Hormonal profile and reproductive performance in lactation deficient (OFA *hr/hr*) and normal (Sprague–Dawley) female rats

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Abstract

Lactation deficiency may have important consequences on infant health, particularly in populations of low socioeconomic status. The OFA *hr/hr* (OFA) strain of rats, derived from Sprague–Dawley (SD) rats, has deficient lactation and is a good model of lactation failure. We examined the reproductive performance and hormonal profiles in OFA and SD strains to determine the cause(s) of the lactation failure of the OFA strain. We measured hormonal (PRL, GH, gonadotropins, oxytocin, and progesterone) levels by RIA in cycling, pregnant, and lactating rats and in response to suckling. Dopaminergic metabolism was assessed by determination of mediobasal hypothalamic dopamine and dihydroxyphenylacetic acid (DOPAC) concentrations by HPLC and tyrosine hydroxylase expression by immunocytochemistry and western blot. OFA rats have normal fertility but 50% of the litters die of malnutrition on early lactation; only 6% of the mothers show normal lactation. The OFA rats showed lower circulating PRL during lactation, increased hypothalamic dopamine and DOPAC, and impaired milk ejection with decreased PRL and oxytocin response to suckling. Before parturition, PRL release and lactogenesis were normal, but dopaminergic metabolism was altered, suggesting activation of the dopaminergic system in OFA but not in SD rats. The number of arcuate and periventricular neurons expressing tyrosine hydroxylase was higher in SD rats, but hypothalamic expression of TH was higher in OFA rats at the end of pregnancy and early lactation. These results suggest that the OFA rats have impaired PRL release linked with an augmented dopaminergic tone which could be partially responsible for the lactational failure.

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Introduction

Lactation insufficiency or failure is relatively common among women. In populations with sufficient economic resources, this may have minor consequences on the growth and health of the infant, because adequate substitute nourishment is available. However, in populations of low socioeconomic status, inadequate lactation can lead to severe health problems in the newborn, whereas a full lactation can assure the normal growth and development of the newborn even in situations of deficient availability of nutrients for the general population.

Most of the ‘nude’ or ‘hairless’ strains of rats and mice, that became very useful experimental tools, are hypoprolactinemic and fail to lactate. The OFA *hr/hr* (OFA) strain of rats, originally developed in Lyon, France and derived from Sprague–Dawley (SD) rats, is also hairless and has deficient lactation but does not present severe impairment of the immune system present in the

‘nude’ strains. A related hypoprolactinemic strain with lactation failure, IPL Nu (Cohen *et al.* 1983, 1985, 1986a, 1986b, 1988, Jordan *et al.* 1987) shows an altered dopaminergic system, which may be responsible for the hypoprolactinemia, since dopamine, originating in the tuberoinfundibular dopaminergic (TIDA) and periventricular hypothalamic dopaminergic (PHDA) neurons of the arcuate and periventricular hypothalamic nuclei (PeN), is the principal negative regulator of Prolactin (PRL) release (Cohen *et al.* 1985). It has been proposed that the diminished TIDA dopaminergic tone and TH expression and activity observed at the end of pregnancy and during lactation (Demarest *et al.* 1983, Grattan & Averill 1990, Hoffman *et al.* 1994, Arbogast & Voogt 1996, Fliestra & Voogt 1997, Li *et al.* 1999, Andrews *et al.* 2001) are the results of decreased sensitivity to PRL negative feedback and the adaptive response of the hypothalamus to allow for the sustained hyperprolactinemia induced by suckling

(Andrews 2005). Alterations in the regulation of the hypothalamic dopaminergic system during the period preceding the initiation of lactation could well underlie the posterior lactation failure of these strains of rats.

The nature of the mutation of the OFA strain has recently been shown to be a large intragenic deletion of the desmoglein-4 gene (*Dsg-4*) encompassing nine exons (Bazzi *et al.* 2004, Kim *et al.* 2004, Meyer *et al.* 2004), which codifies for a protein belonging to the desmoglein family. The products of these genes, expressed in neural and neuroendocrine tissues, are cell adhesion molecules related to cadherins.

The main objective of the present paper is to elucidate the possible factor(s) responsible for the lactational deficit of the OFA strain of rats by characterizing the reproductive performance of the OFA and SD strains. We also attempted to determine whether alterations in the dopaminergic TIDA and PHDA systems are responsible for the lactational deficit. With these aims, we performed a comparative study in OFA and SD rats of the hormonal profile during proestrus, pregnancy, and lactation, mammary gland function, response to the suckling stimulus and of the dopaminergic system through measurements of dopamine (DA) and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) and of tyrosine hydroxylase (TH, limiting enzyme for dopamine synthesis) expression in hypothalamic areas.

Materials and Methods

Animals

Adult female and male SD and OFA (originally purchased from Iffa Credo, France and denominated IFL Nu at that time) rats bred in our laboratory were used. The rats were used between 3 and 4 months of age, weighing 200–230 g (females) or 250–300 g (males). Animals were kept in a light- (lights on 0600–2000 h) and temperature (22–24 °C)-controlled room. Rat chow (Cargill, Córdoba, Argentina) and tap water were available *ad libitum*. Since most of the pups from the OFA rats died within the first 3 days of lactation, they were fostered on the first day *post partum* to SD mothers that had given birth within the previous or subsequent 24 h. The foster mothers were given litters of six to eight pups and the fostered OFA pups achieved normal growth rates. For the experiments, vaginal smears were taken daily from young (3- to 4-month old) female rats and only those showing regular 4-day cycles were used. The rats were made pregnant by caging with a fertile male on the night of proestrus. The presence of sperm on the vaginal smear the following morning indicated day 0 of pregnancy. Two or three days before delivery, the rats were caged individually. The day and approximate hour of delivery and the size and weight of the litters were recorded. On day 1 of lactation, the number of pups in each litter was standardized to eight. Animal maintenance and handling were performed

according to the NIH guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1985 and 1991) and the UK requirements for ethics of animal experimentation (Animals Scientific Procedures, Act 1986). All the experimental procedures were approved by the Institutional Animal Ethics Committee.

