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Tryptophan ingestion by pregnant rats induces pituitary and mammary tumours in the adult female offspring

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The present study was designed to evaluate the long-term consequences of tryptophan treatment on the central serotonergic activity in the female offspring of rats, and particularly on serotonin-controlled hormone release. During the second half of gestation, tryptophan (200 mg/kg/day) was given daily by stomach intubation to pregnant rats and the brain concentrations of serotonin and 5-hydroxyindole acetic acid and the plasma concentrations of prolactin, progesterone, oestradiol and luteinizing hormone were quantified in the adult female offspring. The offspring showed an increase in hypothalamic serotonin and serum progesterone and prolactin. In addition, maternal ingestion of tryptophan induced a marked rise in 665-day-old offspring in the incidence of both pituitary prolactinomas (62%) and mammary adenomas (49%). Present data suggest that tryptophan regulates serotonergic differentiation during early development. A transitory modification of the tryptophan concentration in the fetal brain induces a permanent increase in hypothalamic serotonin level and, in addition to modifying the release of prolactin, increases the incidence of tumours in the hypophysis and mammary gland.

Key words: adenoma/prolactin/prolactinoma/serotonin/tryptophan

Introduction

In animals and humans, the differentiation of mono-aminergic cells occurs prenatally, and their dendritic growth and terminal synaptogenesis occur during postnatal life (Hyyppä 1972; Lauder and Bloom, 1975; Zeisel *et al*., 1981; Wallace and Lauder, 1983; Arevalo *et al*., 1991). In addition to their action as neurotransmitters during adulthood, monoamines act as a differentiation signal during early neurogenesis (Arevalo *et al*., 1987; Mattson, 1988). We have previously reported that when tyrosine is administered to pregnant rats it induces an increase in the levels of tyrosine and catecholamines in the fetal brain (Garabal *et al*., 1988), and a persistent modification of central dopaminergic neurotransmission together with a disruption of dopamine-related behaviour in adult offspring (Arevalo *et al*., 1987; Rodriguez *et al*., 1994; Santana *et al*., 1994). In the same way, tryptophan administered to pregnant rats modifies the central serotonergic neurotransmission, inducing persistent alterations in some serotonin-controlled functions (Arevalo *et al*., 1991; Martin *et al*., 1997). The regulation of the neuroendocrine system is one of the main roles of serotonergic cells during adulthood. Among other hormones, serotonergic neurones regulate prolactin release (Kamberi *et al*., 1971; Chen and Meites, 1975; Woolf and Lee, 1977; Kalra and Kalra, 1983; Gartside and Cowen, 1990), and the activity of the hypothalamic–pituitary–gonadal system (Lu and Meites, 1973). We have previously reported that tryptophan, when administered to pregnant rats, induces a persistent increase in prolactin secretion in the male offspring during adulthood (Martin *et al*., 1997). The present study was designed to evaluate the functional consequences of maternal ingestion of tryptophan on central serotonergic activity, on serotonin-controlled hormone release and morphological characteristics of pituitary and mammary tissues of the adult female offspring.

Materials and methods

Experiments were carried out on female Sprague–Dawley rats (Letica; Barcelona, Spain). In accordance with the *NIH Guide for Care and Use of Laboratory Animals*, rats were housed two per cage with standard light:dark schedule (12:12 with light on 3.00–15.00; 22°C) and free access to food and water. Experiments were performed in agreement with the European Community Council Directive of 24 November 1986 regarding the care and use of animals for experimental procedures.

L-Tryptophan (200 mg/kg; Sigma, St Louis, MO, USA) was given daily by stomach intubation to young pregnant rats (150–200 g body weight before gestation). The amino acid or its saline vehicle was administered during the light cycle from day 15 to day 21 of gestation. Timing of pregnancies was determined by daily vaginal washings checking for spermatozoa; the day on which spermatozoa were found was regarded as day 0. Only the rats that were born on day 21 of gestation were included in the study. Immediately after weaning, 180 female rats were divided into two equal groups, the tryptophan-mother and saline vehicle-mother groups. In order to prevent a litter effect, only two female offspring per rat were used. At 240, 380, 517, 566, 593 and 665 days after birth, the rats were killed by decapitation at the beginning of the dark period of the second day of dioestrous (determined by analysing vaginal smears for at least eight consecutive days). Different samples were obtained for biochemical

