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Chronic exposure to the opioid growth factor, [Met^5]-enkephalin, during pregnancy: maternal and preweaning effects

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Abstract

The opioid peptide, [Met^5]-enkephalin (termed opioid growth factor, OGF), is an autocrine growth factor that serves as a constitutively active inhibitory agent. OGF crosses the placenta and depresses DNA synthesis in the fetus. The role of OGF in pregnancy and parturition, and the influence exerted on prenatal and neonatal features of the offspring, were studied in rats. Females received daily injections of 10 mg/kg OGF throughout gestation; all offspring were cross-fostered to lactating noninjected dams at birth. No effects on the length of gestation, course of pregnancy, behavior of the pregnant dam, maternal weight gain, or food and water intake throughout gestation were recorded in OGF-treated mothers. Moreover, nociceptive response in these females was not altered by chronic OGF exposure, and no signs of physical dependence or withdrawal could be observed following a challenge by the opioid antagonist naloxone. Litter size and the number of live births per litter of OGF-treated mothers were reduced by 25% from control subjects and a fourfold increase in stillborns was noted for mothers receiving OGF compared to control levels. Histopathologic analysis confirmed the stillborns to have died in utero. OGF-exposed neonates were normal in body weight and crown-to-rump length, but these pups were observed to be lethargic and cyanotic, and had subnormal weights of many organs. Body weights of 10-, 15-, and 21-day-old OGF-exposed rats were reduced 11–27% from control levels. Wet and dry organ weights of the rats maternally subjected to OGF were decreased from control values in six of the eight organs evaluated at 10 days. At weaning, some organs were subnormal in weight. These data lead us to hypothesize that a native opioid peptide—OGF—is integral to certain aspects of maternal, neonatal, and postnatal well-being, and that disruptions in this opioid peptide have serious repercussions on the course of pregnancy and fetal outcome.

Keywords: Opioids; Enkephalin; Development; Opioid growth factor; Prenatal; Neonate; Growth factors; Transplacental; Fetus

1. Introduction

Endogenous opioids function as growth factors in neural and nonneural tissues (see Zagon and McLaughlin, 1993), in addition to serving in neurotransmission and neuromodulation (Akil et al., 1984). Opioids may influence growth in a variety of ways including: (i) transduction of effects by way of classical opioid receptors such as μ, δ, or κ (Barg et al., 1993; Bohn et al., 2000; Hatzoglou et al., 1996; Knapp and Hauser, 1996; Linner et al., 1995; Shahabi and Sharp, 1995), (ii) modulation that does not appear to involve receptors (Bartolome et al., 1987; Bayer et al., 1992; Hatzoglou et al., 1996; Kampa et al., 1997; Maneckjee and Minna, 1992; Melzig et al., 1995), and (iii) mediation of activity by receptors that are not opioid receptors (Isayama et al., 1991; McLaughlin, 1996; McLaughlin et al., 1999a,b; Blebea et al., 2000; Wilson et al., 2000; Bisignani et al., 1999; Zagon and McLaughlin, 1991; Zagon et al., 1994, 1995, 1996a,b,c, 1997a,b, 1998, 1999a,c, 2000a). One endogenous opioid, the pentapeptide [Met^5]-enkephalin (termed the opioid growth factor—OGF—to signify its function in neural and nonneural cells), has received attention as a growth factor. OGF is an autocrine-expressed inhibitory growth factor that is broad-based in action and functions in

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development, cellular renewal, cancer, wound healing, and angiogenesis (Isayama et al., 1991; McLaughlin, 1996; McLaughlin et al., 1999a,b; Blebea et al., 2000; Wilson et al., 2000; Bisignani et al., 1999; Zagon and McLaughlin, 1991; Zagon et al., 1994, 1995, 1996a,b,c, 1997a,b, 1998, 1999a,c, 2000a). This endogenous opioid exhibits activity at physiologically relevant concentrations. OGF has a direct, rapid, prolonged, stereospecific, noncytotoxic, and reversible influence on growth in both tissue culture and in prokaryotic and eukaryotic organisms, and is targeted to the G_0/G_1 phase of the cell cycle. Cell proliferation and migration, as well as tissue organization, are associated with OGF activity. OGF action is mediated by a receptor—OGFr. This receptor is an integral membrane protein associated with the outer nuclear envelope (Zagon et al., 1991), and has been cloned and sequenced in rat, mouse, and human (Zagon et al., 1999b, 2000b,c). Blockade of the interaction of OGF with the OGF receptor using antibody neutralization or an opioid antagonist (naltrexone, NTX), as well as by antisense perturbation of OGFr, enhances cell replication, migration, and organizational cues, suggesting that OGF is tonically active.

Both OGF and OGFr have been localized in the embryo and fetus of the rat, and in the human fetus (Zagon et al., 1999c). Moreover, functional experiments have shown that OGF is an inhibitory peptide with respect to DNA synthesis of fetal cells, both under in vivo and in vitro conditions (Zagon et al., 1999c). Recent studies have documented that injection of [Met^5]-enkephalin into the pregnant rat at 20 days of gestation undergoes transplacental passage to the fetus (Zagon et al., 2001). Indeed, these elevated levels of peptide persist in tissues of the fetus for at least 14 h. The present study was designed to take a direct approach to whether OGF functions in the regulation of ontogenic processes. We chose a paradigm that used OGF at a dosage known to alter DNA synthesis in fetal organs (Zagon et al., 1999c). In essence, the fetus was exposed to the peptide throughout the period of gestation. The relationship of OGF exposure to the course and outcome of pregnancy, including maternal food and water consumption and nociception, litter size, neonatal viability, and body weight, and length of the neonate were examined. Additionally, the repercussions of maternal OGF treatment on wet and dry organ weights, and on organ to body weight ratios, of the offspring were ascertained at birth and at 10 and 21 days.

