Developmental changes in the inhibition of cultured rat uterine cell proliferation by opioid peptides

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Abstract. Opioid peptides are negative regulators of cell proliferation in several organs including the uterus. In the present study, the ontogeny of the direct inhibitory action of opioid peptides on the proliferation of cultured rat uterine cells was investigated. Uteri of 7, 14, 21, 28, 35 and 60-day-old rats were removed in a sterile way. Tissue blocks were dispersed by limited digestions with trypsin and collagenase. Cells were cultured in enriched Dulbecco's modified Eagle's medium (DMEM). Treatments were present during the entire culture period. Cell densities of the monolayers were determined by counting the cells following trypsinization and trypan blue exclusion. Rat uterine mixed cell cultures grew to confluence within 10 days. The average population doubling time gradually increased with the age of animals. Epidermal growth factor (EGF) increased cell densities of cultures from all age groups. The oestradiol (E_2)-responsiveness appeared at 21 days of age. The effect of [D-Met²-Pro⁵]-enkephalinamide (ENK) was biphasic. ENK and [Met⁵]-enkephalin (OGF) decreased cell densities of both unstimulated and EGF-stimulated cultures from 7-day-old rats to the same extent. ENK failed to act in 14-day-old animals. From 21 days of age on, the E_2 - or EGF-stimulated proliferation was inhibited only by ENK and DAMGO, while 30 nM DPDPE, Dynorhin-A, OGF, [Leu⁵]-enkephalin, beta-endorphin, and morphiceptin were ineffective. The half-inhibitory concentration of ENK was 0.3 nM. The effects of ENK were prevented by concomitant treatment with naloxone. Our novel data demonstrate two different phases of the inhibitory action of opioid peptides on rat uterine cell proliferation during ontogeny with an insensitive interval in between.

INTRODUCTION

Opioid peptides exert their widespread biological actions via opiate receptors: three classical subtypes as mu-(MOR), delta-(DOR), kappa opiate receptor (KOR), the pharmacologically related so-called opioid growth factor receptor (OGFr) and, the structurally related nociceptin-receptor

(NOR) (for review see Kieffer 1999; Chaturvedi *et al.* 2000; Reinsheid *et al.* 2000; Mogil & Pasternak 2001; Wei & Loh 2002; Zagon *et al.* 2002).

Ample evidence shows that, the opioid peptides are inhibitors of DNA synthesis and cell proliferation in the CNS (Vértes *et al.* 1982; Zagon & McLaughlin 1991; Epifanova & Brooks 1994; Zagon *et al.* 2002) and in several peripheral tissues and cells (Maneckjee *et al.* 1990; Margioris 1993; Agarwal & Glasel 1999; Wilson *et al.* 2000; Zagon *et al.* 2002). Their anti-proliferative effect is mostly associated with the opioid growth-factor receptor in early post-natal age and in cancer (Wilson *et al.* 2000; Zagon *et al.* 2002), and with the mu (Környei *et al.* 1997, 1999, 2001) and kappa (Chatzaki *et al.* 2000, 2001) subtypes of opiate receptors in some normal adult tissues including uterus. The delta opioid receptor can also serve as a negative regulator of cell proliferation (Agarwal & Glasel 1999).

Opioid peptides (Wahlstrom *et al.* 1985; Bardin *et al.* 1987; Li *et al.* 1991; Zhu & Pintar 1998), as well as opioid receptors (Baraldi *et al.* 1985, Vértes *et al.* 1986; Chatzaki *et al.* 2001) and their mRNA messages (Jin *et al.* 1988; Muffly *et al.* 1988; Low *et al.* 1989; Wittert *et al.* 1996; Zhu & Pintar 1998) are present in the female reproductive tract and, are regulated by ovarian steroids (Baraldi *et al.* 1985; Wahlstrom *et al.* 1985; Vértes *et al.* 1986; Jin *et al.* 1988; Muffly *et al.* 1988; Low *et al.* 1989).

Our laboratory also described that the opioid peptides (OP) are negative regulators of both the oestradiol-(E₂) (Ördög *et al.* 1992; Környei *et al.* 1997) and epidermal growth factor-stimulated (EGF) (Vértes *et al.* 2000; Környei *et al.* 2001) mitogenic actions in rat uterine and human myometrial cells (Környei *et al.* 1999). Based on these data, we suggested that, in adult uterus, shared step(s) of E₂ and EGF mechanism of action might be targeted by opioid peptides and not the general machinery of cell proliferation. In search for the molecular target site of the cell proliferation-inhibitory action of opioid peptides in the adult rat uterus, we demonstrated that activator protein-1 (AP1)-DNA binding activity, E₂ and progesterone receptor protein levels, as well as ligand binding capacity, are affected by opioid peptides (Oszter *et al.* 2000a,b).