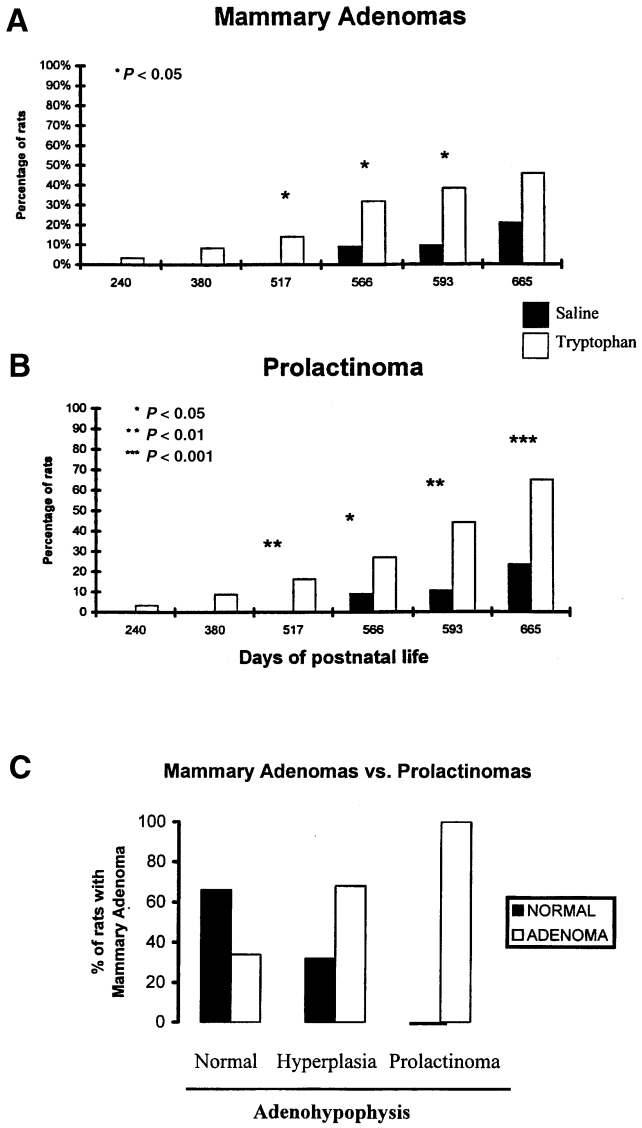

Figure 1. Incidence of (**A**) mammary adenomas or (**B**) pituitary prolactinomas in the saline-mother (black bar) and tryptophan-mother (white bar) female rats (expressed as a percentage of rats that developed a tumour before the date referred to on the *x*-axis). (**C**) Percentage of rats with prolactinoma or pituitary hyperplasia showing mammary gland adenoma. Statistical differences between saline-mother and tryptophan-mother groups are indicated by asterisks [Fisher's exact test in (A) and (B); χ^2 test in (C)].

studies of serum and brain tissue, and for histological studies of pituitary and mammary tissue.

Blood was collected from the trunk and allowed to clot at room temperature. Serum was separated by centrifugation (2000 *g* for 15 min), divided into aliquots, and stored at –40°C until assay. Serum luteinizing hormone (LH) and prolactin radioimmunoassays were performed using materials provided by the National Hormone and Pituitary Program (Rockville, MD, USA), and the results were expressed in terms of the respective standard hormone preparation according to previously validated procedures (Mas *et al.*, 1984). Progesterone and oestradiol were quantified by radioimmunoassay (Coat-a-count kit; Diagnostic Products Corporation, Los Angeles, CA, USA). The intra- and inter-assay variation coefficient was <8% in all the radioimmunoassays, as calculated at the concentration of the 50% displacement.

Immediately after extraction, the brain was laid on its dorsal surface and the hypothalamus and medial preoptic area (MPOA) dissected from the forebrain structures according to a previously described procedure (Gonzalez *et al.*, 1986). The brain pieces were quickly removed and weighed in conical 5 ml test-tubes. A 2 ml aliquot of perchloric acid (PCA) (0.1 mol/l) containing 4×10^{-5} mol/l sodium metabisulphite was pipetted into the tubes to avoid the metabolism or oxidation of monoamines. Thus, at 2 min after killing of the rat the brain monoamines were protected in a stable solution. The mixture was then sonicated at 100 W for about 12 s on ice, and the homogenate centrifuged for 15 min at 15 000 *g*. The supernatant and pellet were kept in separate tubes. All samples were stored at –70°C to avoid deterioration until biochemical measurements were carried out (within 2 months of brain dissection). Using this procedure, the concentrations of compounds studied were not significantly modified before their measurement (Arevalo *et al.*, 1991).

