Comparative Medicine
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Vol 52, No 2
April 2002
Pages 136-142

Development and Vulnerability of Rat Brain and Testes Reflected by Parameters for Apoptosis and Ornithine Decarboxylase Activity

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Background and Purpose: **Awareness of effects of chemicals on brain and sex organs during organogenesis is increasing. Balance between apoptosis and ornithine decarboxylase (ODC) activity has an essential role for final structure and function of these organs. It is important to localize stages in development where these processes may be particularly vulnerable to chemicals. We describe reference data on apoptosis and ODC activity in brain and testes.**

Methods: **Brain and testes specimens were obtained during gestational days (G) 15 to 21 and on postnatal days (P) 1 to 60, and ODC activity and parameters of apoptosis (DNA laddering and Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling-staining) were investigated.**

Results: **Brain ODC activity reaches maximum at G19 and thereafter rapidly decreases until P7. Apoptotic DNA laddering occurs in the brain from G17 to P7. Significant apoptotic ladders were not detected between P9 and 60. In the testes, apoptotic laddering was weak from G21 to P15, but increased significantly from P15 to 60. Histologic examination and DNA laddering analyses revealed low-level germ cell apoptosis from G15 to P11. At onset of spermatogenesis at P15, the number of apoptotic germ cells increased markedly.**

Conclusions: **Brain ODC activity and apoptosis from G15 to P7 and at the onset of testes apoptosis at P15 are relevant markers for chemically induced developmental toxicity in these organs.**

Awareness of the effects of chemicals present as contaminants in the environment and in food on the development of brain and sex organs during organogenesis, including the potential for endocrine modulation and the interplay between brain and sex organs, is increasing (1, 2). Toxicity testing of chemicals previously has mainly focused on toxicity in adult laboratory animals, but the concern for the potential endocrine-disrupting chemicals (xenohormones) and for greater vulnerability of the developing brain (3-5) have changed the focus of interest to include the developing fetus, postnatal animals, and young animals. International regulatory agencies have taken actions on these problems, have already established a guideline for testing developmental toxicity (6), and are about to finish a specific guideline for developmental neurotoxicity (7).

Apoptosis is an active process regulated by molecular mechanisms that activate pre-programmed events. This process is essential for tissue and organ sculpturing, elimination of damaged or undesired structures, and control of cell numbers. It is important to study the effect of various chemicals on usual apoptotic processes, including time dependency, and to accelerate research on these topics because they are only in their early beginning (8).

During spermatogenesis, apoptosis in testicular germ cells is recognized as an important physiologic mechanism to match the number of germ cells with the supporting Sertoli cells (9). The mechanisms involved in germ cell apoptosis are numerous and are investigated, for example, by the removal of follicle-stimulating hormone and luteinizing hormone (9). Regulation of germ cell apoptosis in the normal testis is controlled by the *BCL-2* family, *p53*, and *Fas*-signaling pathway (10).

Increased germ cell apoptosis has been associated with azoospermia, severe oligozoospermia, and infertility in men (11, 12). In rodents, several chemicals such as mono(2-ethylhexyl)phthalate (MEHP), 2,5-hexandione, nitrobenzene, deltamethrin, and hydroxyurea are reported to increase apoptosis in the testes (13-17).

During the development of brain and testes, the apoptotic processes play an essential role in the final structure and function (10, 18). In toxicology, the effect of chemicals on apoptosis frequency during development is of most concern. A priori, we expect that the most critical time window for studying the toxic effects of chemicals on the level of apoptosis is when it is at its highest, as reported recently for the effect of toluene on cells in the cerebellar granular cell layer at postnatal day 21 (19).

It has been reported that ornithine decarboxylase (ODC; EC 4.1.1.17) is a useful marker for cell proliferation in the brain during development (20). The enzyme converts L-ornithine to putrescin, the rate-limiting step in the synthesis of polyamines, which are necessary for stimulation of cell growth and cell differentiation. In an intimate balance, this stimulation is counteracted by cell death. Any disturbance of this balance may be manifested as disturbed brain development. Knowledge about the age variation of ODC activity during ontogenesis of the rat is sparse and relies on interpretation from various studies. Therefore, it is important systematically to reveal the age variation in brain ODC activity and to localize stages in development,

Received: 10/02/01. Revision requested: 11/13/01. Accepted: 1/09/02.
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where ODC may be particularly sensitive to chemical-induced disturbances and how this is related to the frequency of apoptosis.

The aims of the study reported here were: to obtain reference data for apoptosis in the brain and testes of rats from gestational day (G) 15 to postnatal day (P) 60; to compare levels of apoptosis and ODC activity in the brain during ontogenesis; to select an appropriate time window for toxicity studies of chemicals with effect on the brain or testes development; and to discuss the applicability of these parameters in toxicity testing.

