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Prenatal Exposure to Anti-HIV Drugs: Neurobehavioral Effects of Zidovudine (AZT) + Lamivudine (3TC) Treatment in Mice

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ABSTRACT

Background: The new antiretroviral treatments that combine the zidovudine (AZT) regimen with lamivudine (3TC) appear as a cost-effective alternative to the current AZT monotherapy to prevent mother-to-fetus transmission of the HIV-1 virus. Recent evidence in uninfected children raised concern about the long-term effects of perinatal exposure to AZT and 3TC, especially when used in combination. Animal studies indicated behavioral changes in offspring exposed perinatally to both AZT and 3TC, whereas no animal data are available on the effects of the perinatal exposure to the AZT + 3TC combination on neurodevelopment.

Methods: Pregnant CD-1 mice received p.o. AZT + 3TC (160 and 500 mg/kg, respectively) or vehicle solution (NaCl 0.9%) twice daily from gestational day 10 to delivery. Maternal reproductive endpoints such as pregnancy length, abortion, litter size, sex ratio, and offspring viability were assessed. Pups were scored for different somatic and behavioral endpoints, including sensorimotor development, homing performance on postnatal day (PND) 10, passive-avoidance testing (PND 22–23), locomotor activity (PND 23), and social interaction (PND 35).

Results: While no effects were observed on maternal reproductive endpoints, treated pups showed a long-lasting reduction of body weight and a slightly delayed maturation of placing and grasping reflexes and pole grasping. No effects on passive-avoidance or locomotor activity were found. AZT + 3TC-treated mice showed selective alterations in the social interaction test; the treated female offspring also displayed a significant reduction of affiliative interactions.

Conclusions: The combination of AZT and 3TC (1) induced small, but more marked, effects on somatic and sensorimotor development than either of these drugs administered separately; and (2) affected juvenile social behavior.

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INTRODUCTION

Mother-to-child transmission accounts for more than 90% of all human immunodeficiency virus (HIV) infections in infants and children. Vertical transmission rates vary geographically. While transmission rates in developing countries are 20–35%, in the United States and Western Europe the incidence of vertical infection has been reduced radically (5%) since the publication of the results by the AIDS Clinical Trials Group (ACTG) 076 (Leroy et al., '98). This study showed that, in the absence of breastfeeding, zidovudine (azidodeoxythymidine, AZT), as intensive monotherapy during gestation and labor, and given orally to babies for 6 weeks after birth, reduced vertical HIV transmission by two-thirds (Connor, '94). The efficacy of AZT in preventing vertical transmission led to public health guidelines that recommend the implementation of HIV counseling and testing practices, the use of the ACTG 076 AZT regimen, and the implementation of safe alternatives to breastfeeding (CDC, '97).

In view of the complexity and cost of such standard therapy, several multicenter clinical trials are under way to implement other antiretroviral treatment regimens equally effective at preventing vertical transmission but more affordable in developing countries (CDC, '98).

The results from a placebo-controlled trial started in Thailand in 1996 have recently shown that a shorter AZT regimen from the 36th week of gestation until delivery reduced the vertical transmission rate by 50%, in the absence of breastfeeding (Shaffer et al., '99). In 1996, the UNAIDS PETRA study was started in Africa to evaluate whether a more aggressive standard antiretroviral therapy could be safely and effectively adopted. The preliminary results of this study showed

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that the AZT + 3TC (dideoxythiacytidine) combination given from the 36th week of pregnancy and during labor and delivery produced a 50% reduction of the transmission rate. Furthermore, a shorter AZT + 3TC regimen starting during labor and followed by mother and newborn for 1 week reduced transmission to the infant by 37%, even in the presence of breastfeeding (Saba, '99).

The only reported adverse effect in infants exposed perinatally to AZT was a mild anemia, which disappeared after 12 weeks (Sperling, '96). However, the long-term effects of in utero AZT exposure in growing children and adults are still largely unknown. Recently, a follow-up study on uninfected children (age range 0–4 years), born to HIV-infected women exposed perinatally to AZT, did not show any adverse long-term effects on developmental/cognitive parameters (Culnane et al., '99).

The safety and pharmacokinetics of 3TC alone, or in combination with AZT, have been evaluated in phase I trials, indicating that 3TC does not produce toxic effects in either the fetus or the newborn (Johnson et al., '96; Moodley et al., '98). A study of a seropositive cohort of French women has recently reported preliminary results on the safety and efficacy of the AZT + 3TC combination given to women during pregnancy, and to babies after delivery; these results were compared with women receiving AZT monotherapy. The addition of 3TC to the AZT regimen further reduced the transmission rate (from 6.5% to 2.6%) (Blanche et al., '99a). Nevertheless, a recent study (Blanche et al., '99b) of the same cohort reported the presence of symptoms associated with mitochondrial dysfunction in eight exposed, but noninfected, infants. In two of the eight reported cases, convulsive episodes and death were described. In the light of these latest human data, a thorough analysis of the potential adverse effects of perinatal exposure to AZT or 3TC, or their combination, was recommended.

Animal models of prenatal AZT exposure have been used to study the potential toxic effects of this drug. These models suggest that AZT may produce mutagenic and carcinogenic effects in rodents (Ayers et al., '97; Olivero et al., '97; Zhang et al., '98), as well as some reproductive effects (Sikka et al., '91; Greene et al., '96) and alteration in neurodevelopment of rats (Petykò et al., '97; Applewhite-Black et al., '98; Busidan et al., '98, '99), mice (Taylor et al., '92; Calamandrei et al., '99a,b; Rondinini et al., '99), and monkeys (Ha et al., '94; '98).

Animal studies have failed to demonstrate teratological effects of prenatal 3TC exposure in rats and rabbits at doses 130- and 160-fold higher than the human dose, respectively (Minkoff and Augenbraun, '97). However, in a recent multidose study on short-, medium-, and long-term effects on neurobehavioral development of CD-1 mice, we observed some limited but significant effects of in utero 3TC exposure (Calamandrei et al., '99c, '00).

Our results, together with published data on AZT prenatal exposure in animal models, do not identify about the specific targets of the neurotoxic effects of AZT and 3TC. However, they do suggest that the exposure of the developing central nervous system to these drugs might induce minor alterations in some behavioral endpoints, especially those that involve cognitive processes (Ha et al., '94; Petikò et al., '97; Calamandrei et al., '99a,b), coping with a social stressor (Rondinini et al., '99), and locomotor activity levels (Calamandrei et al., '99c), as well as alterations in the response to pharmacological challenges such as scopolamine (Calamandrei et al., '00) or amphetamine (Applewhite-Black et al., '98).

