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Effects of prenatal AZT+3TC treatment on open field behavior and responsiveness to scopolamine in adult mice

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Abstract

Treatment of pregnant seropositive women and their neonates with the nucleoside analogs (reverse transcriptase inhibitors) zidovudine (AZT), lamivudine (3TC) and their combination has become a standard of care in industrialized countries to prevent transmission of the HIV-1 virus. Animal studies indicated limited but significant behavioral changes in AZT or 3TC-prenatally exposed offspring, whereas data on the potential neurobehavioral outcomes of AZT + 3TC combination are still lacking. The aim of the present study was to assess in mice prenatally exposed to AZT + 3TC the functional state of cholinergic muscarinic neuroregulation at adulthood. Pregnant CD-1 mice received per orem twice daily AZT + 3TC (160 and 500 mg/kg, respectively) or vehicle solution (NaCl 0.9%) from gestational day (GD) 10 to delivery (GD 19). Locomotor activity, exploratory behavior and responsiveness to the muscarinic cholinergic blocker scopolamine (2 mg/kg) were analyzed at adulthood (PND 70) in offspring of both sexes in an open field test. Results indicated that prenatal AZT + 3TC exposure does not influence responsiveness to the muscarinic cholinergic antagonist as measured by analysis of the drug's effects on locomotor and exploratory activity and different behavioral items. However, AZT + 3TC-treated mice displayed higher frequency of rearing, and lower frequency and duration of self-grooming behavior, consistent with an effect on dopaminergic neurotransmission. However, this would need confirmatory experiments.

Keywords: Zidovudine; Lamivudine; Cholinergic system; Scopolamine; Rearing; Grooming; Dopaminergic system

1. Introduction

Mother-to-child transmission near the time of birth is the primary route of HIV-1 infection among infants and young children worldwide. In 1994, the Pediatric AIDS Clinical Trials Group (PACTG) protocol 076 demonstrated that the risk of mother-to-child transmission could be reduced by nearly 70% following treatment of seropositive pregnant women and their newborns with zidovudine (AZT) [15,43]. Thus, since 1994, the prophylactic regimen with AZT has been incorporated into general clinical practice in USA and Europe, and this has successfully decreased transmission rate from 25% to 6–9%. In industrialized countries standard protocols of care for pregnant seropositive women and their newborns always include AZT, more often alone or in combination with other antiretroviral nucleoside analogs, mainly lamivudine (3TC) [13,19,20,22,28,32,35]. Despite the dramatic increase in the prenatal use of antiretroviral drugs, in particular of AZT, that has occurred, information about the long-term consequences of these agents on the fetus is limited.

Toxic effects of chronic treatment with AZT and other nucleoside analogs are well documented both in humans and laboratory animals. They include bone marrow toxicity, myopathies and peripheral neuropathies as well as effects on the reproductive and immune system [7,31]. Mitochondrial toxicity is reportedly responsible for most of the adverse effects of antiretroviral nucleoside analogs, since these agents can interfere with mitochondrial replication and function [7].

As for the effects of developmental exposure to antiretrovirals, data mostly concern the effects of AZT monotherapy in animal models [4,21,23,34,42,52], whereas little data are available regarding the pharmacokinetics and safety of antiretroviral agents other than AZT during gestation. In children, observational studies have not revealed excess

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malformations or major abnormalities after prenatal AZT exposure [16]. Yet, AZT induces in prenatally treated newborns a marked though transient anemia, likely caused by blocking the maturation and survival of early erythroblasts. 3TC has been reported to have a lower toxicity than AZT [13]. A very recent report has found alterations in brain morphology, neurological anomalies and evidence of mitochondrial disorders in some children prenatally exposed to AZT or AZT/3TC combination [6]. This last finding raised serious concern on the potential long-term outcome of prenatal antiretroviral therapy, indicating the central nervous system as one of the possible targets for the adverse effects of these agents. Indeed, limited though significant effects on neurodevelopment have been reported in a number of studies carried out in rodents and non-human primates [3,8–12,24,36,39,45].