Materials and Methods

Rats. Wistar rats (MOL:WIST) with specific-pathogen-free health status were purchased from Moellegaard Breeding Centre Ltd. (DK-4623, Lille Skensved, Denmark). Each of 42-month-old females was mated with one of 15 fertile male Wistar rats from our own animal facility. The rats were housed conventionally one per cage (Macrolon, Tecniplast Gazzada S. ar. L., Buguggiate, Italy) with light from fluorescent tubes from 9 p.m. to 9 a.m. Room temperature was 22 ± 1°C and relative humidity was 55 ± 5%. The rats were given a standard diet, Altromin rat No. 1324, and citric acid acidified tap water (to avoid microbiological contamination of drinking water), both ad libitum. After mating, the offspring were either taken by cesarean section (G15, 17, 19, or 21) of mothers under deep CO_2/O_2 anesthesia and killed immediately by decapitation, or the dams were allowed to deliver and the offspring were sacrificed at P1, 3, 5, 7, 9, 11, 15, 19, 22, 30, or 60 by decapitation while under CO_2/O_2 anaesthesia. The day of conception was defined as the day a vaginal plug was observed, as determined on the basis of three daily inspections. Terminal body weight was determined at sacrifice, and the brain from six females per age group and the brain and testes from six males per age group were excised and weighed. The study and all procedures were in accordance with the rules of the National Animal Ethics Committee.

Brain specimens. After decapitation, whole brains were quickly transferred to ice-cold 0.32*M* sucrose and homogenized at 0 to 4°C, using an Ultra-Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany) at full speed. Aliquots of homogenate were immediately frozen at -40°C and stored at -80°C until analyses.

Brain protein analysis. An equal volume of a 2% (v/v) aqueous Triton X-100 solution was added to specimens, which then were incubated at ambient temperature for one hour. After appropriate dilution with a 1% (v/v) aqueous Triton X-100 solution, protein was measured by use of the bicinchoninic acid (BCA) method (BCA Protein Assay Reagent, Cat. No.23225, Pierce, Rockford, Ill.) with bovine serum albumin as the standard.

Brain ODC activity. The ODC activity in the brain was determined by use of a modification of the method described by Bondy in 1986 (21). In a sealed tube, the $[^{14}C]O_2$ liberated by ODC from L-[1-^{14}C]ornithine was collected on filter paper wisps to which was added 200 µl of saturated $Ba(OH)_2$ solution. The incubation medium was composed of 50 µ*M* pyridoxal-5-phosphate, 100 m*M* Tris-HCl, 400 µ*M* L-ornithine, and 5 m*M* dithiothreitol (pH 7.1). The total incubation volume was 250 µl, and the brain protein concentration was between 0.25 (youngest rats) and 10 (oldest rats) mg/250 µl. The specific activity of L-[1-^{14}C]ornithine (CFA 491, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) in the incubation medium was 2 mCi/mmol. Spontaneous and nonspecific decarboxylation was determined after adding 7.5 m*M* (final concentration) difluoromethylornithine (Cat. No. D-193, RBI/Sigma, Natick, Mass.), an irreversible and specific ODC inhibitor, followed by pre-incubation for 10 min. Incubation was carried out at 37°C for 60 min, and was stopped by addition of 200 µl of 4*M* sulfuric acid through the seal. The $[^{14}C]O_2$ was collected for another 60 min at 37°C. The wisps were transferred to counting vials, four milliliters of HighIonic (Packard Instruments, Didcot, Oxfordshire, UK) counting cocktail was added, and radiolabel was counted in a TriCarb 2500 TR Liquid Scintillation Analyser (Packard Instruments), applying quench and fluorescence corrections. The ODC activity was expressed as micromoles per gram per brain per hour.

Detection of nucleosomal ladders in the developing brain and testes. The DNA from the respective organs was isolated according to the method of Strauss (22). Detection of nucleosomal ladders was based on ligation-mediated polymerase chain reaction (PCR) analysis (LM-PCR) (23), using the ApoAlert LM-PCR Ladder Assay Kit (Cat. No. 905-1, Clontec, Palo Alto, Calif.). In short, the first step is the ligation of dephosphorylated adaptors (composed of a 12-mer and a 24-mer) to the ends of the DNA fragments generated during apoptosis. In mammalian cells, such fragments generally have 5`-phosphorylated blunt ends (23); thus, only the 24-mer is ligated to the DNA fragments. When the mixture of ligated DNA fragments is heated, the 12-mer is released. Next, the 5`-protruding ends of the molecules are filled in by a thermostable DNA polymerase. The 24-mer then serves as a primer in a PCR analysis in which the fragments with adaptors on both ends are exponentially amplified. The resulting nucleosomal ladder can be UV-light visualized on a 1.2% agarose electrophoresis gel stained with ethidium bromide. As a positive control, calf thymus DNA provided by Clontech with the kit was used. The negative control did not contain DNA.

The measurements of apoptotic changes in the brain and testes were performed for two animals per age group; however, in some instances (i.e., rat brain ages P15 and P22 and in rat testes at P7, 9, and 11), apoptosis was measured only for one animal because of lack of sufficient material.