Concentrations of tryptophan, serotonin (5-hydroxytryptamine; 5-HT) and 5-hydroxyindole acetic acid (5-HIAA) in brain tissue were measured using liquid chromatography with electrochemical detection, according to previously validated procedures (Afonso *et al.*, 1990; Arevalo *et al.*, 1991; Santana *et al.*, 1994). An aliquot of the supernatant was injected into the chromatographic column (300×3.9 mm stainless steel column packed with Nova-Pack c18, 4 μm particle size; Waters, Milford, MA, USA). The mobile phase consisted of 0.1 mol/l $NaH_2PO_4 \cdot H_2O$, 0.5 mmol/l EDTA, 1 g/l sodium heptanesulphonate (PIC B7) and 6% acetonitrile. The final solution (pH 4.35) was filtered (0.45 μm Millipore filter) before use. Standards of serotonin and 5-HIAA (Sigma) were dissolved in PCA containing 4×10^{-5} mol/l sodium metabisulphite and kept as stock solutions at –20°C. They were diluted with ice-cold PCA/sodium metabisulphite shortly before chromatographic injection. All separations were performed isocratically at a flow rate of 1.0 ml/min at room temperature. The electrochemical detector used was a Waters Model 460 (Waters, Milford, MA, USA). The detector potential was 0.82 V. Quantifications were performed from standard curves of peak height.

Mammary and pituitary tissues were dissected and processed for histological studies with light microscopy. Two processing methods were used, one for epoxy resin-embedded tissue and the other for paraffin-embedded tissue. In the first case, samples were fixed with 2.5% glutaraldehyde in Millonig buffer (monosodic phosphate 1.69%, sodium hydroxide 0.387%, glucose 0.488% and calcium chloride 0.0045% in distilled water), post-fixed with osmium tetroxide, dehydrated in a graded ethanol series and embedded in epoxy resin (EPON®; Tousimis Research Corp., Rockville, MD, USA). Semithin sections (1 μm) were cut and stained with toluidine blue. In the second case, samples were fixed in 10% formaldehyde, embedded in paraffin with an automatic processor (Autotechnicon®; Technicon Instruments Corp., Tarrytown, NY 10591, USA), sectioned at 5 μm and stained with haematoxylin and eosin. Selected slices of paraffin-embedded tissue were used for immunohistochemical detection of prolactin in both pituitary and mammary tissues. Cells with prolactin were detected according to a published procedure (Bratthauer and Adams, 1994) and using the Histostain™ SP Kit (Zymed Lab. Inc., San Francisco, CA, USA). Briefly, after deparaffinization, hydration and blocking endogenous oxidation with 3% hydrogen peroxide (10 min), tissues were incubated overnight in 10% non-inmune goat serum at room temperature. Three drops of the primary prolactin antibody provided by the NHPP (National Hormone and Pituitary Program) (1:100 000) were added to sections. After 1 h, and after washing the tissue with phosphate-buffered saline and goat serum, three drops of the biotinylated secondary antibody were added for 45 min. Finally, slices were washed, covered with horseradish peroxidase–streptavidin for 45 min, developed with diaminobenzidine (Sigma Fast™, 3-3′ Diamino