To determine the pattern of hormonal secretion during the cycle, pregnancy and lactation, groups of eight to ten rats of each strain were bled (0.5–0.8 ml blood) by the tail vein under light ether anesthesia at 1800 or 1930 h on proestrus or at 1200 h on estrus, or on days 5 (at 1600, 1900, and 2200 h), 10, 15, 19, 20 (all at 1200 h), and 21 (1800 and 2200 h) of pregnancy and on days 2, 7, 14, and 21 of lactation (at 1600 h). Since this procedure may produce a mild stress, the blood samples were always taken within 1 min of introducing the rat in the ether jar, thus, the hormonal values measured represent the basal levels. We also compared the results with hormone values obtained from decapitated rats in the different physiological states, and found no significant differences, in special of PRL, thus confirming that the values obtained represent those of unstressed animals. The blood was allowed to clot at room temperature and serum was separated by centrifugation and stored at –30 °C until used. Some rats were decapitated at 1800 h on day 21 of pregnancy and trunk blood and inguinal mammary glands were collected. The mammary glands were washed in a cold saline solution and stored at –70 °C until they were analyzed.

In order to determine the hormonal and neurochemical response to premature luteolysis at the end of pregnancy (Vermouth & Deis 1972, 1974), groups of rats were injected with 25 µg prostaglandin F_{2α} (PGF_{2α}) analog, cloprostenol (Estrumate, Schering-Plough Veterinaria SA, Argentina) in 0.2 ml saline at 0800 and 1200 h on day 19 of pregnancy and decapitated 24 h after the second injection. Trunk blood was collected, allowed to clot at room temperature and serum separated by centrifugation and stored frozen for hormone determinations. Brains were rapidly removed and placed in a cold plate for dissection. The mediobasal hypothalami (MBH; collected from bregma –2.12 to –4.52 mm, which includes the PeN, arcuate nucleus, and median eminence (ME)) were removed, frozen on dry ice, and stored at –80 °C until processing for DA and DOPAC assays.

The expression of TH, determined by immunohistochemistry (IHC) in brain slices or western blot of MBH homogenates was studied in groups of male and female rats on estrus, on days 19 (G19), 21 (G21) of pregnancy at 1800 h or on day 2 of lactation (L2) at 1200 h. The blood and inguinal mammary glands were obtained from the pregnant and the lactating groups of female rats for serum hormone measurements and mammary gland histology.

To determine the response to suckling, groups of eight to ten mothers from each strain were isolated from the litter at 0800 h on days 10 or 11 of lactation. The litters

were completed to eight pups when necessary with foster pups of the same age, weighed, and reunited with their mothers at 1600 h. In order to check whether the fostering procedure altered the response to suckling, the suckling experiments were also performed in SD rats that nursed fostered OFA or SD pups, with no differences in the responses compared with mothers nursing their own litters. After 15 or 30 min of vigorous suckling, the mothers were bled from the tail vein under light ether anesthesia; the litters were weighed again and returned to their mothers. To measure basal levels of hormones, other groups of rats were bled at 1600 h before replacing the pups. Serum was separated and stored at -30°C for RIA of PRL, growth hormone (GH), progesterone, and oxytocin. Other groups of six to eight SD or OFA rats were decapitated at 1600 h after 8 h of separation or at 1630 h after 30 min of suckling, trunk blood was collected and serum obtained for RIA of hormones, the brains were removed and the MBH dissected and processed as described below for the measurement of DA and DOPAC by HPLC.

Hormone determinations

PRL, LH, FSH, and GH were measured by double antibody RIA, using materials provided by Dr A F Parlow and the NHPP (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA, USA). The hormones were radioiodinated using the chloramine-T method and purified by passage through Sephadex G75. The results were expressed in terms of rat PRL RP-3, LH RP-3, FSH RP-3, or rat GH RP-2 standard preparations. Assay sensitivity was $0.5\text{ }\mu\text{g/l}$ serum for PRL, FSH, and GH and $0.2\text{ }\mu\text{g/l}$ serum for LH and the inter- and intra-assay coefficients of variation were $<10\%$ for all hormones. All the samples were measured on the same assay by duplicate.

Oxytocin was measured by double antibody RIA using an antibody and purified oxytocin generously provided by Dr N Hagino and by Novartis-Argentina respectively. The hormone was radioiodinated using the chloramine-T method and purified by passage through Sephadex G50. The standard curve was prepared using the same preparation of purified oxytocin used for radioiodination. To maximize sensitivity of the assay, the standards and serum samples were incubated 24 h at 4°C with appropriate dilution of the antibody, subsequently the labeled hormone ($8\text{--}10\times 10^3\text{ c.p.m.}$) was added and the tubes incubated overnight at 4°C before addition of the second antibody. Assay sensitivity was 8 ng/l serum and the intra-assay coefficients of variation were $<10\%$. All the samples were measured on the same assay by duplicate.

Serum progesterone was measured using a RIA developed in our laboratory (Busmann & Deis 1979) with an antiserum raised in rabbits against progesterone-11-bovine serum albumin conjugate or using commercial kits (DSL-3400 double antibody RIA, from Diagnostic Systems Laboratories, Webster, TX, USA). Assay

sensitivity was $<70\text{ fmol/tube}$ and the inter- and intra-assay coefficients of variation were $<10\%$.

Mammary gland histology

Mammary tissue was fixed in buffered formaldehyde, dehydrated in ethanol, and embedded in paraffin wax. Sections of $3\text{--}5\text{ }\mu\text{m}$ were cut with a Reichert-Jung Hn 40 microtome and stained with hematoxylin-eosin. Images were taken with a Zeiss Axioscop-2 light microscope fitted with a Sony CCD-IRIS/RGB videocamera under 100 and $400\times$ magnifications. For all the morphological analyses, only the inguinal mammary glands were used. Sections were histologically evaluated for changes in the extent of lobulo-alveolar development and supporting adipose tissue and for the extent of ducto-lobular luminal secretions. The morphological state of the alveoli was determined by analyzing serial sections from three different animals per group.

Casein and lactose determinations

Mammary casein and lactose were measured in mammary gland samples of OFA and SD rats as previously described (Jahn & Deis 1991). Briefly, 200 mg mammary tissue were cut into small pieces and homogenized in 2 ml of 50 mM sodium phosphate buffer, 150 mM NaCl, NaN_3 0.1%, Triton X-100 0.1% (pH 7.6) with an Ultraturax homogenizer. The homogenate was centrifuged at 600 g for 30 min and the supernatant used for β -casein determination by a homologous RIA (Busmann & Deis 1985) and lactose concentration was assessed by the method of Kuhn & Lowenstein (1967).