The aim of the present study was to extend these findings by investigating some short- and medium-term effects of maternal exposure to AZT + 3TC in combination during the first 5 weeks of life in mice.

MATERIALS AND METHODS

Animals and breeding procedures

Male and female mice of an outbred Swiss-derived strain (CD-1), weighing 30–35 and 25–27 g, respectively, were purchased from a commercial breeder (Charles River, Calco, Italy). Upon arrival at the laboratory, the animals were housed in an air-conditioned room (temperature 21 ± 1°C, relative humidity 60 ± 10%) with lights on from 8 PM to 8 AM. Adult virgin males and females were housed in same sex pairs in 33 × 13 × 14 cm Plexiglas boxes with a metal top and sawdust as bedding. Pellet food (enriched standard diet purchased from Mucedola, Settimo Milanese, Italy) and tap water were available ad libitum. After 3 weeks of acclimatization, breeding pairs were formed. Females were inspected daily for the presence of a vaginal plug (pregnancy day 0). On gestational day (GD) 10 the studs were removed and 24 females were randomly assigned either to the control or the AZT + 3TC treatment group (n = 12 in each group).

Prenatal treatment

AZT and 3TC (both provided by Glaxo-Wellcome Research and Development Ltd., Middlesex, England) were dissolved in a 0.9% NaCl solution. Pregnant CD-1 mice were treated per os twice daily (between 8–9 AM and 7–8 PM) from GD 10 to delivery with either AZT + 3TC,, at the dose of 160 and 500 mg/kg, respectively (total daily dose 320 and 1,000 mg/kg/day), or saline vehicle. Time, route of administration, and doses were chosen on the basis of our previous studies (Calamandrei et al., '99a,b,c, '00). Specifically, the two doses selected were shown to induce behavioral changes, but no overt toxic effects on somatic growth.

Maternal measures and offspring assignment

Female body weight was recorded daily from GD 10 to delivery. To evaluate food intake, food containers were weighed every second day from GD 10 until delivery. Female body weight and food intake were mea-

sured to the nearest 0.01 g (Mettler PK-300 balance set to automatically compensate animal movements, Mettler Instrumente AG, Switzerland). Proportion of term pregnancies, gestation length, litter size, sex ratio, and neonatal mortality were also measured to assess potential effects on reproductive performance. In addition, the uterus was examined for sites of placental attachment, and the number of sites compared with litter size to reliably assess postnatal maternal cannibalism and/or embryo resorptions.

At birth, all litters were fostered to untreated dams of the same strain which had given birth to healthy litters within 24 hr. Each litter was culled to four males and four females at birth. Pups were weighed to the nearest 0.01 g (Mettler PK-300 balance set to automatically compensate animal movements, Mettler Instrumente AG, Switzerland) every other day from PND 2 to 18. Pups were weaned on PND 24, and the four males and four females of each litter were group housed separately by sex in cages identical to the home cage.

For postnatal assessment, special care was taken to assign animals with the same testing history to each of the following behavioral tests (see Alleva and Bignami, '86, p. 313, for a discussion on the potential interactions between handling and prenatal treatments), and in some cases this led to a reduction of the number of animals assigned to the different tests. Any additional reductions in the number of litters used in each behavioral test are due to missing data (e.g., the two genders were not equally represented in the litter, or some subjects were not used so as to avoid testing the animals beyond the time interval established for each behavioral test). One pup/sex/litter were assessed for somatic and sensorimotor development, and one pup/sex/litter were tested in the homing performance task. The remaining four pups/litter were assigned to the passive-avoidance task. The male and the female used in the no-shock, control condition in this test were also assigned for the locomotor activity testing.

Assessment of somatic and neurobehavioral development (PND 2–18)

Every other day from PND 2 to 18, one male and one female from each litter in each treatment group (Veh (0), n = 11; AZT + 3TC (160, 500 mg/kg), n = 12) were tested for somatic and neurobehavioral development.

Pups were weighed to the nearest 0.01 g (see above). Body and tail length were measured with a flexible ruler to the nearest 0.1 cm. Hair growth and days of eyelid and ear opening and incisor eruption were also recorded.

Pups were then assessed for a number of measures currently used in the study of sensorimotor ontogeny in mice according to a slightly modified Fox battery (Fox, '65; Alleva et al., '85; Calamandrei and Alleva, '89). The tests were conducted during the dark period between 9 AM and 2 PM under red light. Each subject was tested at approximately the same time of the day. The following reflexes and responses were scored:

Righting reflex: Pup turns over with all four feet on the ground when placed on its back.

Cliff aversion: Pup withdraws from the edge of a flat surface when its snout and forepaws are placed over the "cliff."

Forelimb and hindlimb stick grasp reflexes: pup grasps the shaft of a toothpick when the fore- or hind paw is stroked.

Forelimb and hind limb placing reflexes: Pup raises and places its fore- or hind paw on the surface or the edge of an object when stroked on the dorsum of the paw.

Level screen tests: Pup holds onto a wire mesh (5 × 5 mm) when it is dragged across it horizontally by the tail.

Screen climbing test: Pup climbs up the vertical screen using both fore- and hind paws. The full response for this test is attributed when the subject is able to reach the top of the vertical screen (10 × 10 cm), which usually takes about 5 sec.

Pole grasping: Pup is forced to hang from a wooden pencil with its forelimbs to measure his ability to hold on to a bar.

The following scores were used for each of the somatic and behavioral variables so far mentioned: 0 = no response; 1 = uncertain response; 2 = incomplete response; 3 = full response. Animals were timed on the righting reflex (0–3 sec = full response; 3–6 sec = incomplete response; 6–10 sec = uncertain response; more than 10 sec = no response), cliff aversion (0–3 sec = full response; 4–6 = incomplete response; more than 6 sec = no response, uncertain response was attributed when pup turned its snout to the flat surface but it is unable to withdraw) and pole grasping (more than 5 sec = full response; 2–5 sec = incomplete response; 0–2 sec = uncertain response; 0 sec = no response).