2. Methods

2.1. Animals

Nulliparous female (175–225 g) and male (325–400 g) Sprague–Dawley rats (Charles River Labs, Wilmington, MA) were used in this study. Animals were housed in an environment of 21 ± 0.5 °C with a relative humidity of 50 ± 10%. The room had a complete exchange of air 11–15 times/h and a 12-h light–dark cycle with no twilight; water and Laboratory Rodent Diet #5001 (PMI Feeds, St. Louis, MO) were continuously available. All investigations conformed to regulations of the National Institutes of Health and the guidelines of the Department of Comparative Medicine of the Pennsylvania State University, College of Medicine.

Animals were mated (one male to two females) and the presence of sperm (vaginal smears) indicated pregnancy (Day 1 of gestation); all animals were maintained in stainless steel, wire-bottomed cages except where noted. Three days prior to parturition, the pregnant females were separated and placed individually into solid bottom cages to deliver their pups. Some nulliparous female rats were mated in order to serve as lactating females for the cross-fostered rat pups.

2.2. Drug injections

On Day 1 of pregnancy, animals were randomly assigned to two groups and received daily injections (intraperitoneal) of either 10 mg/kg [Met^5]-enkephalin (OGF, 97% purity, HPLC, Sigma, St. Louis, MO) or 0.3 ml of sterile saline. The dosage of OGF chosen for study (i.e., 10 mg/kg) has been documented to influence DNA synthesis in a variety of organs and tissues of the rat fetus and postnatal organisms (McLaughlin, 1996; Zagon and McLaughlin, 1991; Zagon et al., 1996b, 1997a, 1998, 1999c; Wilson et al., 2000). Pregnant rats were weighed daily and the dose of OGF adjusted. OGF was prepared every 3 days and stored at 4 °C.

2.3. Maternal behavior and food and water intake

Pregnant rats were weighed and observed daily before and after injections with respect to their behavior. Periodic food and water intake was monitored by placing the gravid dams in separate cages with preweighed food and premeasured water. After 24 h, food and water were remeasured and the amount of each consumed over the 24-h period was recorded. At least six rats in each group were monitored individually for food and water intake on Days 7 and 14 of pregnancy.

2.4. Maternal analgesia

On Days 7, 14, and 21 of pregnancy, some females were examined for their latency of response (licking of paws and/or rapid lifting of paws) on a 55 °C hotplate (Analgesia Meter, Technilabs Instruments, Pequannock, NJ). Other females were evaluated for nociceptive response approximately 22 h after the last injection of OGF or saline and following parturition. Within 15 min of recording the baseline latency of response, females from both groups were injected with 30 mg/kg ip naloxone in order to examine physical dependence to the endogenous opioid. All females were observed for 1 h for signs of wet-dog shakes, tremors, and/or paw licking.

2.5. Neonatal observations

Within 3 h of parturition, the number of live and dead pups, as well as the gross appearance of offspring were recorded; the length of gestation was noted. Pups born to females receiving OGF or saline were culled to litters of 10 and cross-fostered to lactating untreated mothers within 3 h of birth. Inasmuch as possible, an equal number of male and female pups were included in each litter; pups representative of different litters were randomly distributed to create new litters. Some newborn pups from each group ($n = 18$ control pups and 31 OGF pups) were placed under a warming lamp and observed for 15 min for signs of withdrawal (wet-dog shakes and/or tremors). Behavior and appearance of the pups were recorded.

2.6. Pathology of stillborns and the number of implantation sites

A random sample of eight stillborn pups from OGF-treated mothers were collected, placed in buffered formalin for at least 48 h, and processed in paraffin. To expedite fixation, skin was removed and the thoracic and abdominal cavities were opened; three 15-mm blocks of whole body were embedded—head, thorax, and abdomen. Thin sections (6 μm) from each block, as well as individual organs were stained with hematoxylin and eosin and examined for histopathology. One neonatal pup in the control group was also processed for histopathology.

In addition to examination of stillborn pups, a random sample of OGF- and saline-injected mothers were anesthetized and sacrificed several hours after parturition. The number of implantation sites were counted in each uterine horn at parturition, and in rats sacrificed at E16 and E20 for other studies.

2.7. Newborn body weights and crown-to-rump lengths

Pups were weighed at birth (Day 0) and crown-to-rump measurements were taken by measuring the distance from the base of the skull to the base of the tail using a metric ruler.

2.8. Postnatal body and organ weights

On Postnatal Days 0, 10, and 21, at least 10 pups from each treatment group (an equal number of males and females) were randomly selected from at least three litters, weighed, and killed by decapitation. Separate measures for males and females were recorded on Postnatal Day 21. The brain, heart, kidneys (right and left), liver, lungs (right and left), triceps surae (left only), spleen, and thymus were removed, blotted free of blood and connective tissue, and weighed (wet weights). Lungs and kidneys were paired for a single weight. Organs were placed on preweighed plastic weigh boats, dried in an oven at 90 °C for 48 h, and reweighed in order to obtain dry weights. Relative organ weights were calculated by dividing the wet organ weight by the corresponding body weight. Percentage of fluid composition was determined as the wet weight minus dry weight/wet weight × 100.