Specifically, we have found that prenatal AZT exposure impaired acquisition of a passive avoidance task in weanling mice, and altered intraspecific aggressive behavior at adulthood [9,10,39]. Prenatal exposure to 3TC affected response decrement pattern in a locomotor activity test in weanlings, while altering social behavior in juvenile mice [11,12]. A limited effect on responsiveness to amphetamine has been reported in preweaning female rats following prenatal exposure to AZT [3]. Mice prenatally exposed to 3TC showed some slight alterations in open field behavior, and enhanced sensitivity to the muscarinic blocking agent scopolamine as for sniffing behavior [11]. On the basis of these results, it is hard to identify the specific neuronal targets of these agents in the developing nervous system. Yet, some of the behavioral endpoints altered by either AZT or 3TC alone, namely response inhibition, habituation and arousal levels, are deemed to be partially under cholinergic control. The aim of the present study was thus to assess in mice prenatally exposed to AZT + 3TC combination the functional state of cholinergic muscarinic neuroregulations at adulthood. Locomotor activity, exploratory behavior and responsiveness to the muscarinic cholinergic blocker scopolamine were analyzed at adulthood (PND 70) by an open field test.

2. Methods

2.1. 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 20.00 to 08.00 h. 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 continuously available. After 3 weeks of acclimatization, breeding pairs were formed. Females were inspected daily for the presence of the vaginal plug (pregnancy day 0). On gestational day (GD) 10 the studs were removed and 18 females were randomly assigned either to control or to AZT + 3TC treatment group ($N = 9$ in each treatment group). The experimental protocol was approved by the Italian Ministry of Health, in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Prenatal treatment

AZT and 3TC (both provided by Glaxo Wellcome Research and Development, Middlesex, England) were dissolved in 0.9% NaCl solution. Pregnant CD-1 mice were treated per os twice daily (between 08.00 and 09.00 h and 19.00 and 20.00 h) from GD 10 to delivery (GD 19) with either AZT + 3TC (160 and 500 mg/kg dose, respectively) or saline vehicle. Doses, time and route of administration were chosen on the basis of our previous studies, referring to the human therapeutic range and taking into account interspecific differences [9–12,25,38,51]. Specifically, to assess the behavioral effects of the combined treatment we chose the two highest AZT and 3TC doses inducing mild but significant alterations of different behavioral endpoints, without overt toxic effects on postnatal growth [9–12]. At birth, all litters were fostered to untreated dams of the same strain that had given birth to healthy litters within 24 h. Data on reproductive performance such as gestation length, litter size, sex ratio and offspring viability were also collected, and they did not evidence any significant treatment effects. However, AZT + 3TC-treated animals showed significant reduction of body weight at birth (about 18%), but such a weight difference was fully recovered by adulthood, when the present experiment was performed. From birth to PND 35 offspring were subjected to a number of behavioral tests that are the subject of another study. In this respect, special care was taken to assign animals with the same testing history to the open-field test at adulthood (see in particular Ref. [2], p. 313, for a discussion on the potential interactions between handling and prenatal treatments). Thus, due to the complexity of the experimental design aimed at controlling within-litter variability, it was not possible to perform a multi-dose experiment. The scopolamine dose was selected according to previous experiments carried out in our laboratory [1,2], which showed that it was the most effective in inducing hyperkinesis and impaired habituation in CD-1 mouse strain.

2.3. Open-field and scopolamine challenge

On PND 70, four subjects (two males and two females) from each of nine litters in each prenatal treatment group (vehicle and AZT + 3TC) were assigned to either scopolamine or saline treatment. Both sexes were equally represented in each final group ($N = 9$), namely vehicle/saline,

vehicle/scopolamine, AZT + 3TC/saline and AZT + 3TC/scopolamine. Mice were weighed and injected intraperitoneally (i.p.) with either 2.0 mg/kg of scopolamine hydrobromide (United States Chemical, Cleveland, OH) or an equal volume of 0.9% NaCl solution.

Fifteen minutes after injection, individual mice were transferred from the home cage to an open field arena (35 × 35 cm) made of black Plexiglas with a white bottom subdivided by black lines into 7 × 7 cm squares. The test started by placing the animal at the center of the arena. The behavior of the animals was then observed and videotaped for 15 min under red light. Immediately after each test, the apparatus was thoroughly cleaned with cotton pad wetted with 70% ethanol. Recordings were scored by an observer blind to the treatment received by each animal, and the following behavioral categories were analyzed by "The Observer" (Noldus, Wageningen 6700, the Netherlands), a software package for collection and analysis of data [33]: frequency of Crossing (crossing the squares boundaries with both forepaws), frequency and duration of Rearing, Wall-Rearing, Grooming (rubbing the body with paws or mouth and rubbing the head with paws) and Immobility, frequency of Sniffing (placing the nose against ceiling, wall or floor).