Homing test (PND 10)

One male and one female from each litter of each group (n = 10) were separated from the dam and kept for 30 min in an incubator (Elmed Ginevri 0GB 1000, Roma, Italy) at 28 ± 1°C. Individual pups were then transferred to a Plexiglas arena (36 × 22.5 × 10 cm) maintained at 28 ± 1°C, with the floor subdivided by black lines in 12 quadrants. Wood shavings from the home cage were evenly spread under the wire-mesh floor on one side of the arena (14 × 22.5 cm, goal arena) and the pup was placed close to the wall on the opposite side. The time required for each pup to place both forelimbs within the goal area was recorded (cut-off time 3 min). In addition, the pup's overall activity was measured by counting the number of quadrants entered during the 3-min test period.

Passive-avoidance acquisition and retention (PND 22–23)

Apparatus. The passive-avoidance (PA) apparatus (Cat. No. 7550, Ugo Basile, Comerio, Italy, modified to

increase the distance between the light source and the apparatus floor; see below) consisted of a Plexiglas cage with tilting floor (5-degree angle at maximal excursion), divided into two compartments (18 × 9.5 × 16 cm each) by a partition with a sliding door, and connected to a programming-recording unit. One of the compartments (start) had white walls and no ceiling and was brightly illuminated by a 60-W bulb located 40 cm above the floor. The other compartment (escape) had black walls and ceiling and was not illuminated. The sliding door between the two compartments was made of two panels of low-density cellular PVC. The tilting floor consisted of 40 bars of stainless steel (3-mm diameter) spaced 12 mm apart and connected to a source of scrambled shock. Avoidance tests were performed between 9 AM and 2 PM, that is, during the initial portion of the dark period. The procedure consisted of two phases, acquisition and retention, which took place on subsequent 2 days.

Acquisition. On PND 22, two male and two female mice from each of the ten litters in each prenatal treatment group (n = 10) underwent a multitrial PA acquisition session. One male and one female were assigned to the conditioned group, while the remaining littermates were assigned to the nonreinforced control group. In the conditioned group, the acquisition session consisted of a maximum of ten trials, each started by gently placing the subject in the start compartment; this produced the immediate opening of the sliding door between the two compartments. A trial ended when the mouse stepped with all four paws into the escape compartment, which caused the immediate closure of the sliding door, or after 120 sec had elapsed without such a response. Each step-through response was punished by a mild foot shock (3 sec, 0.4 mA nominal intensity). At the end of each trial, the subject was immediately returned to the home cage for a 60-sec intertrial interval. The session was terminated either after two consecutive trials without a crossing response within 120 sec, or after 10 trials without meeting this acquisition criterion. Mice in the nonreinforced condition were subjected to the same procedure, but received no punishment (shock) for the step-through responses and were given a fixed number of 10 trials. This control group allows one to assess potential response changes due to repeated apparatus exposure per se (habituation).

Retention. The retention session took place on PND 23. The procedure was identical for conditioned and nonreinforced pups and consisted of one trial not punished by foot shock. The retention trial ended when the animal either entered the escape compartment or had remained in the start compartment for 120 sec.

Locomotor activity (PND 23)

On PND 23, one female and one male from each group, previously assigned to the nonreinforced control group of the passive-avoidance test (Veh (0), n = 9; AZT + 3TC, n = 9), were transferred to the experimental room between 12 AM and 3 PM. After 30 min of acclimatization, mice were individually introduced for 20 min into a cleaned cage identical to the home cage but without bedding. The cage was placed on a Varimex Activity Meter apparatus (Columbus Instruments, Columbus, OH) set at a standard level, which automatically counted every horizontal movement (= 1 cm), with movements summarized every 5 min, so as to obtain four counts during each test (Bignami et al., '85, Petruzzi et al., '95).

Social interaction (PND 35)

On PND 35 (juvenile stage), one male and one female from different litters in each group (Veh (0), n = 10; AZT + 3TC, n = 10) underwent a 20-min social encounter with another animal of the same sex and group, but from a different litter (design and procedures as in Terranova et al., '93). Immediately before the beginning of the encounter, they were marked for individual recognition with a nontoxic, odorless, permanent marker. Each encounter took place between 10 AM and 3 PM in a test cage identical to the home cage supplied with clean sawdust bedding.

Behavior was videotaped under red light. Recordings were scored by an observer who was blind to the treatment history of each pair. The data were recorded using a keyboard event recorder system (Noldus, '91) connected to a computer for data collection. Separate scores were obtained for each individual in the cage by running the tapes twice; as the two values, however, were not statistically independent, means of the two subjects were used as the statistical unit (pair) for the analyses.

The behaviors scored and their classification into two main groups (nonsocial and social) are based principally on the ethological profile of mouse behavior described by Grant and Mackintosh ('63) and Van Oortmerssen ('71) (for previous use in our laboratory, see Terranova et al., '93).

Nonsocial behaviors were the following:

Exploring: moving around the cage, rearing, sniffing the air, the walls or the sawdust
Digging: digging in the sawdust, pushing and kicking it around using the snout and/or both the forepaws and hindpaws, mostly moving around the cage and sometimes changing the whole arrangement of the substrate material
Self-grooming: wiping, licking, combing, or scratching any part of own body

Social behaviors were the following:

Social investigation: sniffing the anogenital region, the head, or the snout of the partner
Follow: following the partner around the cage, without any quick or sudden movement
Squire: following the moving partner while maintaining a constant nose contact with its fur (mostly near the anogenital area)
Mutual circle: partners mutually sniffing each other's anogenital region, while describing tight cir-

cles with their reciprocal following movements and maintaining close nose-anogenital contact

Social inactivity: lying flat or standing still (with eyes closed or open) while maintaining close physical contact with the partner

Allogrooming: self-explanatory

Push under: pushing the snout or the whole anterior part of the body under the partner's body, and then resting

Crawl over: crawling over the partner's back, crossing it transversally from one side to the other

Another behavioral category was established:

Aggressive behavior: including behavioral items such as fight (rushing approach carried on over the back of the partner, often accompanied by biting attempts) and aggressive grooming (allogrooming markedly intense and persistent, performed leaning on the partner's back with the forepaws, and accompanied by gross movements of the head of the attacker and by vigorous pulling of the fur of the partner with a marked involvement of the teeth)

Statistical analysis

Each of the statistical analyses was performed by BMDP statistical software (version BMDP/dynamic 7.0, Berkeley, CA). Analysis of variance (ANOVA) was performed on dam body weight, postnatal body weight, body and tail length of pups, locomotor activity, and social interaction data. The model of such analysis included: a between-litter treatment factor (2 levels); litter as random factor nested within treatment and as block with respect to sex and repeated measures; sex as fixed effect factor within-litter; repeated measures as fixed factor within-subjects. Obviously, the sex variable was not considered when analyzing maternal reproductive endpoints (Edwards '85; Chiarotti, '87).