2.9. Statistical analysis

Maternal body weights, hotplate latencies, gestational length, litter size, and number of stillborns, as well as body weights and crown-to-rump lengths, were analyzed at each time point using one- and two-factor analyses of variance (ANOVA). Organ weights (wet, dry, and percentage water) were analyzed using one- and two-factor (Day 21) ANOVA. Subsequent comparisons following ANOVA between OGF-

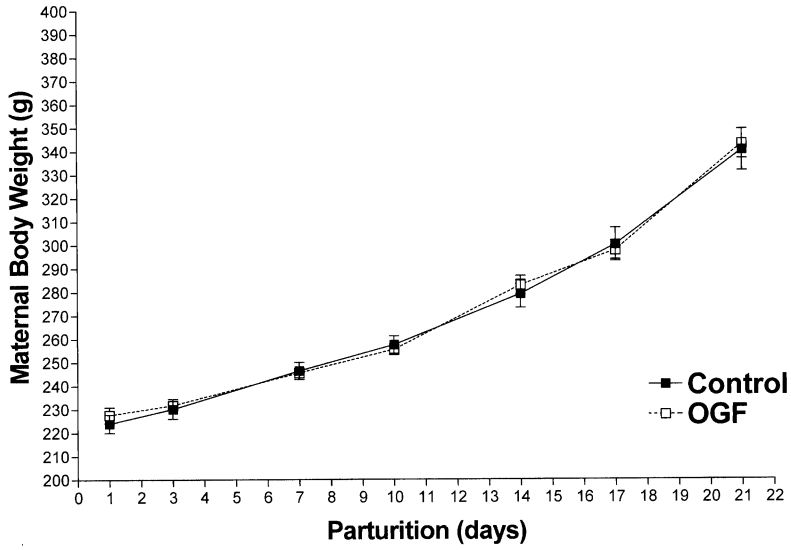

Fig. 1. The body weight of pregnant rats receiving daily injections throughout gestation of 10 mg/kg OGF or saline (control). No differences in body weights were detected between these groups during pregnancy. Data represent means ± S.E.M.

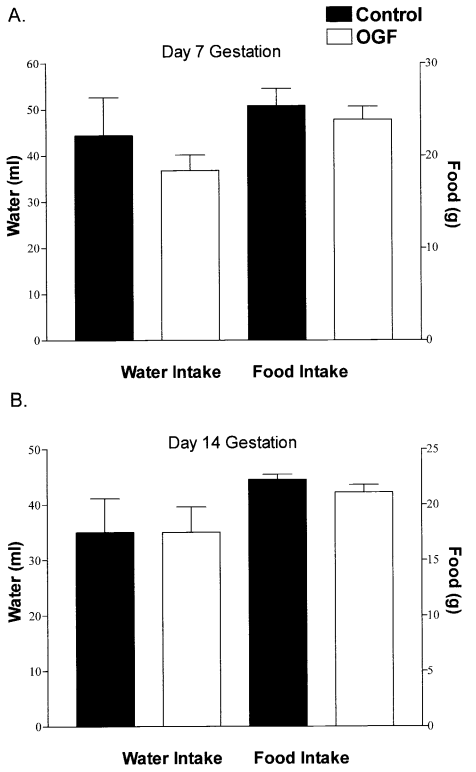

Fig. 2. Food (g) and water (ml) consumption of gravid female rats injected with either 10 mg/kg OGF or saline (control) and measured over a 24-h period of time at 7 (A) and 14 (B) days of pregnancy. Data represent means ± S.E.M. No significant differences between groups were noted.

and saline-injected animals were made using Newman–Keuls tests. Incidence of stillbirths and resorptions was analyzed by the Fisher's Exact Probability Test.

3. Results

3.1. Maternal observations

Thirty-three pregnant rats were injected with OGF and 37 rats received saline. Two independent experiments were conducted and included both OGF- and saline-treated dams; data were analyzed and found to be comparable and reproducible across experiments.

Daily injections of 10 mg/kg OGF throughout gestation had no detrimental effects on the pregnant animal. These rats did not appear to exhibit motor behavior indicative of withdrawal. Chronic injections of OGF or saline did not cause alopecia, erythema, or ulcerative changes in the tissue surrounding the injection site. No mortalities of pregnant rats were recorded.

The body weights of OGF- and saline-treated female rats were similar on the initial day of pregnancy, and no significant differences in body weights were noted throughout gestation (Fig. 1). OGF- and saline-treated females gained approximately 115 and 116 g, respectively, in 22 days.

A two-way ANOVA of food, and for water, intake revealed a significant difference ($P < .02$) for the time factor in water consumption analysis, but not for food intake; no significant interaction was noted for Treatment × Time. Both OGF- and saline-injected female rats consumed approximately 25 g of food and 40 ml of water over a 24-h period of time; these data were recorded after 7 days of pregnancy. On the 14th day of gestation, water intake was 35 ml for both groups, and food intake was nearly 22 g. There were no significant differences between groups in terms of food or water consumption at either time point (Fig. 2).

On the first day of pregnancy, nociceptive tests indicated no differences between the randomly assigned females; controls had a 19.8 ± 1.1-s latency, whereas pregnant females in the OGF group had a mean latency of 19.3 ± 1.0 s. Latency on the hotplate did not differ between the groups at 7, 14, or 21 days of pregnancy, and did not differ within a group across those three time points (Fig. 3).

To determine whether the chronic administration of OGF induced a state of dependency, female rats were challenged with naloxone 22 h following their OGF or saline injection on the day of parturition in order to observe for signs of withdrawal such as wet-dog shakes and/or tremors. Following injection of 30 mg/kg naloxone, no behavioral changes associated with withdrawal or physical dependence were noted in either group. Observations of mothers for up to 3 h following parturition revealed that both OGF and control dams appeared to clean and care for their young in a similar fashion.