In contrast, opioid peptide administered *in vivo* inhibited the unstimulated *in vitro* DNA synthesis in developing rat uterus with a more pronounced effect in younger age animals (Vértes *et al.* 1995, 1996). Preliminary data demonstrated that the unstimulated basal cell proliferation was inhibited in uterine cell cultures from 7-day-old rats (Környei *et al.* 1997). However, it is worth noting that, in cultures of human uterine leiomyoma cells, both the unstimulated basal and the E₂-, or EGF-stimulated cell proliferation could be inhibited by opioid peptides (Környei *et al.* 2000), which might be a sign for partial dedifferentiation in these benign tumours.

Based on available data, it is of interest to know what is the exact timing of the shift between the inhibition of unstimulated basal and stimulated cell proliferation by opioid peptides in rat uterus, whether it might be related to the OGFr (Zagon *et al.* 2002), and whether the inhibition of E₂- and EGF-stimulated cell proliferation by opioid peptides appear simultaneously or separated in the time during early post-natal development.

As no data are available in the literature, the aim of the present study was to investigate the ontogeny of the negative regulatory action of opioid peptides on the proliferation of rat uterine cells in culture.

MATERIALS AND METHODS

Uterine tissue collection, cell dispersion and culture were essentially the same as published in detail previously (Környei *et al.* 1997, 2001). Here we give a short description only.

Materials

The following chemicals and hormones were purchased from Sigma (Budapest, Hungary): Hank's Balanced Salt Solution with calcium ions and magnesium ions, Hank's Balanced Salt Solution containing no calcium ions and no magnesium ions, Trypsin-EDTA, type XI collagenase, type I DNase, Dulbecco's modified Eagle's medium (DMEM), antibiotic-anti-mycotic liquid, kanamycin, HEPES, sodium hydroxide, E_2 and naloxone. Fetal bovine serum was purchased from Gibco BRL Life Technologies (Budapest, Hungary). The following opioid peptides were purchased from Sigma: [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]enkephalin (DAMGO), [D-Pen^{2,5}]enkephalin (DPDPE), porcine dynorphin-A, [Met⁵]enkephalin (OGF), [Leu⁵]enkephalin, beta-endorphin and morphiceptin. [D-Met²,Pro⁵]enkephalinamide (ENK) was a generous gift from Dr S. Bajusz (Institute for Drug Research, Budapest, Hungary), also available from SIGMA as E-8380.

Tissues

Uteri of 7, 14, 21, 28, 35 and 60-day-old Wistar rats were removed in a sterile way following decapitation under light ether anaesthesia. The 35-day-old animals were either separated into two groups according to the closed or opened vagina, or merged into one group. The 60-day-old adult animal pools were either in random cycle phases, or had been ovariectomized and pre-treated with E_2 in parallel experiments (Környei *et al.* 1997, 2001). The number of rats varied from 15 to 46 depending on the age of the animals and the size of the experiment. To compare with previous studies (Környei *et al.* 1997, 2001), some experiments were performed with rats from the CFY strain as well. The internationally accepted principles in the use of experimental animals have been adhered to and our experimental protocol was approved by our institutional ethics committee.