Histologic investigation of the testes. Testis tissue for pathologic studies was fixed in buffered 4% formaldehyde and was embedded in paraffin. Two cross sections were cut from the middle part of the testis. The sections were stained: one with hematoxylin and eosin (H & E), the other with the ApopTag-peroxidase kit (Cat. No. S7100, Intergen Company, New York, N.Y.) (Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling: TUNEL) to visualize the apoptotic cells. The kit procedure was modified (19). In brief, after deparaffinization and rehydration, tissue cross sections (5 µm thick) were incubated with Proteinase-K in phosphate-buffered saline (PBS buffer) for 5 min. A concentration of 5 µl of proteinase-K solution/ml of buffer (corresponding to 0.001 %) was used for the age group G15 to P5, and 10 µl of proteinase-K solution/ml of buffer (corresponding to 0.002 %) was used for the age group from P7 to 60. Sections were washed in PBS, treated with 2 % hydrogen peroxide to block endogenous peroxidase activity, and incubated with the kit reaction mixture containing terminal reaction buffer, digoxigenin-labeled nucleotide strings, and terminal-deoxynuclotidyl-transferase (TdT) following the suppliers' guidelines. Subsequently, sections were treated with anti-digoxigenin-per-

Table 1. Body weight and absolute and relative brain and testis weights of Wistar rats from gestational day (G) 15 to postnatal day (P) 60

	N[a]	Body weight (g)		Absolute brain weight (g)		Relative brain weight (g/100g BW)		N[b]	Absolute testis weight (g)	Relative testis weight (g/100g BW)
Age	Sex M (F)	M	F	M	F	M	F		M	M
G15	4 (10)	0.334 ± 0.023	0.326 ± 0.064	0.0377 ± 0.0027	0.0386 ± 0.0052	11.30 ± 0.54	12.37 ± 3.40	4	-	-
G17	10 (5)	0.479 ± 0.073	0.514 ± 0.062	0.0435 ± 0.0115	0.0501 ± 0.0171	8.97 ± 1.39	9.70 ± 3.21	10	-	-
G19	6 (7)	0.836 ± 0.090	0.854 ± 0.158	0.0698 ± 0.0205	0.0736 ± 0.0043	8.52 ± 2.77	8.91 ± 1.88	6	-	-
G21	7 (5)	3.94 ± 0.382	3.76 ± 0.623	0.165 ± 0.011	0.161 ± 0.015	4.19 ± 0.30	4.36 ± 0.67	7	-	-
P1	6 (6)	7.57 ± 0.75	7.28 ± 1.09	0.295 ± 0.012	0.267 ± 0.036	3.93 ± 0.31	3.70 ± 0.49	11	0.00740 ± 0.00113	0.0996 ± 0.0123
P3	6 (6)	10.3 ± 0.23	9.39 ± 0.78*	0.417 ± 0.016	0.410 ± 0.038	4.05 ± 0.18	4.39 ± 0.50	8	0.0138 ± 0.00130	0.136 ± 0.0116
P5	6 (6)	9.65 ± 1.34	9.80 ± 1.30	0.438 ± 0.051	0.428 ± 0.050	4.56 ± 0.32	4.38 ± 0.17	11	0.0137 ± 0.00282	0.137 ± 0.0129
P7	6 (6)	14.8 ± 0.60	14.2 ± 0.35	0.674 ± 0.020	0.660 ± 0.035	4.58 ± 0.21	4.63 ± 0.22	8	0.0239 ± 0.00197	0.161 ± 0.0166
P9	6 (6)	22.1 ± 1.73	21.6 ± 0.70	0.921 ± 0.032	0.910 ± 0.017	4.19 ± 0.27	4.22 ± 0.17	7	0.0419 ± 0.00375	0.188 ± 0.0150
P11	6 (6)	26.2 ± 5.41	25.9 ± 5.23	1.018 ± 0.081	1.043 ± 0.049	3.98 ± 0.62	4.14 ± 0.67	8	0.0634 ± 0.0209	0.239 ± 0.0492
P15	6 (6)	34.7 ± 2.26	32.6 ± 2.04	1.327 ± 0.069	1.262 ± 0.080	3.83 ± 0.12	3.87 ± 0.13	7	0.130 ± 0.00938	0.371 ± 0.0168
P19	6 (8)	41.1 ± 7.42	45.6 ± 2.30	1.360 ± 0.088	1.372 ± 0.042	3.39 ± 0.50	3.01 ± 0.18	6	0.194 ± 0.0529	0.465 ± 0.0501
P22	6 (6)	47.3 ± 5.85	46.0 ± 5.70	1.458 ± 0.087	1.384 ± 0.093	3.11 ± 0.22	3.04 ± 0.29	11	0.263 ± 0.0273	0.564 ± 0.0245
P30	7 (6)	105.6 ± 14.5	97.8 ± 9.74	1.701 ± 0.097	1.652 ± 0.064	1.63 ± 0.16	1.70 ± 0.13	8	0.803 ± 0.205	0.784 ± 0.139
P60	6 (6)	204.1 ± 69.6	146.1 ± 34.3	1.797 ± 0.226	1.672 ± 0.162	0.941 ± 0.212	1.18 ± 0.18	11	2.13 ± 0.914	1.11 ± 0.0813

[a]No. of animals: M = males; (F) = females used to obtain body weight, absolute brain and relative brain weights.
[b]No. of males used to obtain absolute and relative testis weights.
Non-parametric analysis (Wilcoxon) was used for statistical analysis; $P < 0.05$ indicates significant difference.
Data are expressed as mean ± SD.

oxidase and substrate-chromogen mixture. Finally, sections were counter stained with Mayer's hematoxylin. Controls were run simultaneously. The negative control was processed in identical manner, except that distilled water was substituted for the TdT enzyme. Apoptotic degenerating mammary tissue from a postlactating female rat and testis tissue treated with DNAse-1 were used as positive controls. The number of TUNEL-positive cells was counted in one cross section per testis per animal.