Passive-avoidance and homing data were subjected to nonparametric tests due to the presence of cut-off values. Specifically, the Mann-Whitney U-test (Marascuilo and McSweeney, '77) was applied to evaluate the main effect of treatment, the treatment × sex interaction and, in the case of passive-avoidance data, to evaluate the treatment × condition interaction and the treatment × sex × condition interaction. The Wilcoxon test (Marascuilo and McSweeney, '77) was also applied to evaluate the main effects of sex and training condition within each group.

For somatic and neurobehavioral development data ["Fox scale"; see Bignami ('96)], the original response variables (indicating the developmental level achieved by each pup in each task in the 20 consecutive days of observation) were transformed, considering the first day of adult-like response or full appearance of a somatic feature, choosing, for each response variable, the score (2 or 3). An adult-like response was based on the developmental profile expected in nontreated animals. The resulting new variables were subjected to nonparametric tests, namely the Mann-Whitney U-test, to assess the main effect of treatment and any treatment × sex interaction, and the Wilcoxon test to assess the main effect of sex. The Mann-Whitney U-test was also performed on body weight values recorded on the first day of adult-like response for each sensorimotor response scored. This analysis was applied to assess the potential influence of body weight on the maturation of each of the nine sensorimotor responses scored.

Location estimates or measures of variability were mean and standard error of the mean for parametric data, and median and first and third interquartile for nonparametric data.

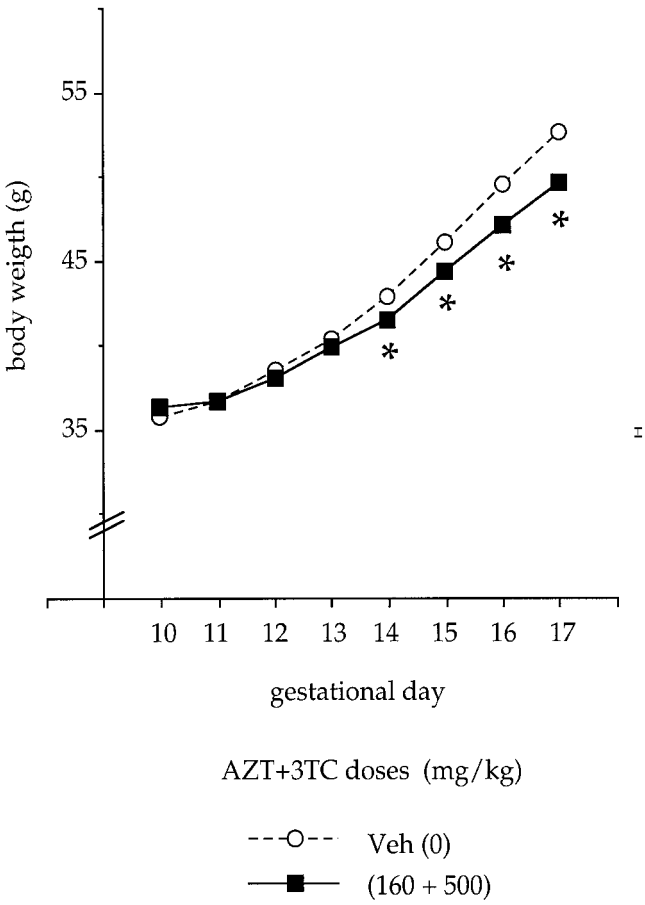


Fig. 1. Body weight of pregnant CD-1 females during zidovudine + lamivudine (AZT + 3TC) exposure. *Significant difference between AZT + 3TC females and controls ($P < 0.01$ by post hoc). Vertical bars to the right indicate pooled SEM; n = 12 in each group.

RESULTS

Effects on maternal reproductive endpoints

No main treatment effect was found on body weight from GD10 to GD17 for pregnant females. However, a significant treatment × day interaction [$F(7, 140) = 7.35$; $P = 0.0038$] indicated a reduced weight from GD 15 onward in the AZT + 3TC group as compared with the vehicle-treated dams ($P < 0.01$ after post hoc comparisons) (Fig. 1). This effect on weight was not accom-

TABLE 1. Reproductive performance data of CD-1 females receiving AZT + 3TC combination treatment from GD 10 to delivery

	AZT + 3TC doses (mg/kg)	
	Vehicle (0)	(160 + 500)
Pregnancy length	17.5 (0.5)	18.0 (0.5)
Litter size	12.25 (0.86)	10.75 (0.85)
Placental attachement sites	13.0 (0.78)	12.75 (0.56)
Sex ratio (no. of males/no. of females)	1.18	0.90

Data are mean ± SEM.
Vehicle (0) n = 8; AZT + 3TC (160 + 500 mg/kg) n = 12.

panied by a decrease in food intake during pregnancy in AZT + 3TC-treated females.

No effect of AZT + 3TC treatment was found on maternal reproductive performance. Specifically, gestation length, litter size, offspring sex ratio, and viability were unaffected by treatment. Furthermore, there were no differences between the number of sites of placental attachment or litter sizes in the two groups, indicating that AZT + 3TC combination did not induce embryo lethality at the doses used in the present study (Table 1).

Postnatal somatic and neurobehavioral development

AZT + 3TC treatment affected offspring somatic development throughout the 5-week period of observation, inducing a significant reduction in several measures of growth (Fig. 2).

The ANOVA on offspring body weight revealed a treatment effect [$F(1, 20) = 45.27$; $P < 0.001$] and an effect of postnatal day [$F(8, 160) = 2549.49$ $P < 0.001$] as well as significant interaction of treatment × postnatal day [$F(8, 160) = 16.27$ $P < 0.001$]. Post hoc comparisons showed a significant delay in body weight in the AZT + 3TC group on every postnatal day measured ($P < 0.001$).

Body length was significantly reduced by prenatal exposure to AZT + 3TC [$F(1, 20) = 15.56$ $P = 0.0008$]. An effect of postnatal day [$F(8, 160) = 579.64$ $P < 0.001$] and a significant interaction of treatment × postnatal day [$F(8, 160) = 2.0$ $P < 0.0496$] were also found; post hoc comparisons revealed a reduced increase of body length in AZT + 3TC pups on every postnatal day considered ($P < 0.001$).

The extent of treatment effect on weight and body length varied over postnatal days (Fig. 2), and this explains the significant interaction treatment × day found in both parameters.

