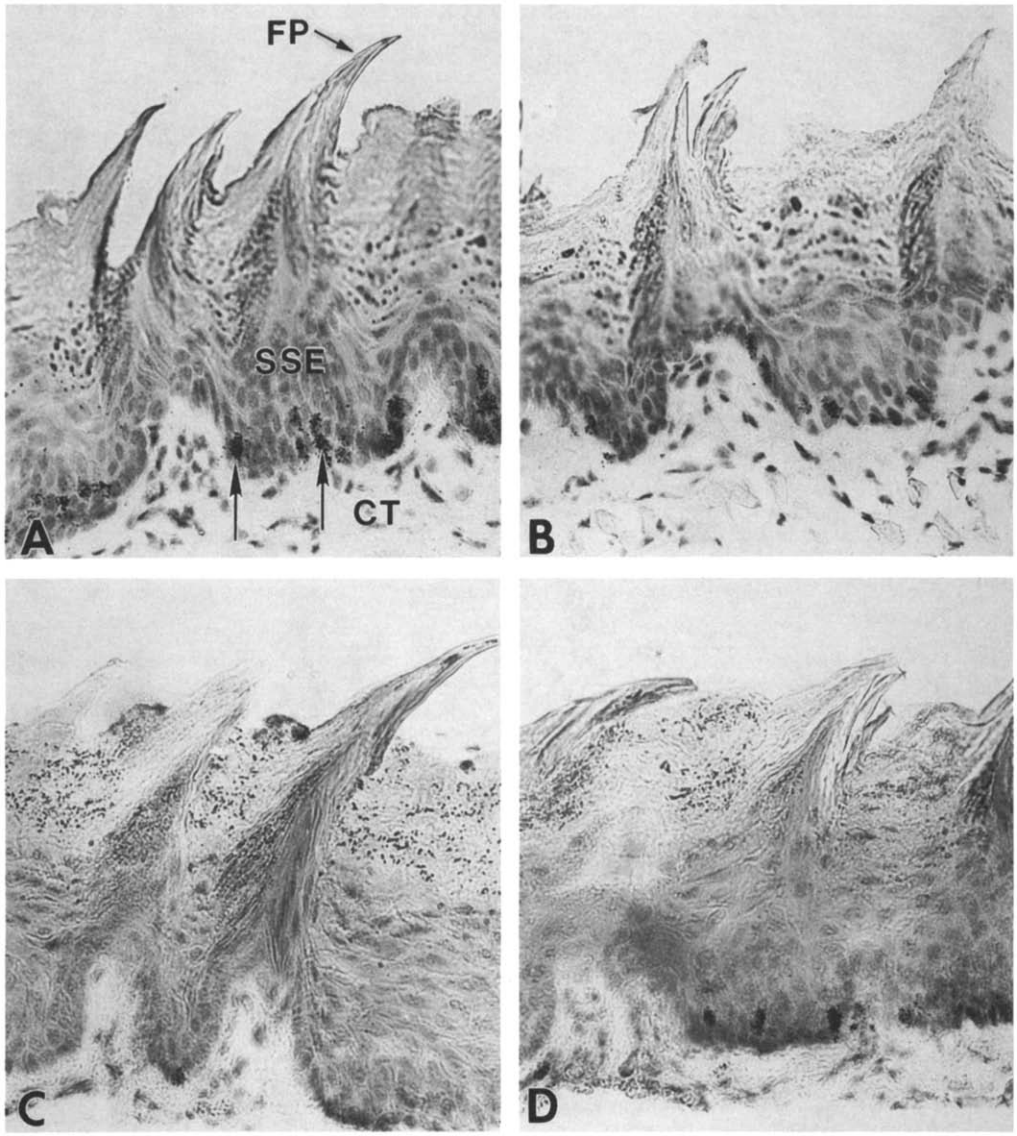

FIG. 1. Photomicrographs of the dorsal surface of the adult mouse tongue sectioned in a plane parallel to the long axis of the tongue. Animals were treated with either 10 mg/kg naltrexone (B,D) or an equal volume of sterile water (A,C) 2 h prior to sacrifice at either 0700 h (A,B) or 1700 h (C,D). At 30 min before sacrifice, mice were injected with [^{3}H]thymidine. Autoradiographs were counterstained with hematoxylin and eosin. At 0700 h, both the control and naltrexone groups had an equivalent number of radiolabeled cells in the basal epithelium. Although both the control and naltrexone groups had a reduced number of labeled cells at 1700 h, the naltrexone group had markedly more cells (45%) incorporating [^{3}H]thymidine than their respective controls. SSE = stratified squamous epithelium; FP = filiform papillae; CT = connective tissue; arrows indicate radiolabeled cells in basal epithelium. ×288.