Statistical analyses. Statistical analysis was performed, using the SAS version 6.12 (SAS Institute Inc., Cary, N.C.). To investigate differences between means in relation to age and sex, a two-way analysis of variance, with age as one factor and sex as another factor, was conducted. To adjust for litter effects, litter was included in the analysis of variance as an independent, random, and nested factor (SAS: Procedure mixed). Non-processed and natural logarithmically transformed data were examined for normal distribution and variance homogeneity. If the data did not fulfil these conditions, non-parametric analysis (Wilcoxon) was used. The general level of statistical significance was set at $P < 0.05$. Therefore, no individual P-values are given.

Results

Body and organ weights. Body and organ weights are presented in Table 1. Generally, the body weight and absolute brain weight of females tended to be lower than those of males. However, only at P3 was the body weight significantly lower in females, compared with males. As expected, for females and males, the absolute brain weight increased, whereas the relative brain weight decreased markedly as the animals developed. From P30 to P60, the absolute brain weight tended to stabilize. The absolute and the relative testis weights increased from P1 to P60. The most remarkable increase in absolute testis weight was from P30 to P60. It was not possible to isolate the testes of male fetuses at G15, and at G17 to 21, extirpation of the testes was too inaccurate to get a reliable weight. The reported body weight, and absolute and relative organ weights were within the range of previous observed data at our institute. Because of the limited number of pregnant dams, the planned 6 animals per sex per age group was not reached at G15, 17, and 21.

Brain protein concentration. The protein concentration was statistically significant lower in females at G15, compared with that in males (Fig. 1). This may be a difference by chance without any biological consequences. Obviously, the protein concentration reached minimal values at G21 and P7 for both sexes. After P7, the concentration increased to a constant value. There was no general concentration difference between males and females.

Brain ODC activity. Age and sex variation of whole brain ODC activity is seen in Fig. 2. There was significantly lower activity in females at P11, compared with that in males. This may be a difference by chance without any biological consequences. There was no general difference between males and females. The ODC activity reached maximum at G19, and thereafter, rapidly decreased until P7, where it was near background value for the analysis. The variation between animals was high at ages G17 and G19. In supplementary measurements conducted to evaluate the ODC analysis, at P1 (medium activity) the intra-sample %SD was 8.0% (n = 8), whereas the day-to-day %SD was 11.2% (n = 8) when measured in the same brain specimen.

The DNA laddering pattern in brain and testes. The results as shown in Fig. 3 indicated that apoptotic laddering occurs in the developing whole brain from G17 to P7. Apoptotic ladders were not detected from P9 to 60. In testes (Fig. 4) developmental apoptosis was weak, with slight to moderate banding from G21 until P15. However, from P15 to 60, there was strong apoptotic banding. It was not possible to obtain relative or absolute values for DNA laddering in either of the two organs, using the applied techniques.

The TUNEL-staining in testes. The level of germ cell apoptosis differed significantly among age groups ($P = 0.001$). In fetal testis from day G15 to 19, germ cell apoptosis was observed at a low level (Fig. 5). At G21 and P1, apoptosis was close to zero, followed by a weak increase until P11. At the onset of spermatogenesis at P15, the number of apoptotic cells in the testis increased markedly. The marked age differences in apoptotic activity are also seen in Fig. 6. Generally, this level of apoptosis was maintained until P60. One male at P30 had many apoptotic cells in the testes, compared with that in the other males of the same age group. This male accounts for the greater mean value and the huge standard variation at P30.