Culturing of uterine cells

Rat uterine cells were dispersed by subsequent limited enzymatic digestions with trypsin, then collagenase as described previously (Környei *et al.* 1997, 2001) for 60-day-old animals. In case of younger rats, modifications of the dispersion methods were needed to achieve best yield in cell attachment. Tissue blocks were dispersed by limited digestions with trypsin and collagenase. Seven-day-old: 0.2% trypsin 37 °C, 5 min; 14-day-old: 0.2% trypsin 37 °C, 5 min; repeated two times. For 21-, 28- and 35-day-old: 0.01% trypsin 37 °C, 5 min, repeated two times, then 0.1% collagenase 37 °C, 40 min. Sixty-day-old: 0.01% trypsin 4 °C, 15 min 3 ×, 20 °C, 15 min 2 ×, then 0.1% collagenase 37 °C, 60 min 2 ×. All enzyme solutions contained 0.0005% DNase as well. The resulting pellets were washed, then plated to reach a final density of 10 000 viable cells (larger than 20-µm diameter) per cm² for 60, 35, 28, 21-day-old animals, 5000 cells/cm² for 14-day-old and, 2000 cells/cm² for 7-day-old animals in 25-cm² Corning culture flasks, yielding about 1000 attached cells per cm² in 1 day. Cells were cultured in enriched DMEM containing 10% fetal bovine serum (Gibco BRL Life Technologies) (Környei *et al.* 1997, 2001). Experiments were performed in primary cultures. Following an initial 24-h period of cell attachment, experimental treatments were present during the entire culture period of 7 days. The following concentrations were present in the culture medium: E_2 , 2.2 nM; EGF, 1 nM; ENK, 30 nM; naloxone (NAL), 30 nM; [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]enkephalin (DAMGO), [D-Pen^{2,5}]enkephalin (DPDPE), porcine dynorphin-A, OGF, [Leu⁵]enkephalin, beta-endorphin and morphiceptin, 30 nM. Culture medium was changed at 48-h intervals. Control cultures received vehicle in the same volume.

Determination of cell densities

Subconfluent phase of cultures was determined under phase-contrast microscope. Uterine cells from subconfluent cultures were detached by trypsinization on day 7 at the latest to ensure the largest amplitude of opiate effect yet. According to the variations in the efficiency of attachment of viable

cells, slightly different cell densities by experiments were reached for the end of the growth period. Viability was routinely determined by trypan blue exclusion. The cell densities were determined by counting the cells using haemocytometers. Counting was performed by two independent investigators who did not know the others' results. Parallel to this, in some pilot experiments counting was performed in a Coulter Counter ZM as well, equipped with a 100- μ m diameter aperture tube. To further control the accuracy of our measuring techniques, in some experiments the DNA content of one flask from the groups was also determined by Burton's method (Burton 1956).

Statistics

Figures are representatives of at least three experiments giving similar results. Not all the experiments could be performed on the same batches of cells. Data are expressed in 10^3 cells/cm² units, with the means and the standard errors of six data points from one representative experiment being presented. Analysis of variance followed by *post hoc* Student-Newman-Keul's multiple range test (Dowdy & Wearden 1983) was used for data analysis. Data sets of experiments on time dependence have been analysed by two-tailed *t*-tests.

RESULTS

Rat uterine mixed cell cultures from 7-day-old animals rapidly grew to confluence in 7–8 days (Fig. 1a). The unstimulated basal cell proliferation was inhibited by ENK (Fig. 1a). This inhibitory effect was most pronounced between 4 and 7 days in culture (Fig. 1a). The half-inhibitory concentration of ENK was 0.3 nM (Fig. 1b). Here, unstimulated cell proliferation means proliferation in the absence of added EGF or E₂. However, the cells were stimulated by serum and possible autocrine growth factors present in the culture medium. EGF but not E₂ increased cell densities from 7-day-old rats (Fig. 2a). ENK decreased the proliferation speed of cells both in unstimulated and EGF-stimulated cultures by the same extent (Fig. 2a). Thus, the EGF-stimulated cell proliferation was significantly, but not fully, blocked here. No alteration of cell densities by the presence of ENK was observed in cell cultures from 14-day-old rats (Fig. 2b). The E₂-responsiveness appeared at 21 days of age (Fig. 3a) and ENK effectively antagonized both the E₂- and EGF-stimulated proliferation, however, ENK did not modify unstimulated cell proliferation (Fig. 3a) any further. In 28-day-old rats (Fig. 3b), E₂ and EGF stimulated cell proliferation to the same extent, and ENK blocked their effects completely. E₂ increased cell densities of monolayers from 35-day-old (Fig. 4a) and 60-day-old (Fig. 4b) animals by 40% and EGF by 100%. ENK blocked these effects, but did not alter basal cell proliferation. No difference was observed when the 35-day-old animal pools were divided into two groups according to open or closed vagina (data not shown). Similar results were obtained in uterine cell cultures from 60-day-old ovariectomized then E₂-treated rats and from cycling rats (data not shown). Naloxone alone did not modify cell densities, but was able to block the effects of ENK in all age groups (Fig. 2–4). Unstimulated cell proliferation was effectively blocked by ENK and OGF, while was only marginally inhibited by morphiceptin in 7-day-old rat uterine cells (Fig. 5a). Stimulated cell proliferation in 60-day-old animals was inhibited by ENK and DAMGO (Fig. 5b). DPDPE, Dynorhin-A, [Leu⁵]-enkephalin, beta-endorphin, and morphiceptin did not alter cell densities at 30 nM concentration used (Fig. 5a and b). Similar results were obtained in some parallel experiments where DNA content (microgram DNA/flask) of the flasks were determined (data not shown). The size and morphological appearance of the attached cells were similar in all age groups (data not shown). Large, flat cells closely attached to each other, thus forming compact