Labeled Thymidine and Autoradiography

At each time point, at least two mice were injected IP 30 min prior to sacrifice with 5 μCi/g body weight of [^{3}H]thymidine (6.7 Ci/mmol, DuPont-New England Nuclear, Boston, MA). Tongues were removed and stored in formalin at 4°C for 24 h, embedded in paraffin, and processed for autoradiography. Longitudinal sections (8 μm) were collected, the slides coated with Kodak NTB-2 emulsion and stored in light-tight boxes at 4°C for 7 days, and developed with Kodak D19. Tissues were counterstained with hematoxylin and eosin.

Labeling Indexes and Statistical Analysis

The number of cells with grains in the basal epithelium were counted from at least three sections/mouse, two mice/treatment group at each time point. Only the region anterior to the sulcus terminalis was scored. Labeling indexes were

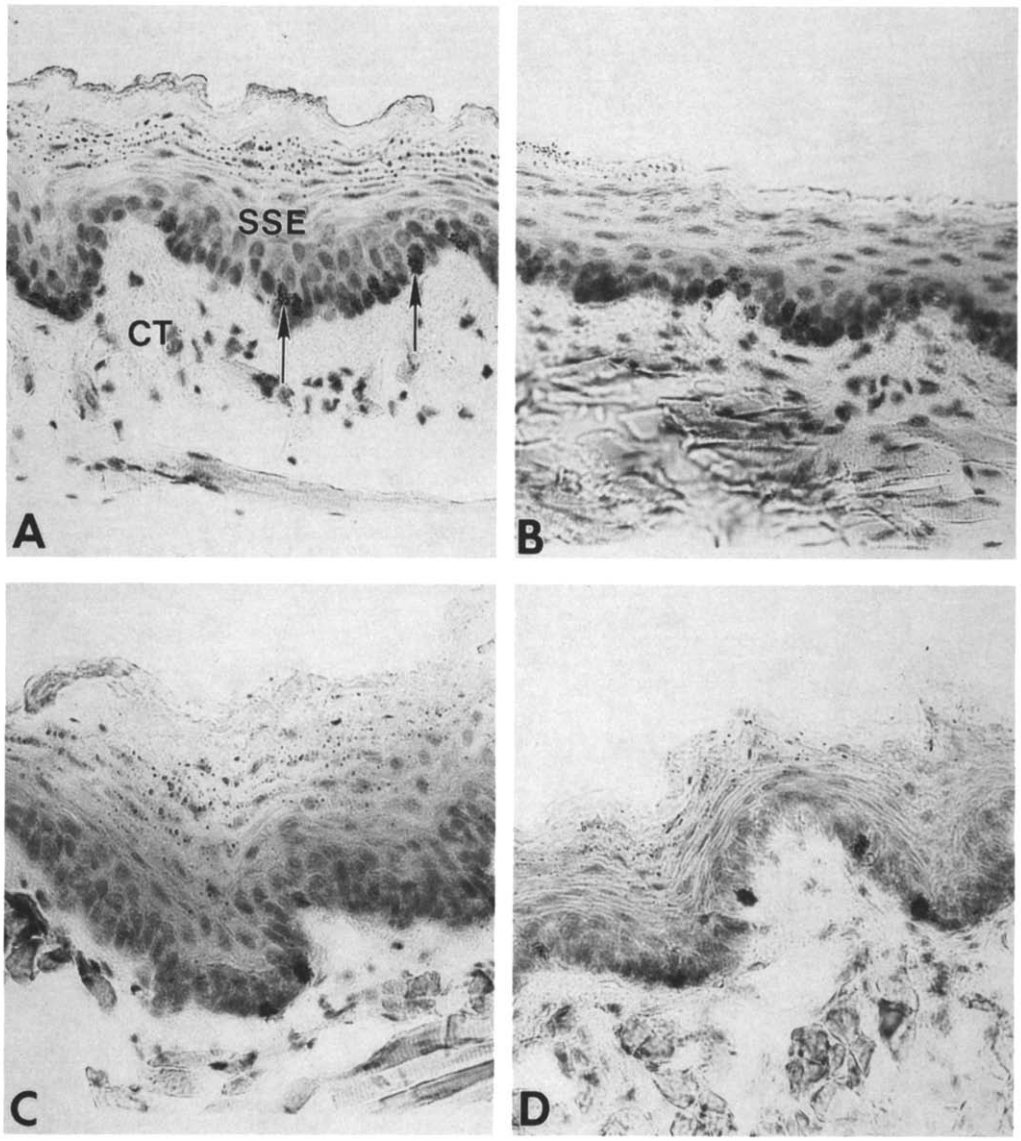

FIG. 2. Photomicrographs of the ventral surface of the adult mouse tongue sectioned in a plane parallel to the long axis of the tongue. Mice were treated with either 10 mg/kg naltrexone (B,D) or an equal volume of sterile water (A,C) 2 h prior to sacrifice at either 0700 h (A,B) or 1700 h (C,D). At 30 min before sacrifice, mice were injected with [^{3}H]thymidine. Autoradiographs were counterstained with hematoxylin and eosin. At 0700 h, both the control and naltrexone groups had an equivalent number of radiolabeled cells in the basal epithelium. Although both the control and naltrexone groups had a reduced number of labeled cells at 1700 h, the naltrexone group had markedly more cells (150%) incorporating [^{3}H]thymidine than their respective controls. SSE = stratified squamous epithelium; CT = connective tissue; arrows indicate radiolabeled cells in basal epithelium. ×288.

computed as the number of cells with four or more grains divided by the total number of cells. Dorsal (containing filiform papillae), ventral, and tip (0.7 mm from the apex extending dorsally and ventrally) surfaces of the tongue were counted separately; 600–1800 cells from each region/mouse were counted. Labeling indexes were analyzed using analysis of variance (ANOVA) (StatQuik software); subsequent comparisons were made using Newman-Keuls tests.