HPLC for DA and DOPAC in the MBH

In addition to the groups of late pregnant and lactating rats described above and to compare the dopaminergic metabolism with them, groups of virgin rats in estrus (1200 h) were also killed and the MBH was dissected and processed for DA and DOPAC determination by HPLC.

The MBHs were homogenized in 0.2 ml of 0.2 M perchloric acid, sonicated briefly in an ice bath to disrupt the tissues, and centrifuged at 12 000 r.p.m. for 30 min. DA and DOPAC were extracted on activated alumina and eluted in 0.2 M perchloric acid. Following centrifugation, the supernatants were separated and kept at -80°C until the assay was performed. Protein content in the remaining pellets was measured using the modified Coomassie blue method of Bradford.

The concentrations of DA and DOPAC in MBH homogenates (extracts) were analyzed by reverse-phase liquid chromatography using C_{18} columns and electrochemical detection (LKB, Bromma, Sweden). The HPLC mobile phase consisted of 45 mM sodium phosphate dibasic buffer (pH 3.5) containing 0.43 mM sodium octyl

sulphonate, 0.34 mM EDTA, and 2% acetonitrile. The oxidation potential was set at 0.55 V. The results are expressed as ng/mg protein for DA and DOPAC and also the DOPAC/DA ratio was calculated as an index of dopaminergic activity.

TH IHC

For TH IHC, the rats were anesthetized at 1200 h with an i.p. injection of chloral hydrate and the brains were fixed by *ex vivo* intracardial perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.01 M borate buffer (pH 7.4) as described by Ezquer & Seltzer (2003). The brains were serially sectioned (40 μ m slides) with a cryostat (Microm) beginning $\sim$ –1.80 mm posterior to bregma, corresponding to the PeN and through the entire rostrocaudal extent of the arcuate nucleus (–2.2 to –4.30 mm posterior to bregma, Paxinos & Watson 1986).

In order to detect the TH immunoreactive (+) cell bodies, their immediate processes in the arcuate nucleus and distal axons in the median eminence free-floating sections were subjected to immunocytochemistry for TH as described by Ezquer & Seltzer (2003) using mouse monoclonal antibody against TH (Semenenko *et al.* 1986) diluted in the ratio of 1:50 in carrier solution and revealed with avidin–biotin–peroxidase (ABC Kit, Vector Corporation) diluted to 1:100 in Tris buffer (TB, 0.1 M Tris–HCl (pH 7.4)). In the control specificity experiments, the primary antibody was omitted in the course of immunostaining.

Immunostained sections were examined in a light microscope with a Nikon Optiphot-2 microscope attached with a Digital Net DN100 camera. Sections from each brain were classified according to location within the MBH, based on planes comparable with plates 25–35 of the atlas of Paxinos & Watson (1986) for the adult rat brain. One section from each of these planes was selected, and the bluish-black TH (+) neurons were counted in the PeN and the complete arcuate nucleus. A total of 11 sections per animal were obtained. The total number of TH (+) cells within the arcuate nucleus or the PeN was calculated as the sum of TH (+) cells of the 11 sections (corresponding to planes described above) from each rat. Computer generated photomicrographs of the different plates were obtained with a Nikon Optiphot with a Digital Net Camera DN100. Images were assembled into figures for publication using Adobe Photoshop (version 7.0: Adobe System) with minimal alteration to the contrast and background. The regions shown in the figures represent some of the areas from which cell counts were obtained.

TH western blots

After decapitation, the brains were rapidly removed, placed on an ice-cold slicer (stainless steel brain slicer,

model RBM 4000C, ASI, Instruments, Inc., Warren, MI, USA), the MBH dissected, and protein homogenates prepared as described by Ezquer & Seltzer (2003).

The samples were analyzed for the presence of TH protein after separation by 12% SDS-PAGE on minigels in parallel with prestained protein molecular weight standards as described by Laemmli (1951), loading 8 μ g cytoplasmic proteins from each homogenate per lane. The samples were run, transferred to nitrocellulose membranes (Hybond C Amersham Life Science), and blotted for TH immunoreactivity as described by Ezquer & Seltzer (2003) using the primary antibody anti-TH (Semenenko *et al.* 1986), at 1:500 dilution and revealed with horseradish peroxidase-conjugated goat anti-mouse (DAKO Corporation, Carpinteria, CA, USA) and detection by chemiluminescence (ECL TM, Amersham Pharmacia Biotech). Multiple exposures of different times were made, to bring the exposures within the linear response range of the film (X-OMAT-AR, Kodak) and quantified by densitometry using digital image processing and the freeware program NIH Image 1.6/ppc (developed at the US National Institutes of Health and available on the internet at <http://rsb.info.nih.gov/nih-image/>). Samples from the two rat strains at the different reproductive states were run and processed simultaneously. To correct for intergel variability, an MBH homogenate control sample from an SD rat on day 19 of pregnancy was run in parallel with the samples in all the gels and the optical densities from each sample were expressed as relative to this control sample that was given an arbitrary value of 100. Three or four independent experiments were made for each reproductive state. To confirm that the observed differences were not due to variations in protein loading, duplicate gels were always run and one of them was stained with Coomassie blue and the other used for transfer and blotting and the values normalized by densitometry of the Coomassie blue-stained gel.

Statistical analysis

Statistical analysis was performed using analysis of variance followed by the τ -test for comparison between means (Snedecor & Cochran 1968). Differences between means were considered significant at the $P < 0.05$ level.

Results

Comparative reproductive performances of OFA and SD rats

Since the OFA rats showed partial or total absence of lactation, for breeding purposes the newborn OFA pups were fostered by SD mothers, who had delivered within the previous or subsequent 24 h. OFA *hr/hr* rats grew approximately at the same rate and had puberty at the

same age as the other strain, showing mostly regular 4-day vaginal cycles. When mated on the night of proestrus at 3 months of age, OFA rats became pregnant and delivered normally between the afternoon of day 21 (~1/4 of the rats) and the early morning of day 22 of pregnancy. The SD rats gave birth somewhat later, between the early morning and the early afternoon of day 22 of pregnancy. The OFA mothers showed a slight but not significant increase in the number of stillborn pups, but when the OFA pups were left with their mothers more than 90% died within 3 days after parturition. When *post partum* OFA rats fostered SD litters of eight pups, approximately half of the mothers lost the entire litter before day 5 of lactation, while most of the remaining mothers had partial lactation, losing between four and five pups on average on the first 2–4 days of lactation, but they were able to nurse the surviving ones until weaning. The surviving pups grew at a rate similar to that of SD pups nursed by their own mothers. Very few OFA *hr/hr* rats (6%) showed normal lactation and were able to nurse a litter of seven to eight pups until weaning.