A main effect of treatment [$F(1, 20) = 5.41$ $P = 0.0306$], as well as a main effect of postnatal day [$F(8, 160) = 1794.18$ $P < 0.001$] were also found for tail length. A significant interaction of treatment × postnatal day [$F(8, 160) = 3.32$ $P = 0.0015$] was also found, and post hoc comparisons revealed a significant delay in tail growth from postnatal day 6 onward ($P < 0.001$) in treated pups.

TABLE 2. Latency time to reach the nest-scent area on PND 10 and number of crossings in a 180-sec test

	AZT + 3TC doses (mg/kg)	
	Vehicle (0)	(160 + 500)
Latency	161.75 (180.0; 50.12)	98.5 (150.0; 58.5)
Crossings	10.0 (15.75; 6.37)	10.0 (21.5; 8.5)

Data are median and interquartile range (Q3; Q1).
Vehicle (0) n = 10; AZT + 3TC (160 + 500 mg/kg) n = 11.

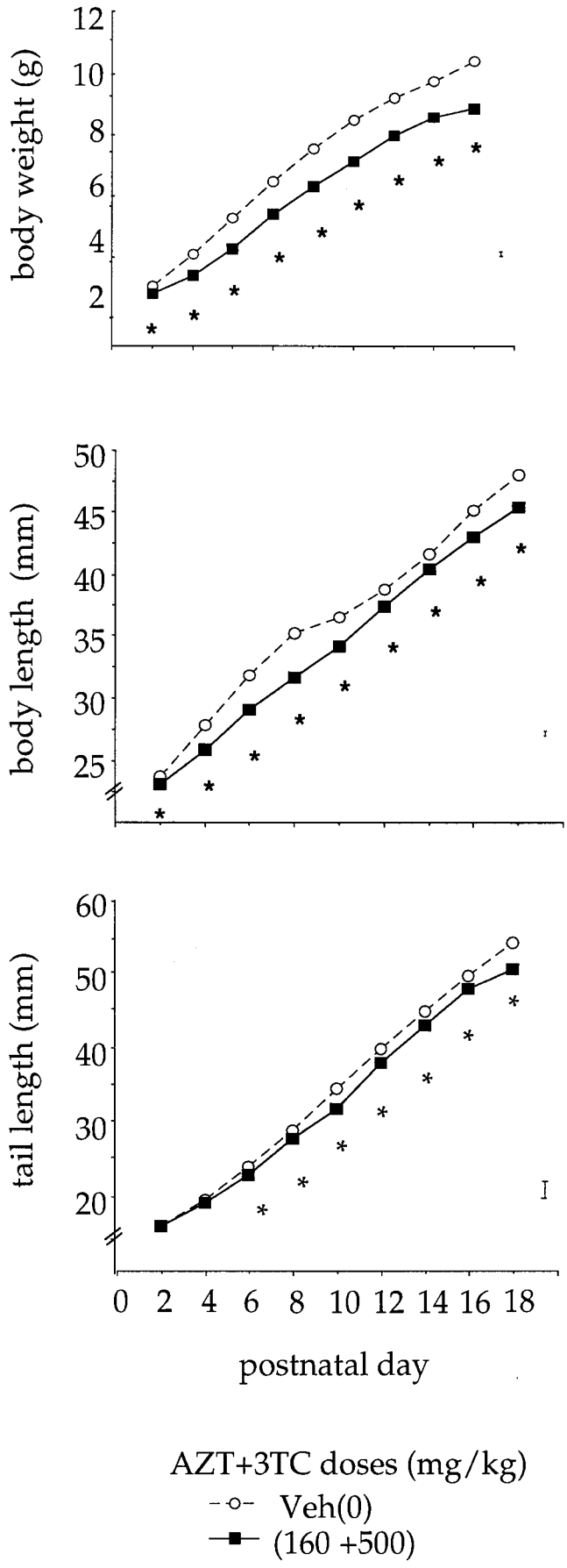
As for eye and ear opening and incisor eruption, they were not affected by the prenatal treatment (Fig. 3). Neither a main effect of sex nor an interaction of sex with treatment or postnatal day was found.

Nonparametric analyses of somatic and neurobehavioral development parameters showed a delaying effect of AZT + 3TC on the maturation of some specific responses (Fig. 3). An effect of treatment was evident for hair growth [$U = 38.5$; $P = 0.0482$], forelimb placing [$U = 25.5$; $P = 0.0095$], forelimb stick grasping [$U = 35.0$; $P = 0.05$], level screen [$U = 24.5$; $P = 0.0093$] and pole grasping [$U = 33.0$; $P = 0.0038$]. Neither an interaction between treatment and sex nor a main effect of sex was found.

To evaluate whether the delay in sensorimotor responses was a consequence of the marked reduction of body weight observed in combination-treated pups, we applied Mann-Whitney's test to individual body weight values recorded on the first day of adult-like response for each of the sensorimotor responses scored. If the maturation of a given sensorimotor response is attained only when each offspring reaches a certain body weight threshold value, body weights of combination-treated pups should not differ significantly from those of vehicle-treated pups when an adult-like response is achieved. Results indicated that on the first day a mature response appeared, body weights of AZT + 3TC pups were significantly lower than those of vehicle pups for seven of the nine responses scored (righting $U = 93.0$; $P = 0.0328$; cliff aversion $U = 92.0$; $P = 0.0383$; forelimb placing $U = 75.0$; $P = 0.3405$; hindlimb placing $U = 98.5$; $P = 0.0453$; forelimb grasping $U = 89.0$; $P = 0.0542$; hindlimb grasping $U = 97.5$; $P = 0.0150$; pole grasping $U = 100.5$; $P = 0.0086$; level screen $U = 76.5$; $P = 0.2930$; vertical screen $U = 102.5$; $P = 0.0058$). Thus, the body weights in the two groups were similar at mature response achievement only in the case of forelimb placing and level screen test.

Homing test (PND 10)

No significant differences between vehicle and AZT + 3TC-treated groups were observed in homing performance assessed on PND 10, although there was an apparently shorter latency to reach the nest-scent area of the arena in treated animals compared with the vehicle controls (Table 2).