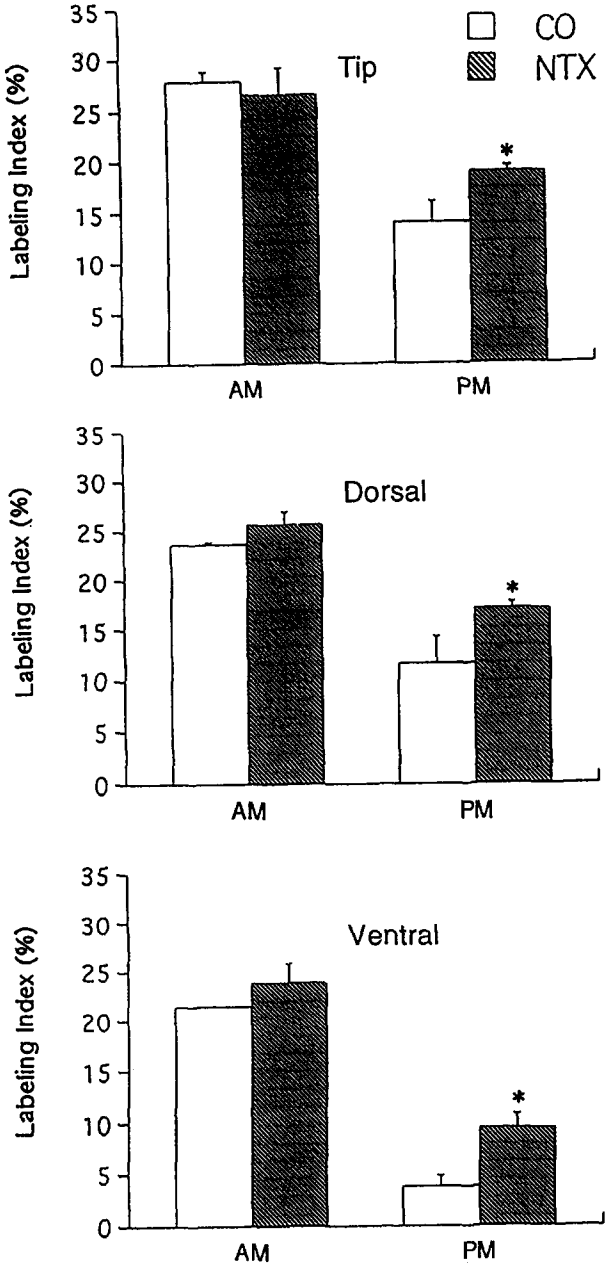

FIG. 3. The effects of naltrexone (NTX) or an equal volume of sterile water (CO) on the labeling index in the tip, dorsal surface, and ventral surface of the mouse tongue at 0700 or 1700 h. Data are expressed as mean ± SEM. *Significantly different from controls at $p < 0.05$.

RESULTS

A distinct circadian rhythm was noted in DNA synthesis for the dorsal surface, ventral surface, and tip of the mouse tongue (Figs. 1–4). In all regions examined from control specimens, DNA synthesis at 0700 h was at least twice that occurring at 1700 h, and in some cases DNA synthesis in the morning was fivefold greater than in the afternoon; the differences in labeling index of control animals at 0700 and 1700 h were significantly different at $p < 0.01$ in all three regions evaluated (Fig. 3). Administration of naltrexone resulted in a stimulation of the labeling index at 1700 h, but had no effect at 0700 h, in all three regions. Thus, in the basal epithelium of the dorsal surface, ventral surface, and tip of the adult mouse tongue, animals given naltrexone had a labeling index at 1700 h that was 45%, 150%, and 43%, respectively, greater than their control counterparts. Comparison of the labeling indexes of naltrexone groups examined at 0700 and 1700 h did reveal a significant difference ($p < 0.01$) in the tip, and ventral and dorsal surfaces. The labeling indexes in naltrexone-treated animals at 1700 h ranged 29–60% of their morning levels; these differences, however, were of considerably less magnitude than those exhibited between the two time points for control animals.