The length of time for gestation did not differ between animals born to females in the OGF- or saline-injected groups (Table 1). The length of gestation ranged from 19

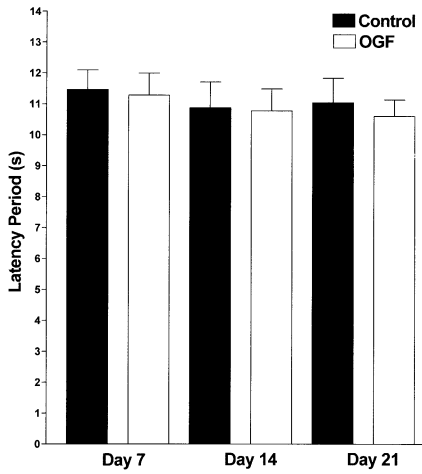

Fig. 3. Latencies in seconds (s) on the 55 °C hotplate of rats treated daily during pregnancy with 10 mg/kg OGF or saline (control); animals were evaluated 4 h after injections on Days 7, 14, and 21 of pregnancy. At least 10 rats in each group were tested. The baseline values recorded prior to rats being mated for NTX and control groups did not differ. Data represent means ± S.E.M. No significant differences between groups were noted.

Table 1
Effects of daily injections of OGF or saline throughout pregnancy on the length of gestation, litter size, and number of stillborns

	Controls	OGF
Gestation length (days)	21.7 ± 0.4	22.1 ± 0.2
Litter size	13.8 ± 0.5	10.4 ± 0.8***
Number of live births per litter	13.5 ± 0.6	10.0 ± 0.8***
Number of stillborns per litter	0.2 ± 0.1	0.9 ± 0.2**

Values represent means ± S.E.M. for the litters of pregnant rats injected daily with 10 mg/kg OGF ($n = 29$) or saline (control) ($n = 32$) beginning at fertilization and continuing throughout pregnancy.

** Significantly different from controls at $P < .01$.

*** Significantly different from controls at $P < .001$.

to 22 days for controls and from 21 to 22 days for the OGF-treated rats.

3.2. Neonatal observations

Within the 15-min period of observation immediately following delivery, no signs of withdrawal (i.e., shakes or shivers) were noted in either group of pups. Control pups were observed to have more sensorimotor activity (e.g., more vocalizations), often huddled together when separated from their mother, and maintained a healthy pink color. OGF-treated pups appeared to be more lethargic, had few vocalizations, failed to huddle when separated, and were often cyanotic in appearance. Gross morphology did not suggest any teratologic effects of prenatal OGF or saline exposure.

3.3. Litter size

Chronic prenatal OGF exposure reduced the number of live births per litter compared to treatment with saline (Table 1). In contrast to the control group, total litter size (dead and live pups) was significantly reduced ($P < .02$) in the OGF-treated females. Of the 33 OGF-exposed mothers included in this analysis, 10 females had one or more stillborn offspring and some mothers had as many as five pups per litter born dead; a total of 26 stillborn pups were detected in the OGF group. Of the 37 mothers receiving saline, only three females had one dead offspring and one mother had two dead pups; thus, five stillborn offspring were associated with the control group. Litter sizes in the OGF-exposed group ranged from 5 to 17, whereas litter sizes in the saline-injected group ranged from 10 to 18. The mean number of live births for the OGF females was decreased 26% relative to the number of control live births (Table 1). The incidence of stillbirth (number of stillbirths per 100 live births) was significantly ($P = .004$) higher in the OGF group than for controls, being 7.6 and 1.2, respectively.

Examination of the uterine horns of some pregnant rats at Gestation Days 16 and 20 revealed an increased number (but not statistically significant) of resorption sites in the mothers chronically exposed to OGF relative to controls. Thus, at 16 days of gestation, the two OGF-treated mothers studied had three resorptions among 16 live pups (a resorption rate of 18%), whereas the one control mother had no resorbed fetuses and 15 live births. At 20 days of gestation, of the two OGF-exposed litters there were three resorptions and 32 offspring (a 6% resorption rate), while two resorptions were noted in four control litters (a resorption rate of 4%). Resorption sites in both females of the OGF and control groups were not localized to a single uterine horn, nor was the entire litter resorbed in any given female.

3.4. Histopathology

Histopathology of the stillborn rats in the OGF group was complicated by postmortem autolysis of the tissues; no

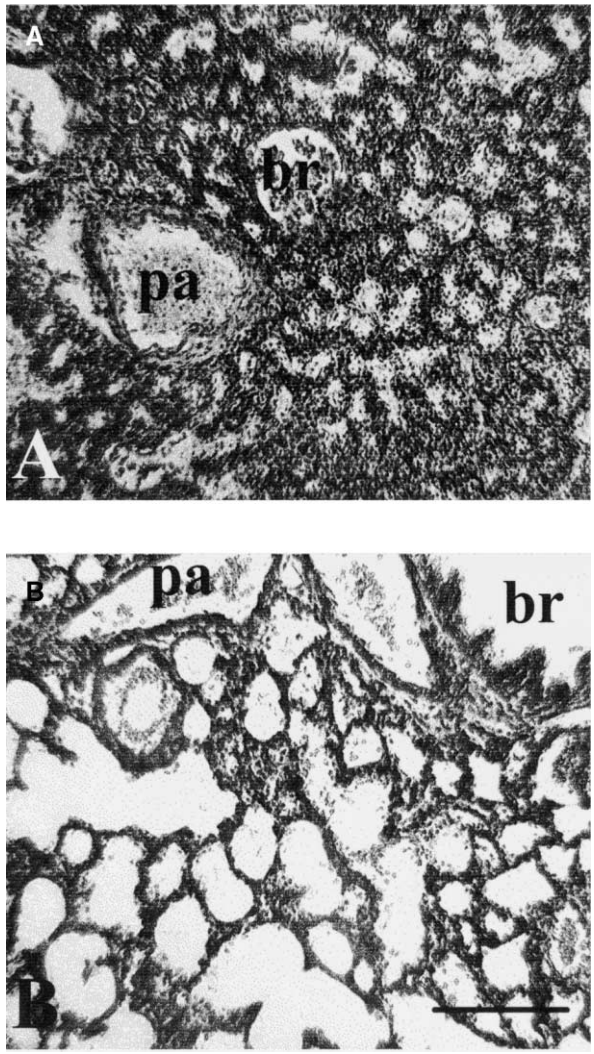

Fig. 4. Photomicrograph of histological preparations of lungs removed from a stillborn OGF-treated (A) and normal neonatal (B) rats. Note the collapsed alveoli, an indication of atelectasis in the stillborn (A) compared to the normal control lungs (B). pa = pulmonary artery, br = bronchiole. Asterisks are placed in the center of alveoli. Bar = 1 mm.