Figure 1. Time-dependent (a) and concentration-dependent (b) inhibitory effect of ENK on the proliferation of cultured uterine cells from 7-day-old rats. ENK was added to the culture medium after an initial 1-day attachment period and was present in 30 nM (a) or 0.01–100 nM (b) concentrations; $n = 6$ each. Two-tailed t -tests in (a) $t = 1.429, 18.627, 17.283, 18.174, 11.864, 6.815, 4.944, 4.976$ for the 2, 4, 5, 6, 7, 8, 10 and 12 days data sets, respectively. *Marks significant difference from the corresponding control value at $P < 0.01$ level. Analyses of variances in (b) $F = 12.36$, post hoc Student-Newman-Keul's multiple range test; values marked by different letters (a, x, y) are significantly different from the corresponding control and from each other at $P < 0.01$ level.

Figure 2. Inhibitory effect of ENK on unstimulated (dotted columns), E₂-stimulated (black columns) and EGF-stimulated (cross-hatched columns) cell proliferation in primary cultures from uterus of 7-day-old (a) and 14-day-old (b) rats, and its blockade by the opiate receptor antagonist naloxone (NAL). Cross marks: 2.2 nM E₂, 1 nM EGF, 30 nM ENK and 30 nM NAL were present. Analyses of variances in (a): $F = 107.83$, panel (b) $F = 98.88$, post hoc Student-Newman-Keul's multiple range tests: values marked by different letters (A, B, C) are significantly different from the corresponding control and from each other at $P < 0.01$ level.

groups, were considered as transformed blast type of normal epithelioid cells. The elongated individual cells, spread all around and moved about and, were taken as myofibroblasts, that were organized to parallel rows in confluent monolayers. Based on photomicrographs, no alteration in the relative amount of epithelioid and myo-fibroblastoid cells was observed in our control or

Figure 3. Inhibitory effect of ENK on unstimulated, E₂-stimulated and EGF-stimulated cell proliferation in primary cultures from uterus of 21-day-old (a) and 28-day-old (b) rats, and its blockade by NAL. Analyses of variances in (a) $F = 63.84$ and (b) $F = 42.73$. Post hoc Student-Newman-Keul's multiple range tests: values marked by different letters (A, B) are significantly different from the corresponding control and from each other at $P < 0.01$ level.

treated cultures (data not shown). Viability of the cells was over 95%. No alterations in viability could be observed according to the several treatment groups or to the different age of the animals (data not shown). Average population doubling time (APDT) gradually increased with the age of the animals from 0.7–1.43 to 2.48–5.17 days for the stimulated and inhibited cultures of 7- and 60-day-old animals, respectively. Practically the same results were obtained in rats from the CFY strain (data not shown) that were occasionally included for comparison with data of previous studies.

Figure 4. Inhibitory effect of ENK on unstimulated, E₂-stimulated and EGF-stimulated cell proliferation in primary cultures from the uterus of 35-day-old (a) and 60-day-old (b) cycling rats, and its blockade by NAL. Analyses of variances in (a) $F = 75.64$ and (b) $F = 46.37$. Post hoc Student-Newman-Keul's multiple range tests: values marked by different letters (A, B) are significantly different from the corresponding control and from each other at $P < 0.01$ level.