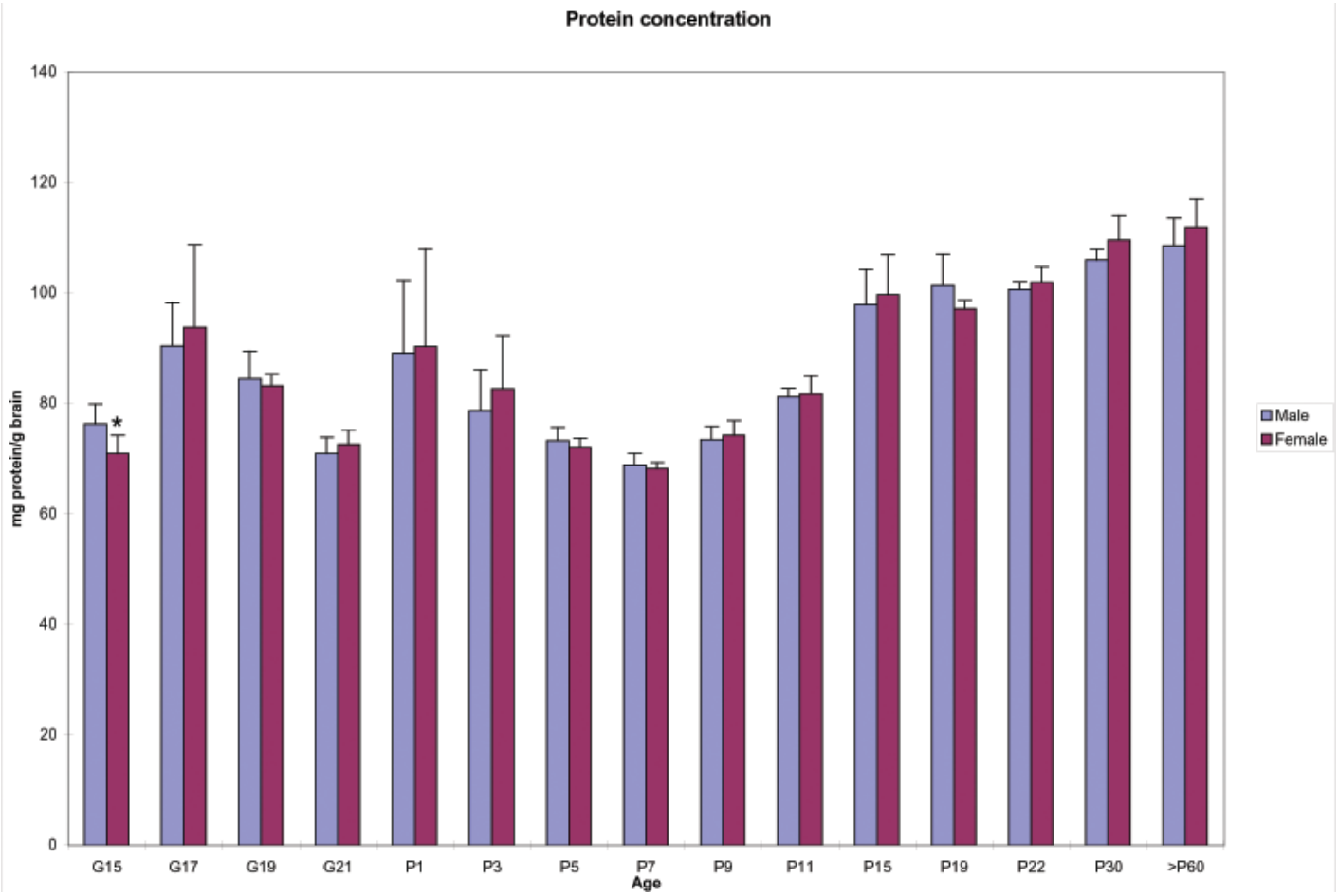

Figure 1. Mean ± SD male and female rat ontogenetic variation in whole brain protein concentration (for N, see Table 1) expressed as milligrams of protein per gram of brain tissue. $^{*}P < 0.05$ between males and females.

Discussion

The main objective of the study was to systematically investigate the age dependence of relevant marker parameters for brain and testes development. Additionally, basal biological parameters were recorded; these included body and organ weights (Table 1). The body and absolute brain weight of females tended to be lower than that of males, however, not significantly lower. The absolute brain weight increased until P30, after which it stabilized. The absolute and relative testis weights increased from P1 to P60, most remarkably from P30 to 60, corresponding to sexual maturation. The body weight and absolute and relative organ weights were within the range of those previously observed at the institute. The observed minimal brain protein concentrations corresponding to G21 and P7 (Fig. 1) were due to increase in brain non-protein components. In that period, substantial myelination of neurons is known to take place and may account for the decrease (24).The ODC analysis indicated a great day-to-day %SD (11.2%, n = 8), as determined in the same P1 specimen. However, other methods, such as quantitative immunoblot (western) analysis and real-time PCR, exist that might also be useful in future determination of ODC in the brain.

There was great interindividual variation in brain ODC activity, especially at G17 and G19. This reflects the different developmental stages between pups even at the same age. The applied method for defining gestational age on the basis of presence of vaginal plugs detected by daily inspections accounts for part of this difference, but also different developmental stage of pups in the same cull might have added to the difference.

The results indicated no overall sex difference in whole brain ODC activity, which increased from G15 to19. Thereafter, it rapidly decreased until P7, after which it was near background value. There was a distinct maximal activity in the brain of pups at G19 (Fig. 2); ODC regulates the coordination of cellular maturation in the brain and it is well known that the ODC activity is greatest in the perinatal period (20, 25). To our knowledge, no publication has reported systematic analysis of the age variation of rat brain ODC activity for such a long period as that involved in the study reported here. During normal ontogenesis, ODC expression and activity in brain, as in other organs, are greatest when the developmental needs for cell growth and differentiation are at maximum. The observed sex difference at P11 might reflect sex differences in regional brain development. However, difference by chance cannot be excluded. In the brain, developmental needs also include axonogenesis and synaptogenesis. Ornithine decarboxylase has been proposed as a marker for cell damage and developmental neurotoxicity (18, 26). Our results suggest that the best period to study the effects of chemicals on whole brain ODC activity is at G19.