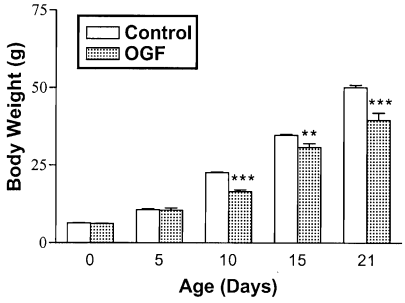

Fig. 5. The body weights of rats on Postnatal Days 0 (birth), 5, 10, 15, and 21. All rats were exposed transplacentally to daily injections of OGF (10 mg/kg) or saline (control) throughout gestation and cross-fostered at birth to untreated lactating rats. Data represent means ± S.E.M. Significantly different from controls at ** $P<.01$ or *** $P<.001$.

stillborn pups were examined in the control group. Of the few specimens that could be processed, pathological examination confirmed that the stillborn offspring exhibited atelectasis, that is, there was a failure of alveoli in the lung to initially expand (Fig. 4). Similar inspection of skin, esophagus, spinal cord, thymus, heart, liver, skeletal muscle, cartilage, brown fat, bone, kidney, intestine, and stomach revealed that the lungs were the only tissues prominently altered in appearance in the stillborn OGF-exposed offspring.

3.5. Neonatal body weights and crown-to-rump lengths

Neonatal weights for OGF-treated rats were comparable to those for control offspring (Fig. 5). Moreover, measurements of crown-to-rump length at birth indicated that pups exposed to OGF (35.9 ± 0.1 mm, $n=23$) did not differ in length from control (36.2 ± 0.2 mm, $n=28$) neonates.

3.6. Preweaning body weights

Body weights of the preweaning OGF-exposed rats were significantly smaller than corresponding controls (Fig. 5). OGF-exposed rats weighed nearly 27% less ($P<.001$) than controls (22.6 ± 0.2 g) at 10 days, 11% less ($P<.01$) than 15-day-old control rats (34.7 ± 0.3 g), and 21% less ($P<.001$) than 21-day-old control rats (50.1 ± 0.8 g). At weaning, no differences in body weight were noted between male and female control rats or male and female OGF-treated rats.

3.7. Organ weights

Wet, dry, and relative organ weights, as well as the percentage of water in organs from offspring exposed in utero to OGF or saline and collected at birth (Day 0), 10, and 21 (male and female) days of age, are presented in Figs. 6–9, respectively. Organs were taken only from offspring that were born alive and from rats with no detectable malformations for any organ.

At birth, the wet weights of the brain, kidneys, lung, muscle, spleen, and thymus were significantly less in the OGF-treated offspring relative to controls (Fig. 6); organs weighed 11–27% less than controls. In contrast, the liver of OGF-exposed offspring was 10% greater in wet weight than in control rats, and the heart was comparable in both groups. After the neonatal organs were dried (Fig. 6), brain, kidney, liver, and lung of rats in the OGF group were all decreased (14–28%) from control levels. Computation of organ to body weight ratios (Fig. 6) revealed that the brain, kidney, liver, and lung were significantly less than control values, with reductions ranging from 16% to 29%. The percentage of each organ composed of water ranged from 73% to 90%, with no differences in water

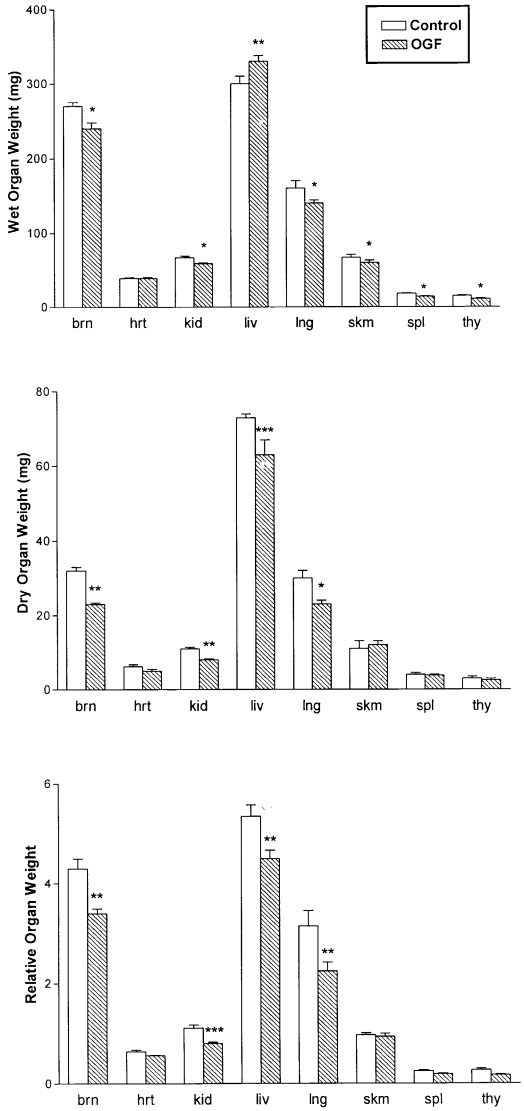

Fig. 6. Wet, dry, and relative organ weights of newborn rat pups born to mothers injected with either 10 mg/kg OGF or saline (control) throughout gestation. Brain (brn), heart (hrt), kidneys (kid), liver (liv), lung (lng), skeletal muscle (skm), spleen (spl), and thymus (thy) were weighed within 12 h of birth. Data represent means ± S.E.M. for 6–10 organs/group, with an equal number of males and females. Significantly different from controls at * $P<.05$, ** $P<.01$, or *** $P<.001$.