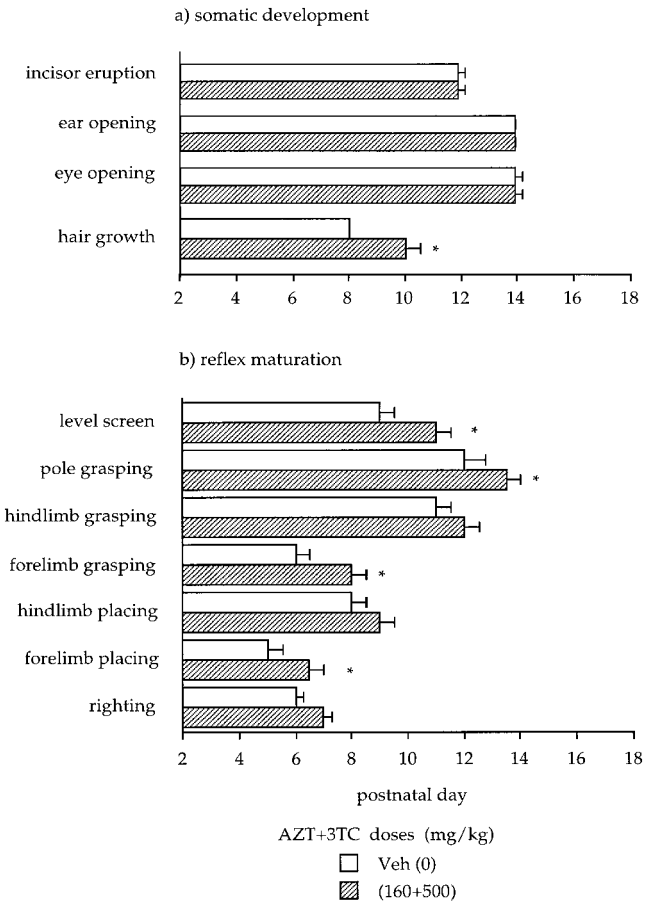


Fig. 3. Somatic (**a**) and neurobehavioral development (**b**) for pups prenatally exposed to zidovudine + lamivudine (AZT + 3TC) or vehicle. Data represent the median first day of the adult-like response on the basis of the developmental profile scored from postnatal day (PND) 2 to18. *Significant difference between the AZT + 3TC pups and controls ($P < 0.01$). Vehicle, n = 11; AZT + 3TC, n = 12.

Passive-avoidance (PND 22–23)

No main effect of AZT + 3TC treatment was found on acquisition and retention performance in the PA task. During the acquisition session, a significant main effect of training condition (conditioned vs nonreinforced mice) was found in all treatment groups (saline vehicle: Wilcoxon = 0.0; $P = 0.0039$; AZT + 3TC: Wilcoxon = 1.0; $P = 0.0039$), indicating that pups acquired the avoidance response regardless of the prenatal treatment received.

In the retest session, a main effect of training condition also was found in both groups (saline vehicle: Wilcoxon = 0.0; $P = 0.0039$; AZT + 3TC: Wilcoxon =

Fig. 2. Body weight, and body and tail growth (from postnatal day [PND] 2 to PND 18) of pups exposed prenatally to zidovudine + lamivudine (AZT + 3TC)or saline (vehicle) starting from GD 10 to delivery. A main effect of treatment was showed for body weight and body length ($P < 0.001$ in both of them). *Significant difference for tail length between AZT + 3TC dams and controls ($P < 0.01$ by post hoc; see Results for details) Vertical bars to the right indicate pooled SEM. Vehicle, n = 11; AZT + 3TC, n = 12.

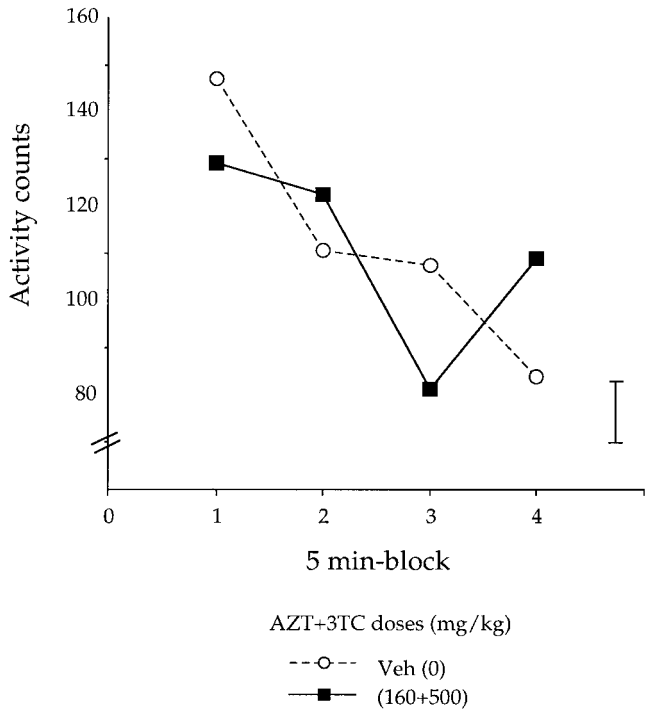


Fig. 4. Activity counts during a 20-min session (four 5-min blocks) in a Varimex apparatus on postnatal day (PND) 23. Male and female data are pooled. Vertical bars to the right indicate pooled SEM; n = 9 in each group.

0.0; $P = 0.0039$), which provides evidence of effective 24-hr retention in both AZT + 3TC and vehicle-treated mice.

Neither a significant main effect of sex nor an interaction between condition × sex was found during acquisition or retest phases of this task.

On PND 22, AZT + 3TC mice weighed significantly less than controls (mean values: AZT + 3TC: males = 12.44 (±0.38), females = 11.33 (±0.37); saline vehicle: males = 14.60 (±0.38), females = 13.4 (±0.28), as indicated by ANOVA performed on body weight [$F(1,18) = 30.98$ $P < 0.001$].

Locomotor activity (PND 23)

The ANOVA did not show a significant main effect of treatment or sex on locomotor activity. The control group showed the expected response decrement during the 20-min session and a response decrement occurred during the first three time blocks in the treated group (Fig. 4). An apparent increase activity during the last 5-min block of the test by AZT + 3TC mice was not statistically significant. There was no significant interaction between treatment and 5-min blocks.

Social interaction (PND 35)

Statistical analyses indicated that only some behavioral parameters of the social interaction repertoire were affected by AZT + 3TC treatment. In the nonsocial response grouping, AZT + 3TC-treated mice devoted less time to digging over the entire session [main effect of treatment: $F(1, 18) = 3.71$ $P = 0.07$] (Fig. 5). In the social response grouping, there was a difference in social investigation behavior in treated and control mice. AZT + 3TC mice displayed a lesser propensity to approach the social mate, as indicated by the higher latency to perform the first social investigation event [main effect of treatment: $F(1, 18) = 3.99$; $P = 0.06$] (Table 3). This difference was confirmed, as significantly less time was spent in social investigation by the AZT + 3TC mice during the first 5-min block [treatment × 5-min block interaction: $F(3, 54) = 3.62$; $P = 0.0186$] (Fig. 5).