The extent of naltrexone's action on DNA synthesis was monitored by injecting animals with the opioid antagonist at either 2, 4, 6, or 8 h before sacrifice at 1700 h (Fig. 4). In the tip of the tongue, the labeling index was significantly increased in the naltrexone-exposed animals at all four time points and ranged 21–35% greater than controls. The labeling index in the dorsal and ventral regions was significantly elevated in the naltrexone group compared to controls at 2, 4, and 6 h (data not shown). In the dorsal region, the labeling index of the group subjected to naltrexone ranged 39–47% greater than controls, and in the ventral surface of this group the labeling index was increased 25–150% from control levels.

DISCUSSION

The present results demonstrate for the first time that an endogenous opioid peptide plays a role in regulating DNA synthesis during epithelial renewal, and does so in a circadian-

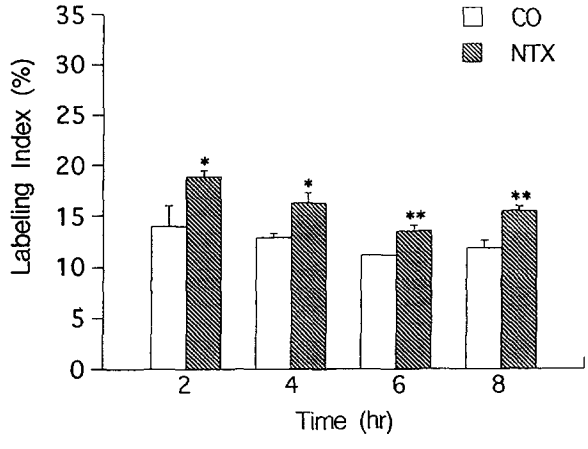

FIG. 4. The effects of naltrexone (NTX) or an equal volume of sterile water (CO) on the labeling index in tip of the tongue 2, 4, 6, or 8 h prior to sacrifice at 1700 h. The labeling index of the NTX group was markedly elevated from control levels at each time point examined. Significantly different from controls at *$p < 0.05$ or **$p < 0.01$.