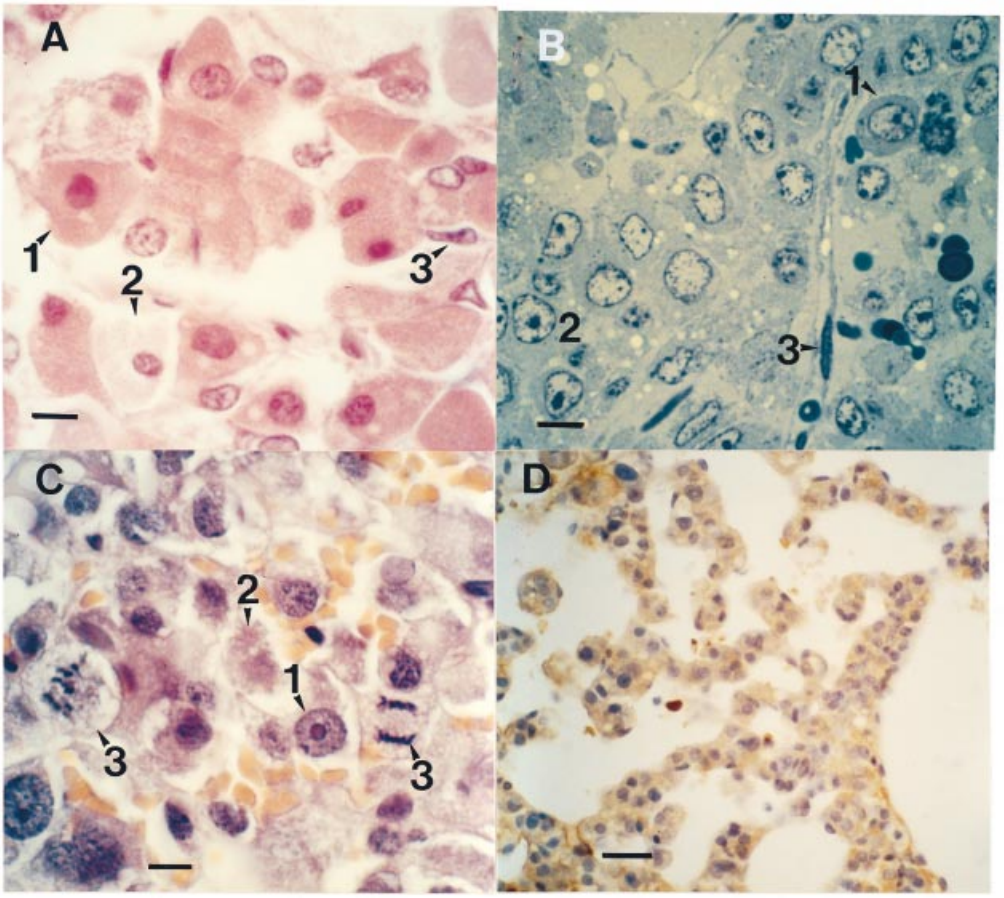

Figure 2. (**A**) Normal pituitary gland with acidophil (1), chromophobe (2) and endothelial (3) cells. (**B**) Normal pituitary gland with basophil cell (1), chromophobe cell (2) and endothelial cells constituting sinusoidal structures (3). (**C**) Pituitary adenoma with basophil (1) and chromophobe (2) cells, mitotic figures (3) and an intense neovascularization (see numerous red blood cells within sinusoidal structures). (**D**) Pituitary adenoma, showing a high number of prolactin-positive cells and dilated sinusoidal structures. (A) and (C) stained with haematoxylin and eosin (5 μm section); (B) stained with toluidine blue (1 μm section); (D) immunohistochemistry for prolactin. Scale bars: (A–C) = 10 μm; (D) = 20 μm.

benzidine tablet sets, product number D-4293), counterstained with Mayer haematoxylin (1 min), dehydrated in an ethanol series, rinsed in xylene, and covered with Eukitt (O.Kindler GmbH & Co., Freiburg, Germany).

The statistical analyses were performed using analysis of variance (ANOVA) followed by Scheffé post hoc test, Student's *t*-test and χ^2 test (Statistica; Statsoft, Tulsa, OK, USA). Differences were considered to be significant when associated with a probability of 5% or less. Values were expressed as the mean ± SEM.

Results

The cumulative incidence of mammary and pituitary tumours at different stages of postnatal development are shown in Figure 1A and B respectively; the co-existence of mammary and pituitary tumours is shown in Figure 1C. At the end of the experiment (day 665 of postnatal life), 49% of the tryptophan-mother offspring initially included in the study had developed mammary adenomas at some stage of their postnatal life (Figure 1A). The percentage showing mammary tumours was higher in the tryptophan-mother offspring than in the vehicle-mother offspring ($P < 0.05$ at days 517, 566 and 593). The percentage of rats showing pituitary tumours (Figure 1B) was also higher in the tryptophan-mother offspring (62.5% of rats before day 665 of postnatal life) than in the vehicle-mother offspring ($P < 0.05$ at day 566; $P < 0.01$ at days 517 and 593; $P < 0.001$ at day 665). Despite the fact that all rats with prolactinoma and most rats with pituitary hyperplasia had mammary adenoma, rats were also found with mammary adenoma and no histological evidence of any abnormality in the adenohypophysis (Figure 1C).