Other items scored (i.e., squire, follow, push under, crawl over, mutual circle, and allogrooming) showed a mean frequency that reached the maximum value of 1. Thus, we applied a Fisher two-tailed test for data from each sex to evaluate differences in percentage of occurrence of each item in the two groups. A significantly lower percentage of mutual circling (Veh (0) 50%, AZT + 3TC 0%; $P = 0.0325$) and allogrooming (Veh (0) 80%, AZT + 3TC 10%; $P = 0.0055$) was shown by AZT + 3TC females, while no significant differences were found for males.

On PND 35 AZT + 3TC mice weighed significantly less than controls (mean values: AZT + 3TC: males = 26.95 (±1.11), females = 21.7 (±0.62); saline vehicle: males = 29.5 (±0.54), females = 23.5 (±0.41), as indicated by ANOVA performed on body weight [$F(1,18) = 5.68$ $P = 0.028$].

DISCUSSION

Prenatal exposure to the combined maternal treatment with the antiretroviral AZT and 3TC drugs resulted in significant, though minor, alterations in neurobehavioral development of CD-1 mice. These effects confirm and extend our previous results separately regarding in utero exposure to either AZT or 3TC (Calamandrei et al., '99a,b,c; Calamandrei et al., '00).

Gestational AZT + 3TC exposure did not appear to affect maternal reproductive endpoints, namely, duration of pregnancy, proportion of pregnancies carried to term, litter size, sex ratio, litter viability, although reduced weights were observed in the AZT + 3TC-treated dams. The combined treatment mainly affected mainly the early development of prenatally exposed mice, delaying somatic growth during the first 19 days of life, and maturation of several sensorimotor reflexes by one or two days with respect to control animals. Some selected alterations of social behavior of AZT + 3TC-exposed mice were also observed in juvenile animal at PND 35, namely a reduced propensity in both sexes to interact with a social mate of the same sex, and a lower occurrence of investigative and affiliative behavior in females (Table 4).

The combined treatment affected overall body growth, including body and tail length, whereas this dose of AZT given alone was found to affect only weight and this dose of 3TC alone had very limited effects on somatic development (Calamandrei et al., '99b,c). Spe-

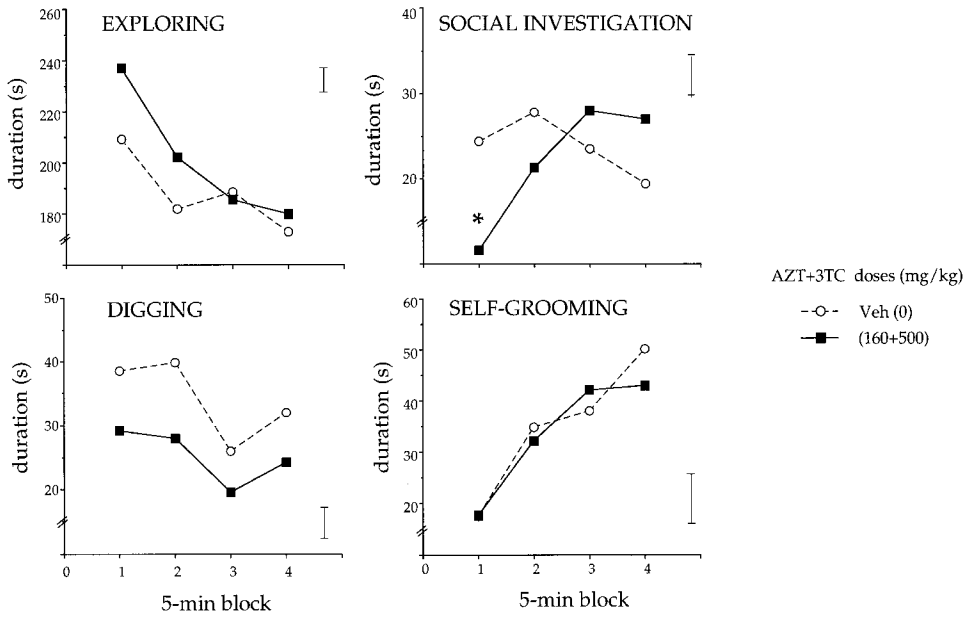


Fig. 5. Mean duration of selected social and nonsocial behavioral items (see Materials and Methods for definition). Pairs of the same sex were observed for 20 min on postnatal day (PND) 35. *Significant difference between groups ($P < 0.05$ by post hoc). The vertical bars to the right indicate pooled SEM; n = 10 in each group.

TABLE 3. Latency time to the first social investigation in a 20-min test of social interaction between pair of the same sex on PND 35

	AZT + 3TC doses (mg/kg)	
	Vehicle (0)	(160 + 500)
Males	39.9 (± 14.75)	77.7 (± 24.46)
Females	17.03 (± 6.2)	42.8 (± 10.58)

Data are mean ± SEM; n = 10 for each treatment group. See Results for more details.