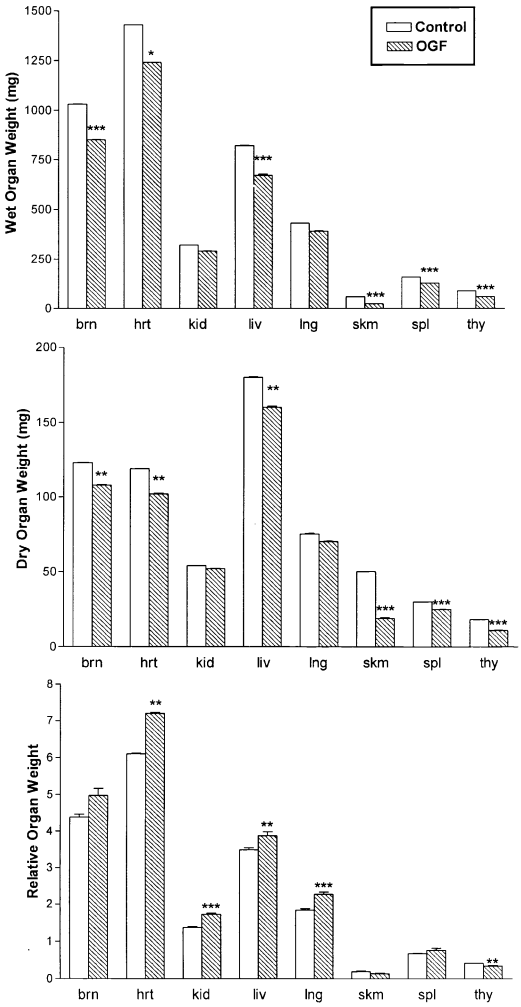

Fig. 7. Wet, dry, and relative organ weights of 10-day-old rat pups born to mothers injected with either 10 mg/kg OGF or saline (control) throughout gestation and cross-fostered at birth to untreated lactating rats. Brain (brn), heart (hrt), kidneys (kid), liver (liv), lung (lng), skeletal muscle (skm), spleen (spl), and thymus (thy) were weighed. Data represent means ± S.E.M. for 6–10 organs/group, with an equal number of males and females. Significantly different from controls at * $P<.05$, ** $P<.01$, or *** $P<.001$.

within an organ for the OGF- or saline-treated neonatal rats (data not shown).

At 10 days of age, pups maternally subjected to OGF during the prenatal period had organs (brain, heart, liver, skeletal muscle, spleen, and thymus) that weighed 13–60% less than corresponding organs from control levels (Fig. 7). Dry organ weights (Fig. 7) of rats maternally subjected to OGF exhibited reductions (range 11–62%) from control subjects in the same organs exhibiting decreases in wet weight. The relationship of organ to body weight (relative organ weight) in 10-day-old rats (Fig. 7) revealed increases ranging from 11% to 26% for the heart, kidney, liver, and lung, but a subnormal ratio (17% decrease) in the thymus. The percentage of water contained in the organs ranged from 78% to 88%. Young rats prenatally subjected to OGF demonstrated significant ($P<.05$) decreases in water for the brain, kidney, and liver as compared to control specimens (data not shown).

At 21 days of age, only two organs—liver and lungs—in male rats maternally exposed to OGF were subnormal in wet (21% and 35%, respectively) and dry (27% and 31%, respectively) weights (Fig. 8). Moreover, only the lungs of the males in the OGF-group had a change in relative organ weight (Fig. 8), with a 24% reduction from control levels noted. In 21-day-old females exposed to OGF in prenatal life, three organs—brain, muscle, and thymus—deviated (reduced 5%, 14%, and 24%, respectively) from control levels in wet weights (Fig. 9). The dry weight (Fig. 9) of the thymus in female rats that were maternally subjected to

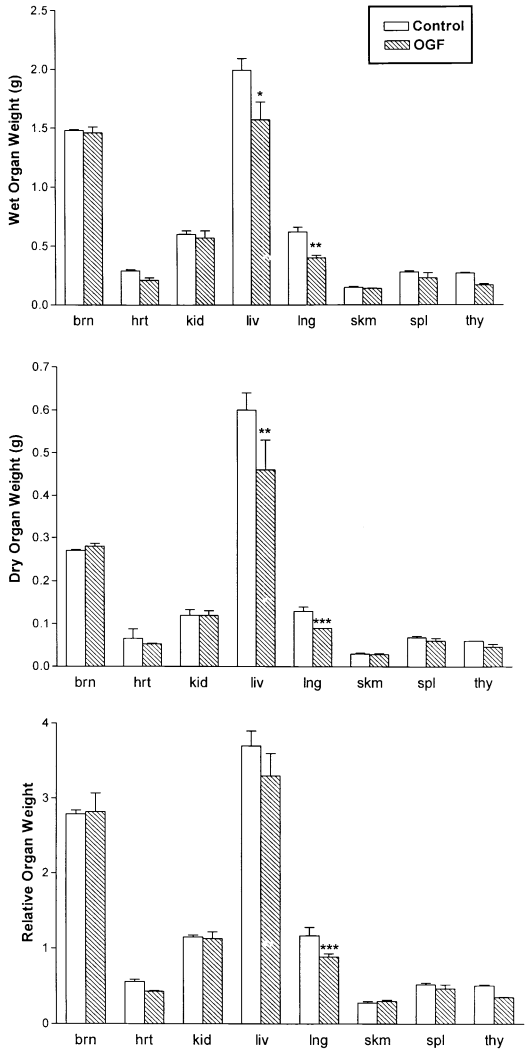

Fig. 8. Wet, dry, and relative organ weights of 21-day-old male rat pups born to mothers injected with either 10 mg/kg OGF or saline (control) throughout gestation and cross-fostered at birth to untreated lactating rats. Brain (brn), heart (hrt), kidneys (kid), liver (liv), lung (lng), skeletal muscle (skm), spleen (spl), and thymus (thy) were weighed. Data represent means ± S.E.M. for at least 10 organs/group. Significantly different from controls at * $P<.05$, ** $P<.01$, or *** $P<.001$.