Pituitary tumours always occurred in the anterior pituitary gland, compressing the posterior pituitary, and often also the pituitary stalk. The histological studies showed a tumoral proliferation mainly composed of basophils and a low percentage of chromophobe cells (Figure 2). These are large-diameter cells with a marked pleiomorphic appearance, irregular nucleus and wide cytoplasm. Most cells in the tumour showed prolactin immunoreactivity and were often found in mitosis. The stromal components were formed by loose connective tissue and abundant new blood vessels with prominent endothelial cells that occasionally showed mitotic figures. Thus, these tumours were classified as prolactinomas.

Mammary tumours were composed of both ductal (Figure 3E) and secretory (Figure 3C, D and F) proliferating cells. These cell groups showed prolactin immunoreactivity and no signs of malignancy (Figure 3F). Secretory tumoral

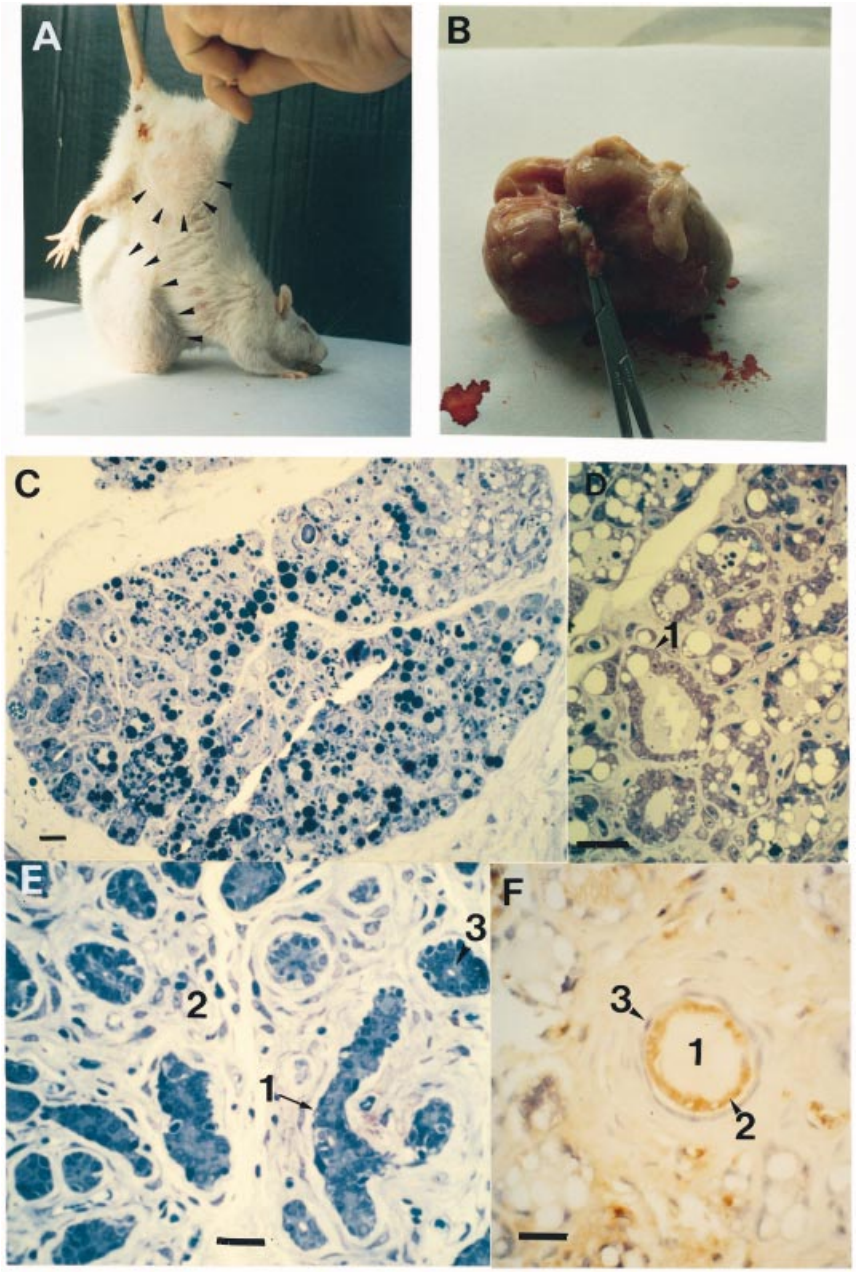

Figure 3. (**A**) Female rat with two mammary tumours (marked by arrowheads); (**B**) macroscopic appearance of rat mammary adenoma; (**C**) mammary adenoma lobes surrounded by loose connective tissue and with numerous lipid droplets within secretory cells; (**D**) detail of mammary adenoma with lactocytes secreting lipid droplets into the ductal lumen (1); (**E**) ductal component of mammary adenoma (1) with a high degree of connective tissue (2) and a constriction of ductal lumen (3); (**F**) cross-section of a terminal end bulb with a dilated lumen (1), tumoral proliferating cells showing prolactin immunoreactivity (2) surrounded by a continuous layer of myoepithelial cells (3). Scale bars: (D–F) = 70 μm; (C) = 20 μm.