cifically, in the present study AZT + 3TC reduced body weight by 20% during the preweaning phase, while a milder decrease in this same endpoint (around 8%) was observed after prenatal administration of AZT alone (Calamandrei et al., '99b). Furthermore, AZT + 3TC combination delayed more developmental milestones of reflex ontogeny than did AZT or 3TC when given separately. Thus, it seems that AZT and 3TC affected the early phase of postnatal development in a synergistic manner. An overall slight, but statistically significant, delaying in neurobehavioral development was, indeed, evident in AZT + 3TC pups, reaching critical levels for several of the developmental milestones considered. In particular, the onset of forelimb reflexes such as placing and grasping as well as the onset of the level screen response and pole grasping behavior were significantly delayed in treated pups. It should be noted that the responses affected by the prenatal treatment were those related to grip strength, whereas neither the righting reflex nor the general locomotor activity (as measured in the homing test and, later on, in the Varimex apparatus) were impaired. Such a profile suggests a pure weight decrement explanation for our behavioral findings is unlikely. Furthermore, the analysis of body weights on the first day of appearance of adult-like sensorimotor responses suggests that the maturation of most of the endpoints considered (7 out of 9) was not significantly affected by delayed growth. Actually, several behavioral teratology experiments have furnished evidence of a dissociation between growth and functional effects, such as those on behavior. Modest behavioral effects have been seen even in the presence of marked weight changes (Vorhees et al., '79). Although it cannot be excluded that the significant delay in somatic growth may have indirectly influenced the maturation of some sensorimotor reflexes, other possible explanations should be considered, such as a specific effect of AZT + 3TC on muscular function (see also Fox, '65, for a discussion of deficiency in pole grasping and muscular weakness). Myopathies have been reported among the adverse effects of chronic exposure to AZT and other nucleoside analogues (Dalakas et al., '90; Arnaudo et al., '91), likely resulting from the interference of these agents with mitochondrial DNA replication (Brinkman et al., '98). Although other organs and tissues are likely to be affected, those tissues with the highest energy requirement, such as nervous and muscular tissues, might be expected to be more vulnerable to inefficient mitochondrial function during critical developmental phases (Brinkman et al., '98). The dissociation between somatic and behavioral effects of the combined treatment is consistent with the results regarding the spontaneous locomotor activity test on PND 21. Although at that age, the weight of AZT + 3TC-exposed offspring was still significantly lower than that of controls, both groups exhibited comparable levels of activity. A slight difference between the two groups was, however, noted in the habituation profile, as an unusual increase in activity counts was observed in the last 5-min block for AZT + 3TC mice. This trend, though not significant, was in agreement with the effects reported for male mouse offspring ex-

TABLE 4. Summary of behavioral and somatic endpoints assessed in developing mice prenatally exposed to AZT + 3TC combination

Day of testing			
PND 2–19	PND 10	PND 22–23	PND 35
Somatic endpoints: reduction of body growth, delayed hair growth	Homing test: no effect	Passive avoidance: no effect	Social interaction: higher latency to perform the first social investigation; less time spent in social investigation
Neurobehavioral endpoints: delayed maturation of forelimb placing, forelimb grasping, level screen, screen climbing, pole grasping		Locomotor activity: no effect	Only females: lower percentage of occurrence of affiliative behaviors (mutual circle, allogrooming)

AZT + 3TC, azidodeoxythymidine (zidovudine) + dideoxythiacytidine (lamivudine); PND, postnatal day.

posed prenatally to 500 mg/kg 3TC only. These mice also decreased habituation in the same test on PND 22 (Calamandrei et al., '99c).

AZT + 3TC exposure did not affect acquisition or retention capabilities in the passive-avoidance task on PND 22–23. In our previous studies on prenatal exposure to AZT (Calamandrei et al., '99a,b), a slight impairment in acquisition rate was found both on PND 14–15 and PND 22–23. However, no effects on performance parameters in this task were observed after prenatal 3TC exposure (Calamandrei et al., '99c). The fact that the present study failed to detect AZT + 3TC effects on passive-avoidance performance does not necessarily lead to the conclusion that this combined treatment does not disrupt learning/associative functions. Studies in primates and rodents (Ha et al., '94; Petykò et al., '97) have shown alterations in some learning paradigms following prenatal AZT exposure. The possibility that the combination of the two drugs might have triggered compensatory mechanisms as to mask the effects of AZT in this specific behavioral task also should be carefully considered. Thus, further investigations using additional dose combinations of AZT + 3TC, as well as other learning paradigms, appear desirable.

The social behavior repertoire analyzed at periadolescent age (PND 35) suggested that prenatal treatment mainly altered the investigative components of social interaction: the motivation to approach the social mate (evaluated as latency to the first sniffing directed to the pair-partner) was lower in AZT + 3TC mice (95% and 147% lower in males and female, respectively). A decrease was also found for the time initially devoted to exploring and sniffing the partner. Furthermore, AZT + 3TC treatment significantly depressed both the investigative (mutual circle) and affiliative (allogrooming) components of social behavior in female offspring. In rats, periadolescence is reported as a critical stage of social behavior development, characterized by an increased propensity to social interactions (Spear and Brake '83). This peak level of social behavior also has been observed in 30-day-old mice (Dyer and Southwick, '74) and largely described by Terranova et al. ('93). Gender-related differences in social interactions consisting in a higher sociality of females were reported both in adult (Mackintosh, '81) and juvenile mice (Terranova et al., '93). Our results suggest that the combined antiretroviral therapy could have affected the ontogeny of social behavior, altering the motivation to interact with a novel social mate and dampening the sexually-dimorphic differences typical of the social behavior repertoire, either by a direct toxic effect during the prenatal period or as a consequence of the impaired postnatal growth of exposed animals. The effects of the combined treatment on social behavior are in line with those previously reported for prenatal AZT exposure. We have shown that sexual differences in investigative behavior tended to be dampened in juvenile mice prenatally exposed to AZT (Calamandrei et al., '99b), and a slight increase in aggressive behavior was displayed by AZT-treated male mice in an agonistic encounter at adulthood (Rondinini et al. '99). By contrast, prenatal 3TC exposure did not affect social behavior (Calamandrei et al., '99c). Thus, the reduced investigative behavior in AZT + 3TC-treated offspring, as well as the diminished affiliative behavior in female offspring, might be ascribed mainly to an AZT effect on the development of sex-dimorphic behavioral patterns, likely by an interference with neurohormonal regulation mechanisms during critical developmental phases (Gogu et al., '92). We are currently studying AZT + 3TC effects on adult behavioral patterns, such as intermale agonistic behavior and maternal behavior, in order to establish whether the alterations shown in the periadolescent age have had repercussions on typical sex-dimorphic behaviors at the adult stage.

In conclusion, this study represents a first step in the assessment of potential adverse effects on neurobehavioral development of prenatal exposure to the AZT + 3TC combinations. Although the effects on somatic growth may confound mechanisms for behavioral effects during the early postnatal life in mice, these data appear relevant in consideration of the recent report published by Blanche and colleagues ('99b), in which eight uninfected children exposed prenatally to AZT, or AZT + 3TC developed neurological anomalies and showed evidence of mitochondrial disorders in different target tissues. Although the assessment of mitochondrial toxicity of antiretroviral drugs was beyond the scope of the present study, the analysis of biomarkers

of mitochondrial function in exposed animals may help to clarify the mechanisms by which these drugs exert their effects on neural and behavioral development. In addition, further multidose animal studies aimed at evaluating the dose-response effects of prenatal exposure to the AZT + 3TC combination appears advisable to delineate overt effects on somatic growth and more subtle effects on behavior.

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