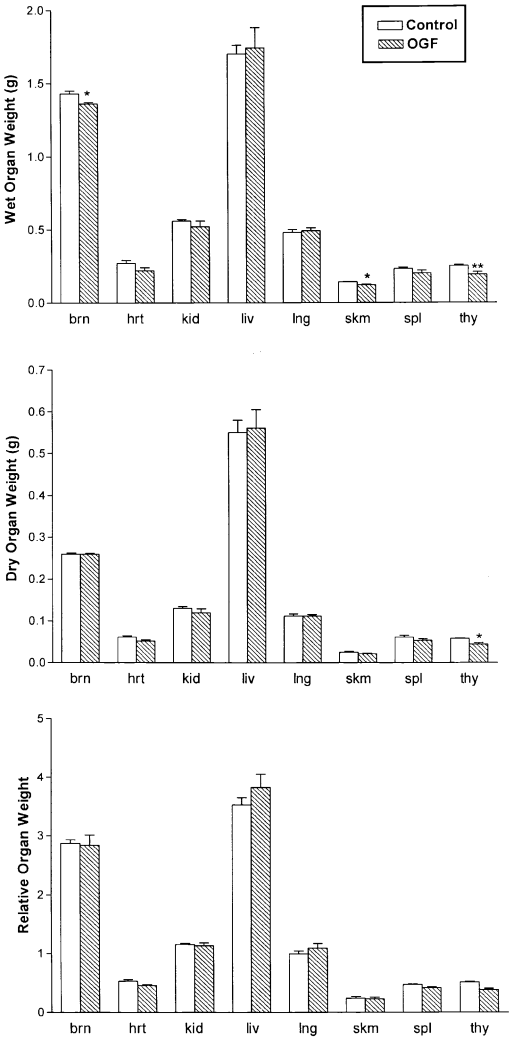

Fig. 9. Wet, dry, and relative organ weights of 21-day-old female rat pups born to mothers injected with either 10 mg/kg OGF or saline (control) throughout gestation and cross-fostered at birth to untreated lactating rats. Brain (brn), heart (hrt), kidneys (kid), liver (liv), lung (lng), skeletal muscle (skm), spleen (spl), and thymus (thy) were weighed. Data represent means ± S.E.M. for at least 10 organs/group. Significantly different from controls at * $P < .05$ or ** $P < .01$.

OGF was reduced 8% from control counterparts. Relative organ weights in female rats of the OGF and control groups were similar (Fig. 9). The percentage of water in each organ ranged from 68% to 81% for males and females in the control group, with no differences in amount of water/organ recorded between the OGF- or saline-treated 21-day-old rats. The only exception was for the thymus in the females of the OGF group, which has a reduction of 5% in water content compared to control females (data not shown).

4. Discussion

The present study was intended to (a) understand the role of an endogenous opioid—OGF, in the process of pregnancy and parturition, (b) inquire as to whether OGF is involved in embryonic/fetal development, and (c) address the implications of OGF exposure in fetal life on preweaning body and organ development. Using a paradigm of continual daily injections of OGF, which is known to elevate the peptide level in fetal tissues for 14–24 h (Zagon et al., 2001), the effects of accentuating the basal level of OGF–OGFr interaction on the maternal–fetal unit were examined. We discovered that excess OGF has no effect on the length of gestation, course of pregnancy, behavior of the pregnant dam, maternal weight gain, or food and water intake throughout gestation. Moreover, nociceptive response was not altered by chronic OGF exposure, and no signs of physical dependence or withdrawal could be observed with a challenge by the opioid antagonist, naloxone. Thus, despite using a dose of OGF that is known to inhibit DNA synthesis in replicating cells (Zagon et al., 1999c), and a regimen that is associated with the persistence of this opioid, chronic exposure to OGF did not have any overt toxicity on maternal well-being.

The data in this investigation revealed that chronic exposure to OGF had a marked influence on the outcome of pregnancy. Litter size and the number of live births per litter of OGF-treated mothers were reduced 25% from control subjects. Moreover, the number of stillborns in litters from mothers receiving OGF was fourfold greater than control levels. The stillborn pups were suspected of dying in utero, as their lungs were not expanded when inspected at birth. Although the OGF-exposed neonates were normal in body weight and body length, subjective impressions revealed that these offspring did not exhibit the same behavior as control neonates. Pups of mothers receiving OGF were lethargic and cyanotic. Therefore, elevated levels of OGF during pregnancy have a marked detrimental influence on in utero viability.

Repercussions of being subjected to OGF in prenatal life were recorded in newborn animals. Organ weight often was markedly subnormal, with changes in both wet and dry weights observed, suggesting that although overt physical parameters such as body weight and crown-to-rump lengths were normal (possibly as a result of a decreased litter size in OGF-treated groups), transplacental exposure to OGF has a detrimental influence on fetal growth. In addition, calculation of organ-to-body weight ratios revealed that some organs (i.e., brain, kidney, liver, and lung) were more affected than body weight. Thus, the influence of OGF in embryogenesis appears to shift the proportionality of some organ weights in relation to body weight, and a "brain sparing" effect was not observed. Given that OGF is known to be an inhibitor of DNA synthesis, it may be conjectured that an excess of this peptide in a period of intense cell replication does not allow for the generation of a full complement of cells to occur. Hence, organs would be compromised at a crucial time in development. The repercussions of a subnormal cell population in these organs on physiology is presently unclear nor do we understand

whether increased number of stillborns is related to dysfunction in DNA synthesis that leads to a compromised state.