dependent manner. Blockade of the interaction of opioid peptides with opioid receptors using an opioid antagonist stimulated DNA synthesis in basal epithelial cells of the tongue, inferring that opioid peptides inhibit processes related to cell replication. This influence on DNA synthesis also must be tonically active, because disruption of peptide-receptor interfacing resulted in a marked effect. The modulatory activity of the opioid antagonist was apparent for a considerable time (i.e., up to 8 h) after drug administration, indicating the profound nature of endogenous opioid perturbation on cellular renewal. Finally, the effects of opioid receptor blockade on DNA synthesis in the mammalian tongue were restricted neither to a singular tissue composition nor individual region, but rather occurred in tissues containing different structural entities (e.g., dorsal surface with filiform papillae), and in the tip as well as the dorsal and ventral surfaces.

In concordance with other workers (4,8,9,12), we found that the mouse tongue demonstrates a distinct pattern of DNA synthesis on a daily cycle, with a considerably higher level of DNA synthesis occurring in the morning than in the afternoon. Other workers have reported that the effects elicited by such agents as ACTH 1-17 (15), adriamycin (3), irradiation (7), and epidermal growth factor (14) on DNA synthesis in tongue epithelium are circadian phase dependent. A major finding in this report is that growth-related opioid action also obeys the circadian rhythm found in DNA synthesis for the tongue. Importantly, opioid antagonists could only modulate DNA synthesis in the afternoon, not in the morning. Moreover, the magnitude of stimulation of DNA synthesis in the basal epithelial cells of the tongue during the afternoon never reached the levels of synthesis found in the morning. Finally, the degree of influence of opioid antagonists on cell replicative processes in the tongue was not unlimited, and appeared to be dependent on the region examined. These data would suggest that a) opioid peptide influence of epithelial renewal is only one factor that governs cellular proliferation in the tongue, and b) opioid peptides have limitations as to their capabilities of altering DNA synthesis.

Naltrexone has long been regarded as a pure antagonist to the many biological actions of opioid substances and devoid of significant intrinsic activity (1). A number of studies (18) have demonstrated that naltrexone's effects on growth were not the result of a direct action by the antagonist, but rather disruption of opioid-receptor interfacing. In the present investigation, further evidence substantiated that opioid antagonists indirectly act by disturbing opioid peptide-receptor events. If, indeed, naltrexone was acting as a stimulator of DNA synthesis, then a population of proliferating cells should have increased levels of thymidine incorporation whenever subjected to this antagonist. However, basal epithelial cells of the tongue only responded to naltrexone in the afternoon, and not in the morning, even though cell proliferation occurred at both times. Thus, these data would affirm and extend earlier observations that naltrexone serves to work within the domain of a native system of cellular regulation that involves opioid peptides and receptors. The reason(s) why opioid antagonists have no effect on thymidine incorporation in the morning when DNA synthesis is highest can be interpreted in a number of ways. First, in the morning the levels of growth-related opioid peptides and/or receptors may be low, or receptor affinity may be altered. Alternatively, mechanisms involved with the transduction of opioid-receptor interaction and the machinery for DNA synthesis, or those aspects of cell regulation that transcend the level of opioid control, may not be responsive. These possiblities concerning the regulation of DNA synthesis by endogenous opioid systems will require further delineation.

The stimulatory effects of naltrexone on DNA synthesis in the basal epithelium of the mouse tongue reported here are consistent with a wide variety of other studies concerned with neural and nonneural cells in culture or in vivo, tumor cells and neoplastic tissues, models of wound healing, and bacteria (11,13,16,21-23). A novel finding in the present study was that tissues undergoing cellular renewal, and in the adult organism, are also guided by endogenous opioid substances. It will be of interest to further inquire whether endogenous opioid peptides also modulate cellular renewal processes occurring in other tissues such as skin, esophagus, and stomach. Moreover, identification of the opioid peptide and receptor that mediate the growth-related activities recorded in the lingual basal epithelium also is needed.

ACKNOWLEDGEMENT

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