Hormonal profiles in male rats and cycling, pregnant, and lactating female rats

In order to determine the cause of the lactational deficit of the OFA *hr/hr* rats, we measured the serum concentration profile of reproductive hormones related to pregnancy and mammary development during pregnancy and lactation. Additionally, we measured circulating gonadotropin concentrations during proestrus and estrus. We also included the values for male rats of the two strains, representing hormonal concentrations unaffected by the cyclic variations in ovarian hormones seen in females.

Figure 1A shows that there were no differences in basal PRL levels on male rats. In the females during proestrus, circulating PRL in the OFA rats was significantly lower at 1800 h and significantly higher at 1930 h compared with the SD rats, suggesting a slight shift in the timing of the preovulatory hormone surge. On estrus, SD rats had significantly higher values compared with the OFA strain. We measured hormone levels on the afternoon of day 5 of pregnancy as representative of the semicircadian rhythm of PRL secretion observed during

Figure 1 Circulating hormones in male, virgin, pregnant, and lactating females of SD and OFA strains. A, PRL; B, GH; C, Progesterone. Hormones were measured by RIA. Unless stated otherwise, samples were taken at 1200 h in virgin, pregnant, and male rats, and at 1600 h during lactation. Values represent the mean $\pm$ S.E.M. of groups of eight to ten rats. * $P < 0.05$ compared with SD rats of the same sex and reproductive state.

early pregnancy (Jahn *et al.* 1986). There were no differences between the strains at 1600 and 1900 h; however, at 2200 h, PRL concentrations were decreasing in SD rats, while in the OFA strain they were still elevated, suggesting that the PRL peak is prolonged in this strain. There were no differences in the pattern of serum PRL during the rest of pregnancy, but during early lactation (days 2 and 7), the OFA rats had values that were <10% of the values in SD rats. On days 14 and 21, serum PRL values on the SD strain had decreased and were similar to those of the OFA strain.

The pattern of circulating GH (Fig. 1B) was similar between the strains, with the exception of proestrus day, when the OFA rats showed values significantly higher than the SD rats at 1800 h and significantly lower at 1930 h, showing an inverse pattern with PRL.

There were significant differences in circulating progesterone between both strains in female rats (Fig. 1C), but not in males. On proestrus, there were no differences at 1800 h, while at 1930 h on proestrus and on estrus OFA rats showed significantly higher circulating progesterone concentrations compared with SD rats. There were no differences on early pregnancy but at the end of pregnancy (day 21) and early lactation (day 2) circulating progesterone was lower in SD rats, suggesting that, in this strain, luteolysis started somewhat earlier and that the activation of progesterone production by the corpora lutea of the *post partum* ovulation started somewhat later compared with the OFA rats. As mentioned above, the OFA rats delivered a few hours earlier than the SD rats, in spite of an apparently later fall in circulating progesterone. This may indicate a reduced sensitivity to progesterone in the OFA rats. SD rats also showed higher circulating progesterone on days 7 and 14 of lactation, although only on the later day the difference achieved statistical significance.

Circulating LH and FSH, determined in males and during proestrus and estrus in females showed only slight differences in timing of the preovulatory surge, but achieved comparable levels that were capable of inducing full ovulation (data not shown).

The differences in serum hormones observed in the two strains may reflect differences in pituitary function. We measured pituitary hormone content in males, females on estrus, and on late pregnancy (days 19 and 21) and early lactation (day 2). There were no differences in pituitary weight between the two strains. PRL content was also similar, except on day 19 of pregnancy SD 5.8 ± 0.5 vs OFA 8.2 ± 0.7 $\mu\text{g}/\text{mg}$ fresh tissue, $P < 0.025$) and day 2 of lactation (SD 4.4 ± 0.6 vs OFA 11.8 ± 1.6 $\mu\text{g}/\text{mg}$, $P < 0.005$), when OFA rats had higher pituitary PRL content than SD rats. There were no differences on pituitary GH content in males and estrous female rats but on day 19 (SD 7.5 ± 0.5 vs OFA 13.2 ± 1.9 $\mu\text{g}/\text{mg}$, $P < 0.025$) and day 21 (SD 8.3 ± 1.1 vs OFA 13.5 ± 1.5 $\mu\text{g}/\text{mg}$, $P < 0.05$) of pregnancy and day 2 of lactation (SD 6.6 ± 0.6 vs OFA 13.0 ± 1.4 $\mu\text{g}/\text{mg}$,

$P < 0.005$), the OFA rats had higher values than those of the SD rats. Pituitary LH values were between two- and sixfold higher in SD rats, with the smaller difference observed on day 2 of lactation due to a diminution in the levels of the SD rats (not shown). The pattern of pituitary FSH concentrations was similar to that observed in LH. Again, pituitary content of this hormone in the SD rats was higher in males (threefold) and on late pregnancy (twofold), with no differences on estrus and day 2 of lactation, when the values in the SD rats were the lowest (not shown).

To determine whether there were any differences in MBH dopaminergic activity between the SD and the OFA virgin rats, we measured DA and DOPAC concentration in the MBH of rats on estrus day at 1200 h. There were no significant differences in DA (SD 117 ± 7 and OFA 132 ± 14 ng/mg protein, NS) or DOPAC (SD 126 ± 5 , OFA 110 ± 4 ng/mg protein, NS) concentrations between the strains, nor in the DOPAC/DA ratio. However, these concentrations were several times higher than those observed in late pregnant or lactating rats of both strains (see below and Fig. 6).