It is interesting to note that measures of organ development beyond birth showed that exposure to OGF in prenatal life could markedly alter body and some organ weights in the preweaning period. The brain, skeletal muscle, spleen, and thymus were prominently depressed at 10 days of age. Even at 21 days, females in the OGF group had notable decreases in brain, skeletal muscle, and thymus weight compared to control subjects, whereas OGF-treated males had marked weight reductions of the liver and the lung in contrast to control males. These data indicate that changes observed at birth as a result of OGF exposure may persist long after birth. Moreover, some organs that were not altered at birth, such as the heart and skeletal muscle, were found to be markedly decreased in weight at 10 days of age. These observations may reflect differences in the pattern of organ development, with some organs undergoing robust cell proliferation and differentiation later than others, so that manifestations from prenatal peptide exposure may have to await a point in postnatal ontogeny to be noticed. These results suggest that sex effects as a result of being prenatally subjected to excess quantities of OGF require further investigation. It also should be mentioned that many of the changes in organ weights observed during preweaning life and associated with prenatal OGF exposure were not observed at weaning (21 days); whether this resolution in weight gain can be interpreted as a delay but the regaining of normal parameters, or whether we are seeing a quantitative but not qualitative compensation, needs to be investigated. Finally, the significance of the deviations in postnatal ontogeny recorded herein with respect to immediate and long-term organ function and capability needs to be addressed in future research.

Recent studies using HPLC have shown that OGF, [Met^5]-enkephalin, undergoes transplacental passage from the maternal to the fetal circulation (Zagon et al., 2001). These earlier studies revealed that OGF persists in fetal tissues for at least 14 h, and no longer than 24 h, after acute exposure of the female on Gestation Day 20. Therefore, although metabolic studies have documented that OGF, [Met^5]-enkephalin, undergoes rapid proteolysis by endoproteases and that the half-life of this peptide in plasma and brain is only a few minutes (Hambrook et al., 1976; Dass and Mahalaksmi, 1996), animals subjected daily to excessive levels of this peptide must be looked upon as being immersed in this opioid pentapeptide for an extended period each day. We now see that such an environment involving exposure to levels above basal of a naturally occurring opioid peptide during gestation can have serious consequences on the outcome of pregnancy and the neonate, at least at the dosage utilized.

The effects of chronic maternal exposure to OGF throughout gestation as reported herein affords the opportunity for comparison to previous studies with opiates such as morphine, methadone, heroin, and buprenorphine (White et al., 1978; Zagon and McLaughlin, 1977a,b; Ericksson and Ronnback, 1989; Enters et al., 1991; Hutchings et al., 1992, 1995; Kunko et al., 1996; Zhu and Stadlin, 2000; Ford and Rhines, 1979; Davis and Lin, 1972; Tempel et al., 1995; Basheer et al., 1992; Seatriz and Hammer, 1993). Unlike studies with opiates, prenatal exposure to OGF does not produce physical dependence in either the mother or the neonate, analgesia in the pregnant female, or decreases in maternal weight gain and water intake. Moreover, the marked reduction in neonatal birth weight often observed in offspring delivered by mothers receiving opiates was not recorded in progeny of females treated daily with OGF. However, some similarities do exist in comparing the results of studies using opiates and OGF, such as no disturbance in the length of gestation. Additionally, studies with OGF are similar to investigations of opiates in which a decrease in litter size and an increase in the number of stillborns (e.g., Zagon and McLaughlin, 1977a,b; Kunko et al., 1996) are observed, although the literature is uneven as to whether opiates alter litter size and/or number of stillborns (e.g., Enters et al., 1991). Thus, there appears to be some substantial differences between the consequences of exposure to opiates and the native opioid peptide—OGF, perhaps suggesting that the pathways may be different with respect to maternal and offspring outcome.

The present studies exploring the influence of endogenous opioids on mother and offspring also can be compared to previous studies that addressed questions about the role of native opioid peptides and their receptors in perinatal development (McLaughlin et al., 1997; Nieder and Corder, 1982; Seatriz and Hammer, 1993; Shepanek et al., 1989, 1995; Vorhees, 1981). Some of these investigations employed an opioid antagonist paradigm, whereby NTX continuously blocked endogenous opioids from opioid receptors—including OGFr. The results of these studies showed that chronic opioid receptor blockade invoked throughout gestation had no effect on the length of gestation, course of pregnancy, litter size, or the viability of the mother or offspring. Moreover, the body weights and crown-to-rump lengths of neonates delivered by NTX-treated rats were substantially elevated compared to newborns of saline-injected mothers. In the present report, challenge with OGF differed from the opioid antagonist by disrupting litter size and yielding neonates of a weight and size comparable to control levels. These data speak to the fact that endogenous opioids influence litter size, the incidence of stillborns, and the size of neonates.

It would appear that the pregnant rat and the prenatal organism are dependent upon the interaction of an endogenous opioid—[Met^5]-enkephalin—for litter size and fetal viability. Excessive peptide in the maternal–fetal circulation has an adverse effect on these processes, suggesting that an opioid peptide must be in a delicate balance as to concentration, and presumably interaction with its receptor(s) (e.g., OGFr), in developmental biology. In a clinical view, this information would indicate that elevated levels of OGF—

[Met^5]-enkephalin—during pregnancy might have an untoward effect on outcome. Whether these increased levels are induced by disturbances such as those involving metabolism and/or from outside influences (e.g., ingestion of [Met^5]-enkephalin-rich substances), the hypothesis can be put forth that alteration in the levels of this peptide may pose a hazard to pregnancy and neonatal well-being.

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