A recent study (24) involved investigation of regional apoptosis

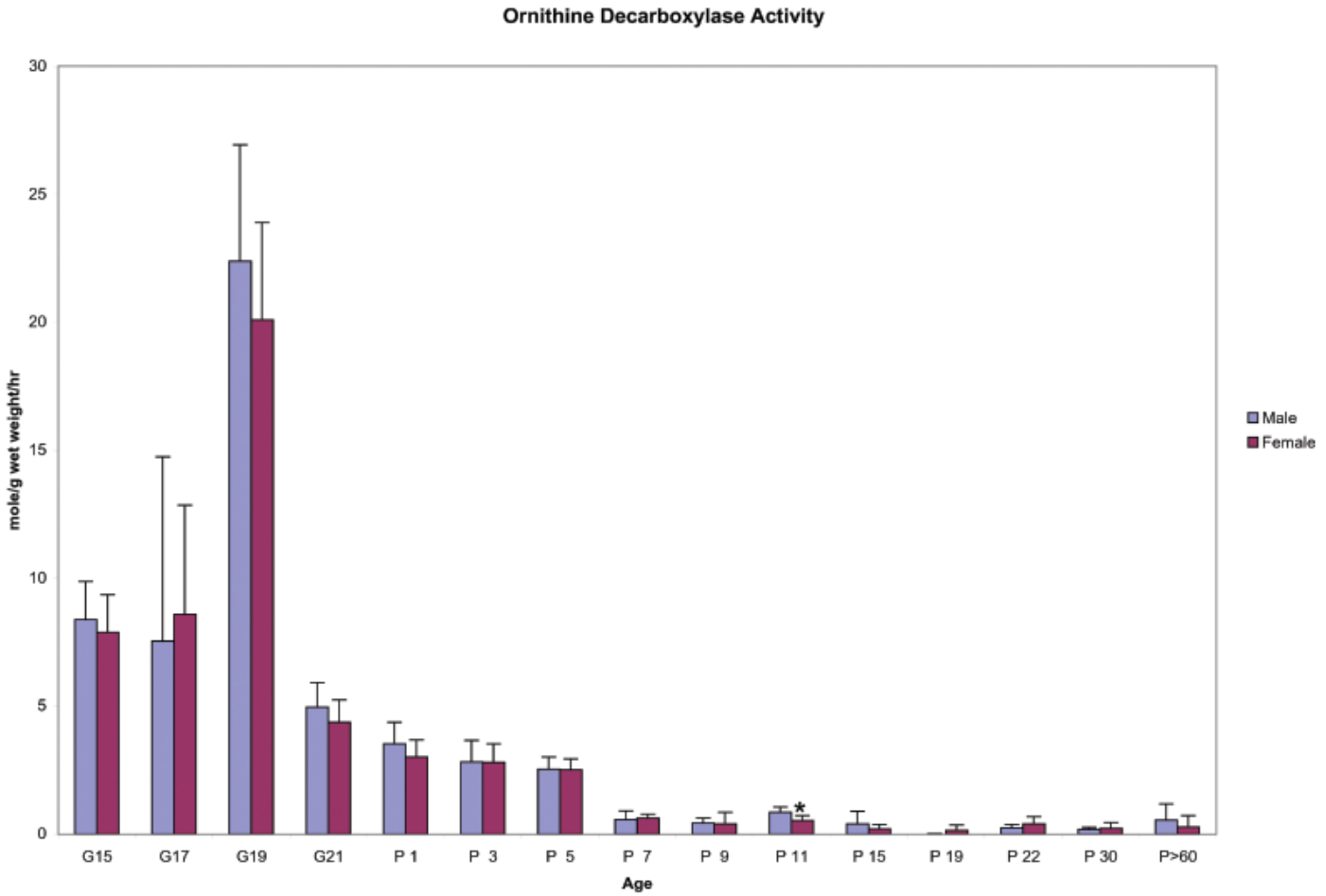

Figure 2. Mean ± SD male and female rat ontogenetic variation in whole brain ornithine decarboxylase (ODC) activity (for N, see Table 1) expressed as micrograms of CO_2 per gram of brain tissue per hour. $^*P < 0.05$ between male and females.