Effect of $\text{PGF}_{2\alpha}$ on serum hormones and hypothalamic DA and DOPAC content

It is well known that the elevated circulating progesterone observed at the end of pregnancy produces a tonic inhibition of PRL release, and conversely, when its levels decay on day 21 of pregnancy or after removal of the corpora lutea or induced luteolysis, a surge of PRL occurs (Vermouth & Deis 1972, Bussmann & Deis 1979, 1985, Jahn *et al.* 1986, 1993). We investigated whether the response to the progesterone decay 24 h after a luteolytic regimen of the $\text{PGF}_{2\alpha}$ analog, cloprostenol, was different in OFA and SD rats. The treatment produced a decrease in circulating progesterone to <20% of control values (Fig. 2) and a surge of PRL that were similar in both strains. We also observed a fall in circulating GH in SD but not in OFA rats.

In order to explore whether changes in DA transmission play a role in the release of PRL induced by progesterone fall, we also measured DA metabolism on MBH in this experimental model, observing differential responses on the two strains (Fig. 2). DA concentrations were similar in both strains, and were not modified by $\text{PGF}_{2\alpha}$ treatment. DOPAC levels were unchanged after $\text{PGF}_{2\alpha}$ administration in SD rats while in the OFA rats the values increased significantly after $\text{PGF}_{2\alpha}$ treatment, suggesting the establishment of the short negative feedback loop by PRL. The DOPAC/DA ratios showed changes similar to those shown for DOPAC.

Mammary gland function and lactogenesis

In order to establish the causes for the failure of lactation in OFA rats, we explored mammary histology and casein

Figure 2 Circulating PRL, GH, progesterone (Pg), and MBH dopamine (DA) and DOPAC concentrations and DOPAC/DA ratio at 1200 h on day 20 of pregnancy in rats of SD and OFA strains, after vehicle or PGF_{2α} treatment (25 µg/rat x 2, 24 h previously). Results are expressed as ng/ml for hormone values and ng/mg protein for DA and DOPAC. Values represent the mean ± S.E.M. of groups of eight to ten rats. **P* < 0.05 compared with SD rats of the same group. †*P* < 0.05 compared with vehicle-treated groups of the same strain.

and lactose contents at the end of pregnancy and early *post partum* in SD and OFA rats. Table 1 shows that in both strains, casein values were similar on days 19 and 20 of pregnancy and increased significantly on the afternoon of day 21, a time when lactogenesis is taking place. Mammary lactose concentrations increase later than casein in normal lactogenesis (Deis *et al.* 1989), and did not increase significantly before delivery but on days 2 and 12 of lactation, both casein and lactose were elevated (Table 1). The casein and lactose concentrations

in the OFA rats were similar to those of the SD rats, with the exception of casein concentrations on day 21 of pregnancy, when the SD rats had significantly higher values (Table 1). These values indicate that lactogenesis at the end of pregnancy and milk synthesis during established lactation seem to proceed normally in the OFA rats.

Advancement of luteolysis induced by PGF_{2α} treatment triggered increases in casein and lactose 24 h later levels that were similar in the OFA and SD rats (Table 1). We also performed histological (stained with hematoxylin–eosin) examination of the mammary glands on day 19 and 21 of pregnancy and days 2, 7, 14, and 21 of lactation on the two strains. There were no differences in the degree of mammary development among the strains, although the inguinal mammary glands of the OFA rats tended to be smaller. On day 19 of pregnancy, the mammary glands of the OFA and SD rats showed comparable development, with a slight distension of some alveoli, which was filled with material; on day 21, a greater distension of the alveoli was observed, indicating that lactogenesis had taken place normally; and on day 2 of lactation, the images were typical of lactation. There were no differences between the strains (results not shown). Taken together, these results indicate that mammary function does not seem to be responsible for the deficit in lactation of the OFA rats.

Response to the suckling stimulus

OFA rats had diminished circulating PRL on days 2 and 7 of lactation (Fig. 1A), suggesting an impaired suckling reflex. To explore this hypothesis, we performed an acute suckling experiment in mid-lactating rats (days 10–11 *post partum*), consisting in measuring serum PRL, GH, progesterone, and oxytocin concentrations as well as the amount of milk ingested by the litter (estimated by the increase in weight), after 15 or 30 min of suckling by a litter of eight pups to mothers that had been separated from the litter for 8 h. As indicated in Materials and Methods, the OFA mothers nursed litters of foster SD pups, while the SD mothers nursed their own litters or fostered OFA or SD pups. The OFA mothers used were

Table 1 Mammary βcasein and lactose concentrations in SD and OFA *hr/hr* rats on day 19 (1200 h) and day 21 (1800 h) of pregnancy, days 2 and 12 (1200 h) of lactation and on day 20 of pregnancy (1200 h), 24 h after treatment with PGF_{2α}.

	βcasein (µg/mg tissue)		Lactose (nmol/mg tissue)	
	SD	OFA	SD	OFA
Day 19 of pregnancy	5.92 ± 1.14	5.10 ± 0.56	0.09 ± 0.04	0.81 ± 0.15
Day 21 of pregnancy	18.93 ± 2.21*	9.40 ± 0.81*	0.04 ± 0.02	2.35 ± 0.77
Day 2 of lactation	12.06 ± 1.67*	8.44 ± 1.06*	7.40 ± 1.16*	8.93 ± 0.24*
Day 12 of lactation	22.78 ± 0.63*	26.00 ± 4.21*	9.47 ± 4.26*	19.41 ± 4.91*
Day 20 of pregnancy				
+ Saline	4.49 ± 0.77	4.81 ± 1.10	0.06 ± 0.2	0.23 ± 0.09
+ PGF _{2α}	18.63 ± 2.57†	18.42 ± 0.65†	5.41 ± 1.03†	7.22 ± 2.57†

Values represent the mean ± S.E.M. of groups of eight to ten rats. **P* < 0.05 compared with day 19 of pregnancy. †*P* < 0.05 compared with saline.

those that had been able to nurse at least four pups of normal growth rate until the day of the experiment and, if necessary, the litters were completed to eight with additional hungry foster pups for the experiment.