cells often showed large lipid droplets (Figure 3C and D). The lumen of the terminal end bulb was dilated (Figure 3F), while the ductal lumen was constricted, having the appearance of a virtual space (Figure 3E). Thus, these were classified as mammary adenomas. These were fast-growing tumours (1 g/day; Figure 4A), and reached a weight of about 20 g on the day that the rats were killed (Figure 3A and B; Figure 4B). No statistical differences were found between saline-mother offspring versus tryptophan-mother offspring for either the growth rate ($P = 0.77$) or final weight ($P = 0.33$) of mammary adenomas.

The prolactin (ANOVA $F_{(2,38)} = 5.12$, $P < 0.01$; $P < 0.01$ saline-mother versus tryptophan-mother with adenoma; $P < 0.01$ tryptophan-mother without adenoma versus with adenoma) and progesterone (ANOVA $F_{(2,24)} = 3.73$, $P < 0.05$; $P < 0.01$ saline-mother versus tryptophan-mother with adenoma) concentrations were higher in the plasma of tryptophan-mother rats with mammary adenoma than in saline-mother rats or in tryptophan-mother rats without adenoma (Table I). No statistical differences were found for LH (ANOVA $F_{(2,32)} = 1.54$, $P = 0.22$) or oestradiol (ANOVA $F_{(2,23)} = 1.27$, $P = 0.29$). The hypothalamic serotonin level was higher in

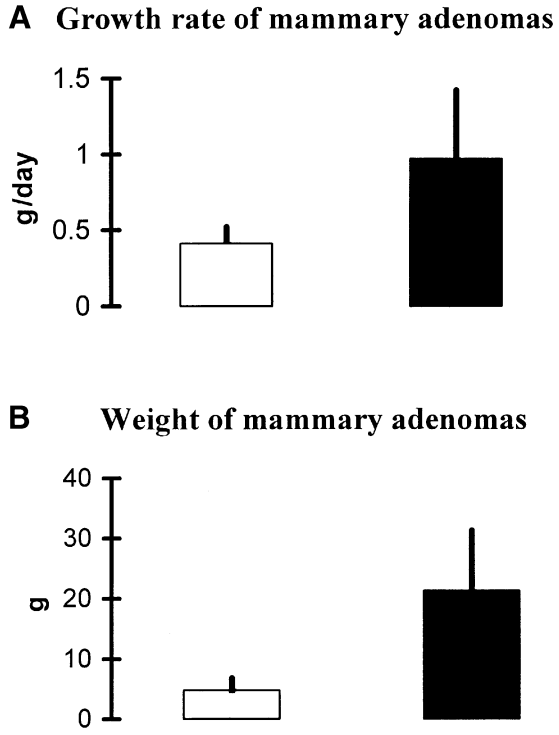

Figure 4. (**A**) Growth rate (g of tumour per day after tumour detection) and (**B**) weight (g) of mammary adenomas in the saline-mother (open bars) and tryptophan-mother (filled bars) female rats. Student's *t*-test showed no statistical differences between saline-mother and tryptophan-mother groups.

the tryptophan-mother rats with mammary adenoma than in saline-mother rats or in tryptophan-mother rats without adenoma (ANOVA $F_{(2,23)} = 5.07$, $P < 0.05$; $P < 0.05$ saline-mother versus tryptophan-mother with adenoma; Table I). No statistical differences were found either for tryptophan (ANOVA $F_{(2,23)} = 2.13$, $P > 0.05$) or 5-HIAA (ANOVA $F_{(2,26)} = 0.6$, $P > 0.05$) in the hypothalamus or for tryptophan (ANOVA $F_{(2,25)} = 0.67$, $P > 0.05$), serotonin (ANOVA $F_{(2,27)} = 1.61$, $P > 0.05$) or 5-HIAA (ANOVA $F_{(2,27)} = 2.18$, $P > 0.05$) in the MPOA (Table I).