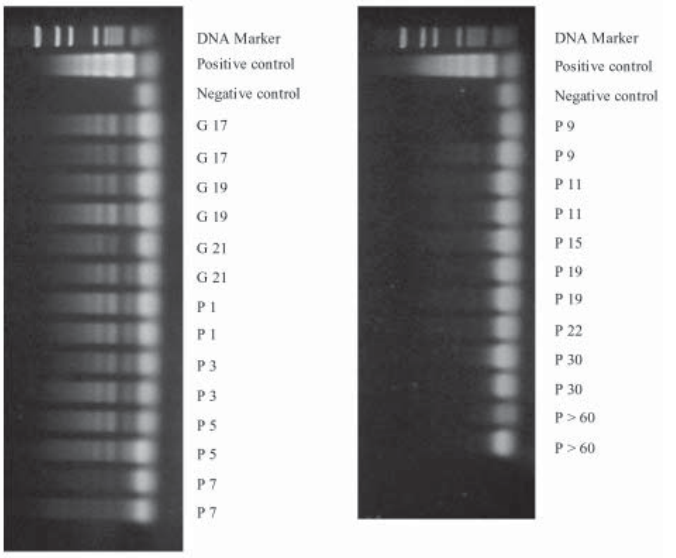

Figure 3. Ontogenetic variation in the apoptotic banding pattern of rat whole brain DNA. Assayed by use of 1.2% agarose electrophoresis.

in developing rat brain neocortex, brain stem, hippocampus, and cerebellum. That study indicated that, during the first two weeks after birth, apoptosis frequency decreased to a basic value except in the cerebellum where two peaks at about seven and 21 days after birth were seen. In another study (19) in control rats, we documented a specific peak in apoptotic activity in the cerebellum at day P21, compared with P11 and P90, whereas apoptotic activity in the hippocampus was about zero at P11, P21, and P90, in agreement with results seen by others (24).

The DNA-laddering in whole brain indicated intense apoptotic activity from G17 to P7, after which the activity decreased to low values until G30 where zero activity was observed (Fig. 3). However, with the applied techniques, it was not possible to obtain relative or absolute values for the intensity of DNA laddering. Therefore, it was not possible to specify maximal activity in the interval G17 to P7.

The developing brain is a heterogeneous organ with great regional variations in the stage of development. Therefore, investigations in the whole brain represent a mean value, in which great regional variations can be concealed. Anyway, we conclude that a striking similar pattern of age dependency exists in the whole brain between ODC activity (Fig. 2) and apoptotic changes as measured by use of LM-PCR (Fig. 3). There are clear detectable apoptotic changes in the whole brain from G17 until P7, paralleling the changes of ODC activity in the same period. From P7 on, both measures decreased.

These results indicate an intimate balance between two opposing processes at high activity during development, compared

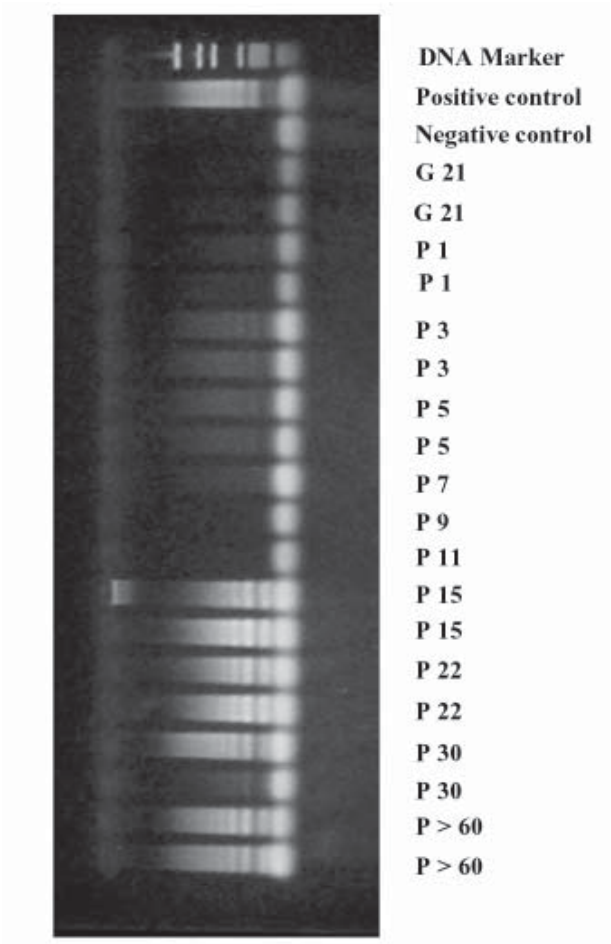

Figure 4. Ontogenetic variation in the apoptotic banding pattern of rat testis DNA. Assayed by use of 1.2% agarose electrophoresis.

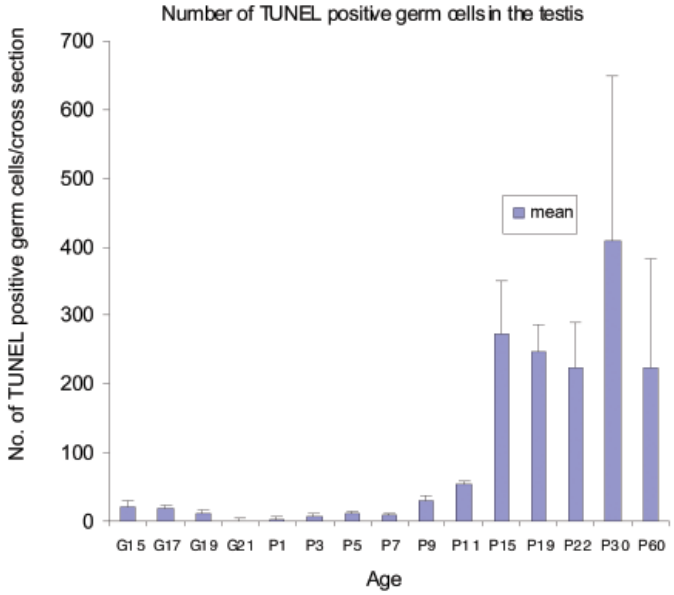

Figure 5. Ontogenetic variation in numbers of apoptotic germ cells in the seminiferous tubules of rat testis as scored by use of the Terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) technique. Data originate from the mean of TUNEL-positive germ cells in right and left testis per male rat. N = 3 to 6 male rats per age group. Data represent mean ± SD.