Figure 3A shows the pattern of hormone release and the increase in litter weight after 15 and 30 min of suckling. While at 15 min of suckling, serum PRL had increased to similar levels in both groups, at 30 min the values of the OFA rats were significantly lower than those of the SD rats. Circulating oxytocin levels also showed different patterns. In both groups, hormone levels were increased after 15 min of suckling, but the OFA rats had significantly lower values than SD rats. After 30 min of suckling, the oxytocin levels in the SD rats remained elevated while in the other strain there was a decrease, arriving to values not different from time 0. The litters from the SD group obtained significant amounts of milk, resulting in a litter weight increase of more than 7 g after 30 min of suckling. On the other hand, the litters suckling the OFA group increased <1 g/l, indicating a significant impairment in the milk ejection reflex. Serum GH levels were not modified by suckling in SD rats, but after 30 min of suckling they were significantly increased in the OFA rats when compared with the other strain. Interestingly, circulating progesterone, measured only at 0- and 30-min suckling, increased in SD rats at 30 min but did not change in the OFA strain, suggesting that the levels of PRL achieved in the former strain were capable of eliciting a response from the luteal tissue, while the lower levels of PRL observed in the OFA rats were not sufficient to stimulate luteal progesterone release. We

also allowed some rats to suckle for longer periods (1, 2, and 4 h) to determine if the OFA rats were able to eject milk after more suckling time. While the pups suckling the SD rats had obtained approximately two thirds of the available milk during the first 30 min of suckling (7.8 ± 0.9 g/l), with most of the rest at 2 h (3.8 ± 1 g/l) and marginal increments at later times, the litters suckling OFA rats showed a modest positive increase in weight only after 2 h of suckling (4.0 ± 0.7 g/l) and no further increase at 4 h. Thus, the total milk quantity that the OFA rats were able to eject after 4 h of suckling was $<50\%$ of the values of the SD mothers. After 1 h of suckling, circulating PRL and oxytocin levels continued to be significantly lower ($\sim 50\%$) in the OFA compared with the SD mothers (results not shown).

We also measured the MBH dopaminergic activity in SD and OFA rats after 30-min suckling. Figure 3B shows that after 30-min suckling neither DA or DOPAC concentrations nor the DOPAC/DA ratio were different from the values in non-suckled rats in both strains. On the other hand, the lactating OFA rats had significantly higher concentrations of DA and DOPAC compared with the SD rats, and this may be at least partially responsible for the reduced suckling-induced PRL levels in the OFA compared with the SD strain.

TH expression on hypothalamic areas in virgin (estrous) rats and on late pregnancy and early lactation

The diminished PRL secretion observed in the OFA rats in different experimental situations, such as lactation or

Figure 3 A: Effect of 8-h separation and 15- or 30-min suckling on pup weight increase, serum concentrations of PRL, oxytocin, GH, and progesterone. B: Effect of 8-h separation and 30-min suckling on MBH dopamine (DA), DOPAC and DOPAC/DA ratio of SD and OFA rats on days 10–11 of lactation. Rats were separated from their litters at 0800 h, the litters were replaced at 1600 h and pups weighed and blood sampled from the mothers after 15 and 30 min of vigorous suckling. See Materials and Methods for further details. Results are expressed as ng/ml for hormone values and ng/mg protein for DA and DOPAC. Values represent the mean $\pm$ S.E.M. of groups of eight to ten rats. * $P < 0.05$ compared with SD rats at the same time. † $P < 0.05$ compared with time 0.

proestrus at 18 h, along with the differential effect of $\text{PGF}_{2\alpha}$ on hypothalamic dopaminergic metabolism and the different DA and DOPAC concentrations in MBH in lactation, may suggest alterations in the dopaminergic neurons of the arcuate nucleus, that are the main regulatory system for pituitary PRL secretion. To investigate changes in the dopaminergic system, we determined the localization and the number of dopaminergic neurons in the periventricular and arcuate nuclei and the hypothalamic expression of TH, on female rats of both strains on estrus, days 19 and 21 of pregnancy and day 2 *post partum* and on male rats (for western blots only), using immunohistochemistry and western blot respectively. Figure 4 shows that there were no differences in the localization of TH (+) neurons between the strains or among the different groups. In contrast, the number of TH (+) neurons was very variable. The SD rats had more TH (+) neurons in the PeN and in the different regions of the arcuate nucleus compared with the other strain, in all the

reproductive states studied, but the differences achieved statistical significance only in virgin and 19-day pregnant rats in the PeN and in the medial arcuate. In the PeN, the number of TH (+) neurons decreased significantly on day 21 of pregnancy in both strains, when compared with the virgin rats, while in the arcuate nucleus there were no significant differences between the different reproductive states. When the values of the arcuate nucleus for all the reproductive states were pooled, the difference between the OFA and the SD rats was significant. The medial arcuate nucleus had more TH (+) neurons than the rostral or caudal part of the nucleus, and accounted for the differences observed between the SD and the OFA strains (Fig. 4B).

Western blot for TH of MBH protein extracts had maximal and similar values in virgin female rats of both strains (Fig. 5). The male OFA rats had slightly but significantly higher TH immunoreactivity compared with the SD rats. In SD rats, there were significant decreases in TH expression on late pregnancy (days 19

Figure 4 Tyrosine hydroxylase immunoreactivity (TH+) in neurons of the arcuate and periventricular nuclei of estrous virgin (Virgins), 19- and 21-day pregnant (G19 and G21) and 2-day lactating (L2) rats of SD and OFA strains. A: Representative microphotographs of stained arcuate and periventricular neurons from virgin and G21 rats of both strains. There were no differences in neuron size or morphology between the two strains. The sections shown correspond to plates 26 and 27 and to plate 25 of the Paxinos–Watson atlas for arcuate and periventricular nuclei respectively. B: Total number of TH (+) neurons in periventricular (A14), entire, rostral (1.8–2.1 mm posterior to bregma, pB), medial (2.1–3.6 mm), and caudal (3.6–4.3 mm, pB) arcuate (A12) nuclei of estrous virgin (Virgins), 19- and 21-day pregnant (G19 and G21) and 2-day lactating (L2) rats of SD and OFA strains. See Materials and Methods for further details. Values represent the means $\pm$ S.E.M. of groups of three to four rats with the exception of the groups designated as total, which represent the value for all the pooled groups, regardless of the reproductive state. * $P < 0.05$ compared with SD rats of the same sex and reproductive state. † $P < 0.05$ compared with virgin female rats of the same strain.