DISCUSSION

Our laboratory described that, the opioid peptides are negative regulators of both the E₂ (Ördög *et al.* 1992; Környei *et al.* 1997) and EGF (Vértes *et al.* 2000; Környei *et al.* 2001) mitogenic actions in rat uterine and in human myometrial cells (Környei *et al.* 1999). Data from the literature

Figure 5. Opioid receptor subtypes involved in the cell proliferation-inhibitory effect of opioid peptides in uterine cell cultures from 7-day-old (a) and 60-day-old (b) cycling rats. Present in the culture medium were 30 nM ENK, DAMGO, DPDPE, dynorphin-A (DYN-A), [Met⁵]-enkephalin (ME), [Leu⁵]-enkephalin (LE), beta-endorphin (ENDO), morphiceptin (MOC) and 2.2 nM E₂ (black columns). Analyses of variances in (a) $F = 29.42$ and (b) $F = 22.62$. Post hoc Student-Newman-Keul's multiple range tests: values marked by different letters (A, B) are significantly different from the corresponding control and from each other at $P < 0.01$ level.

are consistent in that the EGF- and E_2 mitogenic signalling systems strongly co-operate at the molecular level and even mimic each other's action in uterus (Nelson *et al.* 1991; Ignar-Trowbridge *et al.* 1992; Curtis *et al.* 1996; Smith 1998). However, the exact molecular site of the anti-mitogenic action of opioid peptides is still unclear.

In contrast, opioid peptide, administered *in vivo*, inhibited the unstimulated *in vitro* DNA synthesis in developing rat uterus with a more pronounced effect in younger age animals (Vértes *et al.* 1995, 1996). Preliminary data demonstrated that the unstimulated basal cell proliferation was inhibited in uterine cell cultures from 7-day-old rats (Környei *et al.* 1997). It is of interest that, in cultures of human uterine leiomyoma cells, both the unstimulated basal and the E_2 - or EGF-stimulated cell proliferation could be inhibited by opioid peptides (Környei *et al.* 2000).

We were led to the present experiments by the lack of data on the ontogeny of the negative regulatory action of opioid peptides on the proliferation of rat uterine cells in culture. We wanted to clarify the exact timing of the shift between the inhibition of unstimulated basal and stimulated cell proliferation by opioid peptides in rat uterus, the possible involvement of the OGR (Zagon *et al.* 2002), and whether the inhibition of E_2 - and EGF-stimulated cell proliferation by opioid peptides appear simultaneously or separated in time during early post-natal development.

Our data presented here show, for the first time, that the cell proliferation-inhibitory action of opioid peptides in uterus can be separated into two different phases during rat ontogeny. Rat uterine mixed cell cultures from 7-day-old animals grew to confluence very fast, in about 7–8 days, with a lower average population doubling time compared with cells obtained from adult animals (Környei *et al.* 1997, 2001). EGF but not E_2 increased cell densities from 7-day-old rats, demonstrating that the growth of uterus at this age is mainly stimulated by the fully functioning growth factor systems, while the oestrogen receptor signalling system is not yet mature (Kaye *et al.* 1972). ENK decreased the proliferation speed of cells both in unstimulated and EGF-stimulated cultures to the same extent. However, it is worth noting that the EGF-stimulated cell proliferation was significantly, but not fully, blocked at this age. These findings might indicate that an element (or elements) of the general machinery of cell proliferation is partially hit by opioid peptides here, for example by decreasing the amount or the activity of it. At this age, the DNA synthesis and the speed of cell proliferation are very intensive (Kaye *et al.* 1972; Vértes *et al.* 1982) due to the action of active paracrine effects of local growth factors. As our results suggest, ENK might be able to significantly decrease the effects of both exogenous and endogenous growth factors on cell proliferation. We have described previously that this can happen at least with the EGF/EGF-receptor system *in vivo* (Vértes *et al.* 2000).

Similar to previous observations on uterine cells from adults (Környei *et al.* 1997, 1999, 2001), the half-inhibitory concentration of ENK was found to be 0.3 nM, i.e. within the physiological concentration range. Unstimulated cell proliferation was effectively blocked by ENK and OGR, while was only marginally inhibited by morphiceptin in 7-day-old rat uterine cells. DAMGO, DPDPE, Dynorphin-A, leucine-enkephalin, beta-endorphin were ineffective at 30 nM. Though there is no available data in the literature on the presence of functioning OGR in the uterus (Zagon *et al.* 2002), as methionine-enkephalin is the endogenous ligand for the OGR, that is expressed in early post-natal age (Zagon *et al.* 2002), the present findings might suggest that the OGR is involved at this age in our system as well.

Neither the basal unstimulated nor the EGF-stimulated cell proliferation was altered by the presence of ENK in cell cultures from 14-day-old rats representing an opioid-insensitive interval. This phase is synchronous to the most intensive growth period of rat uterus during ontogeny.