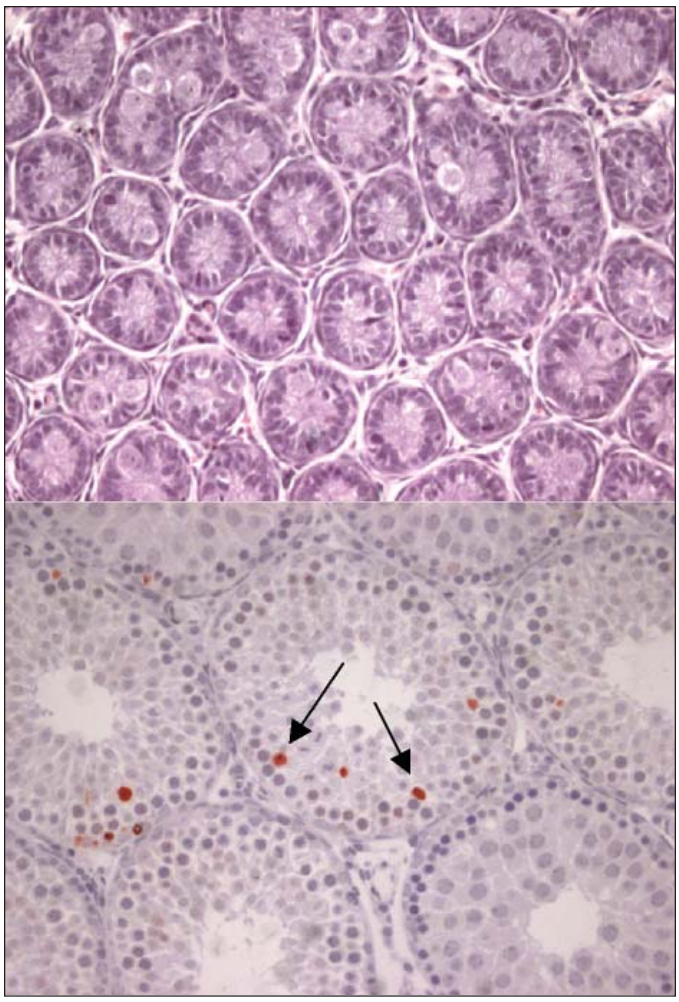

Figure 6. Seminiferous tubules in the testes of rats at postnatal day (P) 5 (upper part) and P30 (lower part) stained by use of the TUNEL technique. In the section corresponding to P5, no TUNEL-positive cells are seen, in contrast to several TUNEL-positive cells as seen in the P30 testis specimen (arrows). Counter stained with hematoxylin; magnification, 20×.

with the activity in mature individuals. Disturbance of any one of these parameters might result in disturbed development. A priori, vulnerability of organs to chemicals is supposed to be greatest when the activity is at its highest, as reported recently for the apoptotic effect of toluene specifically on cells in the granular cell layer of the cerebellum (19). The results of the study reported here suggest that measurements of the effect of chemicals on whole brain ODC activity and DNA laddering in the high activity interval for both parameters (i.e., from G15 to P7) may be used to study the effect of chemicals on whole brain development. Regional specificity can be increased by studying brain regions when regional developmental needs are greatest.

In the testes (Fig. 4), developmental apoptosis is weak from G21 until P15. However, from P15 to P60, there is a strong apoptotic DNA laddering banding pattern. Likewise, using the TUNEL-staining technique, we observed marked increase in the level of germ cell apoptosis in the testes from P15 on (Fig. 5). Data from one animal were responsible for this maximum at P30. This might be a clear outlier, but biological variation could not be excluded. In a previous study, germ cell apoptosis was

investigated in young rats between eight and 70 days of age (9). That study also indicated that the amount of germ cell apoptosis in male rats increases during the first spermatogenetic cycle, from P16 to P32. Our current results (Fig. 4-6) indicate that apoptosis is a natural physiologic process in the testes during spermatogenesis, as described (27). Any disturbance in the amount or the pattern of testes apoptosis, accelerated or delayed, may be an indication of affected testes development.

In conclusion, we propose to study developmental toxicity of perinatally administered chemicals in whole brain as ODC and apoptotic activities measured from G15 to P7, and in testes as germ cell apoptosis at the onset of spermatogenesis at P15.

Acknowledgments

We thank Karen Roswall, Ulla Baroudy, Vibeke Stenberg, and Annette Landin for their excellent technical work.

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