Discussion

The main findings of this study may be summarized as follows. The ingestion of tryptophan by the mother during the second half of gestation produces a marked increase in the percentage of the adult female offspring with mammary and pituitary adenomas. The subgroup of tryptophan-treated rats which developed tumours also showed a hypothalamic serotonergic hyperactivity and hyperprolactinaemia. These data suggest that tryptophan administration during gestation increases hypothalamic serotonergic activity in a significant number of rats (though not all), and it is probably only these sensitive animals which eventually develop hyperprolactinaemia and mammary tumours.

We have previously reported that tryptophan administered to pregnant rats crosses the placental barrier and induces a dose-related increase in tryptophan concentration in the placenta, body and brain of the fetus (Arevalo *et al*., 1991) and a parallel increase in 5-HT and 5-HIAA levels in the fetal brain (Arevalo *et al*., 1991). In the present study it was found that, in addition to this global action on the serotonergic system, maternal intake of tryptophan induced a permanent facilitation of serotonergic neurotransmission in the hypothalamus of female offspring (Table I?). Serotonergic neurones differentiate during early ontogenesis and their fibres reach the hypothalamus in the last days of prenatal life (Hyyppä 1972; Zeisel *et al*., 1981; Wallace and Lauder, 1983; Arevalo *et al*., 1991). However, the final termination density and the formation of precise termination patterns are not reached until adulthood (Jacobs and Azmitia, 1992). Thus, present data show that during early neurogenesis and before synapsis formation, the central availability of tryptophan can modulate serotonergic cell differentiation, inducing permanent functional consequences in the hypothalamic serotonergic system (Arevalo *et al*., 1987; Mattson, 1988; Santana *et al*., 1994).

During adulthood, serotonin is involved in the neural regulation of prolactin release by the anterior pituitary (Kamberi *et al*., 1971; Kordon *et al*., 1973; Lu and Meites, 1973; Chen and Meites, 1975; Woolf and Lee, 1977; van der Kar *et al*., 1980). The peripheral administration of 5-hydroxytryptophan (Jacobs and Azmitia, 1992), the intravenous administration of 5-HT (Pilotte and Porter, 1979) or the administration of serotonergic agonists (Jacobs and Azmitia, 1992) facilitate prolactin release to plasma. The drug-blockade of serotonergic neurotransmission decreases the serum prolactin concentration (Gid-Ad *et al*., 1976). Thus, it has been proposed that hypothalamic 5-HT activates prolactin release from the anterior pituitary gland (Simpkins *et al*., 1977; Meites, 1980, 1982). We observed an increase in both the serum prolactin and the serotonergic activity of the hypothalamus in tryptophan-mother offspring who developed a mammary adenoma. These data suggest that the prenatal administration of tryptophan induces not only a long-lasting activation of serotonergic neurotransmission but also a persistent enhancement of the facilitatory activity of 5-HT on prolactin release in a significant number of rats.

It has been reported in rats that treatments resulting in increased prolactin secretion (medial eminence lesion, reserpine administration) enhance the development of spontaneous mammary tumours. On the other hand, treatments that reduce prolactin secretion inhibit the development of spontaneous mammary tumours (Simpkins *et al*., 1977; Welsch and Nagasawa, 1977; Meites *et al*., 1978; Meites, 1980, 1982). In the present study, a 400% increase in plasma prolactin was found in rats that developed a mammary adenoma. Thus, it is possible that the increase in incidence of mammary adenoma found in the offspring of tryptophan-mothers could be induced by an enhancement in the plasma prolactin concentrations produced by a permanent facilitation of the hypothalamic serotonergic cells. Despite the fact that this hypothesis is supported by the presence of lactorrhoea in most mammary tumours, the epithelial component of adenomas is not necessarily induced by the chronic hyperprolactinaemia. In addition to the high prolactin concentrations, the female offspring of tryptophan-mothers that developed mammary adenomas showed a high concentration of plasma progesterone. As

Table I. Tryptophan, serotonin and 5-hydroxyindole acetic acid (5-HIAA) concentrations in the medial preoptic area and hypothalamus, and prolactin, luteinizing hormone (LH), progesterone and oestradiol concentrations in the serum of the female offspring of saline-mothers without tumours (saline), tryptophan-mothers without tumours (tryptophan) and tryptophan-mothers with mammary adenomas (tryptophan/adenoma). Values are mean ± SEM.