Figure 5 Western blot for tyrosine hydroxylase in MBH homogenates of male and female virgins in estrus (Virgins), 19- and 21-day pregnant (G19 and G21) and 2-day lactating (L2) rats of SD and OFA strains. A: Representative western blots with three samples for each strain in each of the studied reproductive states. All the blots were exposed in the same film. B: Densitometric quantification of the bands. See Materials and Methods for further details. Values represent the means $\pm$ S.E.M. of groups of four to six rats. * $P < 0.05$ compared with SD rats of the same sex and reproductive state. † $P < 0.05$ compared with virgin female rats of the same strain.

and 21) and lactation, compared with virgin rats. In contrast, in the OFA rats, TH expression only fell significantly on day 21 of pregnancy compared with virgins of the same strain (Fig. 5B). Thus, during late

pregnancy and lactation the TH values were significantly higher in the OFA rats when compared with the SD strain (Fig. 5).

Discussion

The present results show that OFA rats, when compared with their strain of origin, the SD strain, were hypoprolactinemic during the course of lactation, but did not show impaired PRL secretion on other physiological states, such as pregnancy or the sexual cycle (Fig. 6). Thus, the surges in PRL secretion on the afternoons of proestrus and day 5 of pregnancy (which we took as representative of the diurnal PRL surges during early pregnancy) seemed to be even more prolonged in the OFA rats, while on day 21 the pattern of secretion was similar in both strains. PRL levels in males, which are not subject to the cyclic ovarian hormone influences, only showed a non-significant tendency to be lower in the OFA rats. OFA rats showed a profound impairment in the PRL response to suckling, observed in the continuously lactating rats (Fig. 1) and in the acute suckling experiment (Fig. 3). In addition, this last experiment demonstrated partial blockade of oxytocin secretion and a profound impairment in milk ejection. In contrast, mammary gland development and lactogenesis proceeded normally, indicating that there seems to be no deficit in mammary gland function. This suggests that the lactational failure of the OFA rats may be exclusively neurogenic through an increased hypothalamic dopaminergic tone that may play a role in the reduced PRL response to suckling.

Thus, the decrease in MBH TH immunoreactivity (which includes PeN, arcuate nucleus and also the ME, that may contribute a significant proportion of the measured protein in the western blots) and in DA and DOPAC concentrations in the MBH during late pregnancy and early *post partum* compared with the values in virgin rats was attenuated in the OFA rats in comparison with the SD strain (Fig. 6), suggesting an increased dopaminergic tone in this strain. There are reports that show increases in dopaminergic activity in other brain areas in this (Estrella *et al.* 2002), and a similar strain (Cohen *et al.* 1985), suggesting that the altered dopaminergic tone is not exclusive of the hypothalamus. Since the diminished hypothalamic TH expression and activity may be essential for the maintenance of the full PRL response to suckling during lactation (Andrews 2005), the increased MBH TH immunoreactivity and dopaminergic activity (DA and DOPAC concentrations) observed on late pregnancy and early lactation in the OFA rats may indicate that this adaptation is impaired, thus compromising suckling-induced PRL release (Fig. 6).

It has been well demonstrated that in normal rats, the responsiveness of tuberoinfundibular DA neurons to PRL feedback is markedly attenuated in late pregnancy and

Figure 6 Relationship between the PRL levels, hypothalamic indices of dopaminergic activity (TH expression and DA and DOPAC concentrations), and the number of TH (+) neurons in virgin, pregnant, and lactating rats of the OFA and SD strains. In SD rats, the decrease in hypothalamic TH expression and DA and DOPAC concentrations observed on late pregnancy and lactation is inversely correlated with increased circulating PRL. In contrast, in the OFA rats, the low circulating PRL during lactation can be correlated with elevated TH expression and dopaminergic activity. * $P < 0.05$ compared with SD rats of the same sex and reproductive state. [†] $P < 0.05$ compared with virgin female rats of the same strain.

lactation (Arbogast & Voogt 1996, Andrews *et al.* 2001, Andrews 2005). This disruption of the normal PRL negative feedback appears to be fundamental for the development of the lactational hyperprolactinemia necessary for optimal milk synthesis (Andrews 2005). In contrast, the OFA rats seem to retain a higher capacity to activate the DA system in response to elevated circulating PRL at the end of pregnancy compared with the parent strain. Thus, when PRL release was stimulated by treatment with $\text{PGF}_{2\alpha}$, the OFA rats showed an increase in DA turnover that was not observed in the SD rats (Fig. 3).

However, in spite of the marked changes in MBH TH expression and DA concentrations, the number of TH (+) neurons in the arcuate nucleus remained constant in all the reproductive situations examined (Fig. 6). Surprisingly, the SD rats seemed to have more TH (+) neurons in the medial area of the arcuate nucleus and also in the PeN than OFA rats (Fig. 4), although their localization was similar. It is interesting to note that compared with the SD strain, Wistar rats also have less TH (+) neurons in arcuate and PeN nuclei, but show a comparable decrease in MBH TH immunoreactivity during late pregnancy and early *post partum* (unpublished results from our laboratory).

The decreased TH immunoreactivity and DA concentrations observed during late pregnancy in SD rats when compared with the virgin values correlates well with the previously reported reduction in TH gene expression on these stages compared with early pregnancy (Arbogast & Voogt 1991, Fliestra & Voogt 1997, Andrews *et al.* 2001). This decrease precedes at least by 3 days the increase in PRL that occurs on day 21 after the progesterone fall, suggesting that other factors may be able to stimulate PRL secretion at this stage, in the presence of a permissive action of the decreased dopaminergic tone. In contrast, although the OFA rats had diminished TH immunoreactivity only on day 21, they showed a similar increase in PRL, suggesting that this decrease in the dopaminergic

tone is sufficient to allow the *prepartum* PRL surge in this strain. There are many evidence showing that the effect of PRL-releasing factors (PRFs), such as TRH (Samson *et al.* 2003), are dependent on a decrease in the dopaminergic tone that is not sufficient to stimulate PRL release *per se*, but that multiplies the release elicited by the PRF (Freeman *et al.* 2000, Ben-Jonathan & Hnasko 2001, Voogt *et al.* 2001). There are other neurotransmitters involved in the release of PRL at the end of pregnancy, such as norepinephrine (Jahn & Deis 1991), serotonin (Jahn & Deis 1987, Jahn *et al.* 1999), and opioid peptides (Soaje & Deis 1994, 2004, Soaje *et al.* 2004, Andrews & Grattan 2003) that participate in the stimulation of PRL release after the progesterone fall and that may be responsible for the similar response observed in the two strains, in spite of the elevated dopaminergic tone of the OFA rats at the end of pregnancy.