The full E_2 -responsiveness appeared at 21 days of age in our experiments. In uteri of 21-day-old and older animals the opioid peptides turned both the E_2 - and EGF-stimulated cell numbers to the control, untreated level. However, ENK did not modify unstimulated cell proliferation any

more. The timing of the appearance of E_2 responsiveness at post-natal 21 days of age in our system correlates well with findings of previous ligand binding (Kaye *et al.* 1972), immunohistochemistry and *in situ* hybridization (Fishman *et al.* 1996) studies on the ontogeny of oestrogen receptor in rat uterus. This finding also confirms that rat uterine cells in short-term primary cultures preserve the major functional age-related characteristics of the tissue they are originated. The simultaneous appearance of the full blockade by opioid peptides of both E_2 - and EGF-stimulated uterine cell proliferation suggests that common step(s) in their mechanism of action might be targeted by opioid peptides later on.

In 28-day-old rats, E_2 and EGF stimulated cell proliferation to the same extent, and ENK blocked their effects completely. E_2 increased cell densities of monolayers from 35 and 60-day-old animals by 40% and EGF by 100%. ENK blocked these effects, but did not alter basal cell proliferation. No alterations of the results was observed according to vaginal opening in 35-day-old rats, and no difference of the effects was found between ovariectomized then E_2 -treated- and cycling 60-day-old animals. Naloxone alone did not modify cell densities, but was able to block the effects of ENK in all age groups, demonstrating that tonic opioid peptide secretion and activity is not present in this system, and the proliferation-inhibitory effect is mediated by opiate receptors.

Similar to previous results (Környei *et al.* 1997, 1999, 2001), the stimulated cell proliferation in 60-day-old adult animals was inhibited by ENK and DAMGO, suggesting that the mu opioid receptor is involved in the anti-proliferative effect of opioid peptides. These findings are consistent with the presence of mainly μ , less δ and κ receptor mRNA messages in rat uterus (Wittert *et al.* 1996) and with the μ -preferring binding characteristics of ENK as well (Bajusz *et al.* 1977; Rónai *et al.* 1981). DPDPE, Dynorhin-A, methionine-enkephalin, leucine-enkephalin, beta-endorphin, and morphiceptin did not alter cell densities at 30 nM in our system. This seems to be in partial contradiction with previous findings, as methionine-enkephalin and beta-endorphin are also potent agonists of mu opiate receptors. However, these endogenous opioid peptides have short half-life due to their fast degradation by proteases present in serum or produced by cells. However, both of the effective synthetic peptides are sufficiently resistant to protease degradation. Further experiments may clarify this virtual discrepancy and, may give more details on, for example, IC_{50} values for these and other opioid peptides as well.

As fetal bovine serum used in our experiments contain numerous biologically active molecules that might bias the observed effects, we performed some of them under serum-free conditions as previously published (Környei *et al.* 1995). Although at very low cell densities, the same effects could be demonstrated (data not shown). As our present results show, the presence of 10% FBS, that is needed to maintain normal growth of cell cultures, does not mean a maximal size mitogenic signal yet, because E_2 and moreover EGF were able to stimulate cell proliferation. Meanwhile, it is worth noting that, in our experimental set-up, we used full-scale mitogenic stimulation, and inhibitory effects were investigated.

Data in the literature suggest that the anti-proliferative effect of opioid peptides might even be related to the stimulation of apoptosis (Chatzaki *et al.* 2001). However, in our system, the viability was detected routinely at the final counting of cells in all experiments. Viability was above 95% and no difference was observed according to the several treatments, thus, no measurable apoptotic or necrotic effect of ENK could be detected by the methods used here.

In summary, we demonstrate here, for the first time, that the inhibitory action of opioid peptides on rat uterine cell proliferation appears to be biphasic during ontogeny, with an insensitive interval in between. During the first phase, the action of opioid peptides might be related to the OGR, while the synchronized appearance of the inhibition by opioid peptides possibly via mu opiate receptors of both E_2 - and EGF-stimulated uterine cell proliferation suggest that common step(s) in their mechanism of action might be targeted by opioid peptides later on. Based on our novel data, a

defensive role of opioid peptides against the over-stimulation of cell proliferation could be indicated, with different mechanisms of action during organ development, compared with that in reproductive age, and possibly in different pathological disturbances. However, to date, the evidence to support this hypothesis is limited. Further experiments are required to clarify the molecular details of the anti-proliferative action of opioid peptides in uterus that may even lead to a better understanding of the patho-mechanism of human uterine disorders like leiomyoma and endometrial cancer.

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