	Saline	Tryptophan	Tryptophan + adenoma
Medial preoptic area			
Tryptophan[a]	732 ± 320.45	512 ± 314.09	1656 ± 1084.55
Serotonin[a]	30.3 ± 13.94	122.4 ± 45.97	87.3 ± 49.37
5-HIAA[a]	160 ± 70.45	68 ± 30.75	179 ± 102.9
Hypothalamus			
Tryptophan[a]	838 ± 436.27	627 ± 455.96	1487 ± 756.61
Serotonin[a]	109 ± 43	98 ± 55.78	454 ± 208.73*/**
5-HIAA[a]	176 ± 95.28	201 ± 117.78	247 ± 94.08
Serum			
Prolactin[b]	83.8 ± 37.3	77.2 ± 28.04	364.1 ± 117.47*/**
LH[b]	1.54 ±0.29	2.17 ± 0.31	1.65 ± 0.21
Progesterone[b]	3.49 ± 0.79	7.14 ± 2.55	13.4 ± 6.38*
Oestradiol[c]	39.13 ± 6.2	38.55 ± 8.26	20.22 ± 2.11

*$P < 0.01$ versus saline-mother without tumours. **$P < 0.01$ versus tryptophan-mother without tumours.
[a]Concentrations given as ng/mg protein.
[b]Concentrations given as ng/ml serum.
[c]Concentrations given as pg/ml serum.

progesterone causes growth of the lobules and proliferation of alveoli cells, this hormone may also be involved in the high incidence of mammary adenomas observed here.

Pituitary adenomas have been found in 8–25% of human autopsies, a percentage that increases with age (Post *et al*., 1980). This high age-related incidence of prolactinomas has also been found in rats and mice (Post *et al*., 1980; Meites, 1982). A high age-related incidence of spontaneous mammary adenomas has also been reported in women and female rats or mice (Welsch and Nagasawa, 1977). As far as we know, and despite the fact that mammary adenoma has been associated with a high plasma concentration of prolactin (Brown *et al*., 1982), the relationship between mammary adenomas and pituitary prolactinomas (particularly in the case of prolactin-secreting microadenomas) has not been established. In addition, we have not found any information about the aetiology of spontaneous pituitary prolactinomas or the possible action of the long-lasting activation of hypothalamic serotonergic systems on lactotroph proliferation. Present data suggest that a persistent increase in hypothalamic serotonin due to age (Meites *et al*., 1978) or other factors (high levels of tryptophan during prenatal life) may cause both pituitary prolactinomas and, in a second stage, mammary adenomas in rats. However, the morphological difference between the mammary adenomas found here in rats and the mammary fibroadenomas reported in the human species suggests that the result presented in this study must not be directly extrapolated to women.

In conclusion, the present study shows that excessive ingestion of one amino acid, tryptophan, during gestation could have important consequences on the brain development of the offspring. It has been reported that in humans transient tyrosinaemia during neonatal life can induce permanent intellectual impairment 8–10 years later (Menkes *et al*., 1972; Mamunes *et al*., 1976). Further studies are warranted to evaluate the functional consequences of the neonatal increase in serum tryptophan on human development. Present data show that prenatal administration of tryptophan induces a permanent facilitation of central serotonergic neurotransmission, disrupting the serotonergic regulation of pituitary prolactin release and increasing the incidence of pituitary prolactinomas and mammary adenomas. We used a tryptophan dose 10–20 times higher than the mean requirement for this amino acid in humans (Lazaris-Brunner *et al*., 1998). However, tablets with a high dose of this amino acid can be obtained in most countries without medical authorization. This study suggests that it is necessary to act cautiously when tryptophan is prescribed as a dietary supplement, particularly in the case of pregnant women or low-weight newborns. Finally, and according to the hypothesis previously proposed for tyrosine and the catecholaminergic systems (Arevalo *et al*., 1987; Garabal *et al*., 1988; Mattson, 1988; Rodriguez *et al*., 1994; Santana *et al*., 1994), present data support the idea that serotonin may have a neurotrophic role before the ontogenic start of neurotransmission.

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