There are also a number of PRL-releasing factors, such as TRH or cocaine- and amphetamine-regulated transcript peptide, (CART) that have been implicated more or less directly in the PRL response to suckling (Freeman *et al.* 2000, Voogt *et al.* 2001, Samson *et al.* 2003). We cannot exclude that alterations in one or more of these systems may also play a role in the impaired hormonal response to suckling.

While suckling-induced PRL secretion is impaired, it seems that GH secretion may be augmented in the OFA rats, which had elevated GH levels after 30 min of suckling. Similarly, at the end of pregnancy, after $\text{PGF}_{2\alpha}$ treatment, circulating GH decreased in parallel with the increase in PRL in the SD rats, but did not change in the OFA rats, indicating a differential regulation of GH secretion in this strain. We have described (Jahn *et al.* 1993) an inverse regulation of GH and PRL secretion at the end of pregnancy in Wistar rats. We showed that circulating GH is elevated and PRL is very low during the last days of pregnancy and when PRL release is induced spontaneously or by progesterone fall, there is a concomitant fall in GH secretion (Jahn *et al.* 1993).

This pattern of secretion seems to be present also in the SD rats (see Figs 1 and 2). Although the pattern of circulating PRL and GH on the last days of pregnancy was similar in the OFA and SD rats, the differential GH response to PGF_{2α} treatment and to suckling may be the product of the same hypothalamic alterations that are responsible for the increased dopaminergic activity. Since GH has some lactogenic actions in the mammary gland in the absence of PRL (Caron *et al.* 1994), the higher GH levels induced by suckling may have provided an additional stimulus for milk synthesis during lactation, providing some compensation for the reduced PRL.

There were important differences in pituitary hormone content between the two strains. The female SD rats had consistently lower PRL and GH contents, while the OFA rats had higher values compared with the SD rats during pregnancy and early lactation, suggesting that the impairment in PRL release may produce accumulation of the hormone in the hypophysis.

It is interesting to note that although OFA rats at 1800 h had lower preovulatory LH secretion, at 1930 h the levels achieved were similar to those of the SD rats and sufficient to insure a normal ovulation rate; furthermore, their overall fertility was similar to that of the SD rats.

In contrast to the relatively minor differences observed in the circulating gonadotropins, their pituitary contents showed marked variations between the two strains, in particular in the male rats, where SD rats had values several times higher than those of the OFA strain. The differences observed in the pituitary gonadotropin content between the two strains, particularly in the females, may reflect a differential sensitivity to estrogen action between both strains, since estrogen is an important factor regulating synthesis and secretion of gonadotropins. The significantly higher PRL values during estrus in the SD compared with the OFA rats may also be indicative of a different sensitivity to estrogen. In this respect, it has been shown that SD rats have differential sensitivity to estrogens or estrogenic substances in comparison with other strains, such as the Wistar (el Abed *et al.* 1987, Garcia-Segura *et al.* 1992), that accounts for their heightened susceptibility to mammary cancer (Isaac 1986, el Abed *et al.* 1987). Our colony of OFA *hr/hr* rats shares the susceptibility to carcinogens with the SD strain, with 70% of the rats developing tumors between 1 and 4 months after administration of the carcinogen DMBA (Jahn *et al.* 2003, Ezquer *et al.* 2003). On the other hand, while in our colony of SD rats, 46% had spontaneous mammary tumors at 18 months of age, only 3 out of 50 OFA rats showed mammary tumors between 12 and 18 months of age, of which only one was epithelial and two were fibrous and composed of connective tissue. Most probably, the altered PRL regulation of the OFA rats contributed to this low incidence of mammary tumors.

The mutation affecting OFA *hr/hr* rats is a deletion of the *Dsg-4* gene, a member of the cadherin family of cell adhesion proteins (Jahoda *et al.* 2004). This protein participates in the assembly of desmosomal protein and has calcium-binding sites (Jahoda *et al.* 2004). The product of this gene has been shown to be present in the hair follicle but its presence in other tissues has not yet been investigated, although it may presumably be present in most tissues that present tight junctions, the nervous tissue among them. Besides disrupting hair follicle function and structure, mutation of the *Dsg-4* gene produces increased cell proliferation in epithelial tissue (Jahoda *et al.* 2004). It is interesting to note that mutations as different as those of the *hairless* and the *Dsg* genes give phenotypes that are quite similar, involving hairlessness, hypoprolactinemia, and lactational failure. The *hairless* gene encodes a protein that is expressed in hair follicles and in nervous tissue and its pattern of expression in developing brain tissue, as well as some described neural abnormalities, suggest that it may have a role in CNS development (Potter *et al.* 2002). Although at present, *Dsg-4* has not been localized in the brain and there are no reports on any relationship between this protein and signaling pathways related to nervous tissue function, there are a number of reports in the literature that show interactions between proteins belonging to cell adhesion families, such as cadherins and catenins and neuron survival, synaptogenesis, and stabilization of synapses (Brose 1999, Tanaka *et al.* 2000, Ferreira & Paganoni 2002, Yu & Malenka 2003, Junghans *et al.* 2005). They are also key players in signaling pathways relevant to neuronal functions and synaptic differentiation such as Wnt (Caricasole *et al.* 2005). Disruption of members of the cadherin family (Gubkina *et al.* 2001, Rubinek *et al.* 2003) has also been linked to alterations in hypothalamic and pituitary regulation of hormone secretion. Thus, the possible participation of *Dsg* proteins in neuroendocrine and neuronal regulation and plasticity opens a new field of investigation that is being pursued at present in our laboratory.

In conclusion, we found that the OFA rats show a profound impairment in the neuroendocrine response to suckling that may account for the deficit in lactation and may be caused by increased hypothalamic dopaminergic tone at the end of pregnancy and during lactation (Fig. 6). We also found subtle differences between the OFA and the SD strains in the pattern of hormone secretion in cycling and pregnant rats that did not affect ovulation or fertility. The alterations in MBH dopaminergic metabolism described in the present paper may indicate that at least in the OFA strain, the *Dsg-4* deletion produces a subtle alteration in the regulation or function of hypothalamic dopaminergic neurons, perhaps mediated through abnormal cytoarchitecture, that results in an elevated dopaminergic tone at the end of pregnancy and during lactation that produce hypoprolactinemia and lactational failure as a consequence.

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