Immediately after the 15-min period, a stimulus object (a 35-mm black cartridge box) was placed in the middle of the arena for 5 additional min [1]. During this last observational period the latency to make the first contact with the stimulus object and the number of contacts with it were also scored.

2.4. Statistical analysis

Parametric analysis of variance (ANOVA) was applied to open-field data (first 15 min of observation), considering prenatal treatment as between-litter factor, sex as within-litter factor, and scopolamine challenge as within-litter and within-sex factor. Kruskal–Wallis analysis of variance was used to evaluate the main effect of prenatal treatment, the prenatal treatment by sex interaction, the prenatal treatment by scopolamine challenge interaction, and the three-way interaction on latency to approach the novel object. Wilcoxon test was also applied to evaluate the main effect of scopolamine and the main effect of the sex within each prenatal treatment group.

Post hoc comparisons after parametric results were performed by Tukey's HSD test and, where appropriate, were also used in the absence of significant ANOVA results [49]. In the case of non-parametric results post hoc comparisons were done using the Mann–Whitney *U*-test with Bonferroni's correction.

3. Results

Fig. 1 shows the frequency of the different behavioral items analyzed during the 15-min open field test, while durations of these items as well as latency to approach the novel object in the last 5 min of the test are shown in Table 1.

AZT + 3TC treatment failed to affect frequency of crossing, frequency and duration of wall rearing, frequency and duration of immobility and frequency of sniffing behavior. A main significant effect of the prenatal treatment was instead found on open rearing and grooming frequency, AZT + 3TC-treated animals showing enhanced rearing [$f(1,16) = 6.48$, $P < .05$] and less self-grooming behavior when compared to vehicle controls [$f(1,16) = 7.29$, $P < .05$].

As for the effects of scopolamine, scopolamine-treated mice were markedly more active than saline controls [$f(1,16) = 9.34$, $P < .01$], and showed increased wall rearing frequency [$f(1,16) = 34.53$, $P < .001$]. On the contrary, scopolamine significantly decreased frequency and duration of open rearing [$f(1,16) = 172.38$, $P < .001$; $f(1,16) = 108.9$, $P < .001$, respectively], grooming [$f(1,16) = 14.18$, $P < .001$; $f(1,16) = 24.44$, $P < .001$, respectively], immobility behavior [$f(1,16) = 30.44$, $P < .001$; $f(1,16) = 15.15$, $P < .01$, respectively]. The effect of scopolamine on sniffing behavior just missed statistical significance [$f(1,16) = 3.94$, $P = .06$], scopolamine-injected animals displaying more sniffing behavior than saline controls.

The two-way interaction between prenatal treatment and scopolamine challenge was not significant for any of the behavioral items considered, and just missed statistical significance for rearing frequency [$f(1,16) = 4.1$, $P = .06$].

Time blocks were significant for crossing [$f(2,32) = 6.62$, $P < .01$], wall rearing [$f(2,32) = 6.15$, $P < .01$ for frequency; $F(2,32) = 8.79$, $P < .001$ for duration], open rearing [$f(2,32) = 7.01$, $P < .01$ for frequency; $f(2,32) = 24.35$, $P < .001$ for duration], grooming duration [$f(2,32) = 20.51$, $P < .0001$], crossing, wall and open rearing decreasing over time whereas grooming duration increased.

The interaction between challenge and time blocks (repeated measures) was significant for crossing [$f(2,32) = 16.72$, $P < .001$], wall rearing [$f(2,32) = 28.81$, $P < .001$], rearing [$f(2,32) = 7.67$, $P < .01$; $f(2,32) = 23.32$, $P < .001$ for frequency and duration, respectively] grooming [$f(2,32) = 3.75$, $P < .05$; $f(2,32) = 16.91$, $P < .001$ for frequency and duration, respectively] and immobility [$f(2,32) = 4.08$, $P < .05$; $f(2,32) = 5.34$, $P < .05$, for frequency and duration, respectively]. The response decrement pattern observed in saline-injected animals for crossing, wall and open rearing and immobility, was disrupted in scopolamine-injected animals over the 15 min of the test. Frequency and duration of grooming, that increased over time in saline-injected mice, decreased in scopolamine-injected mice over the 15 min of the test.

The three-way interaction among scopolamine challenge, time blocks and prenatal treatment was significant only for sniffing frequency [$f(2,32) = 4.47$, $P < .05$]. In AZT + 3TC-treated mice, scopolamine failed to enhance sniffing behavior in the second and third time blocks (P's < .05 after post hoc comparisons).

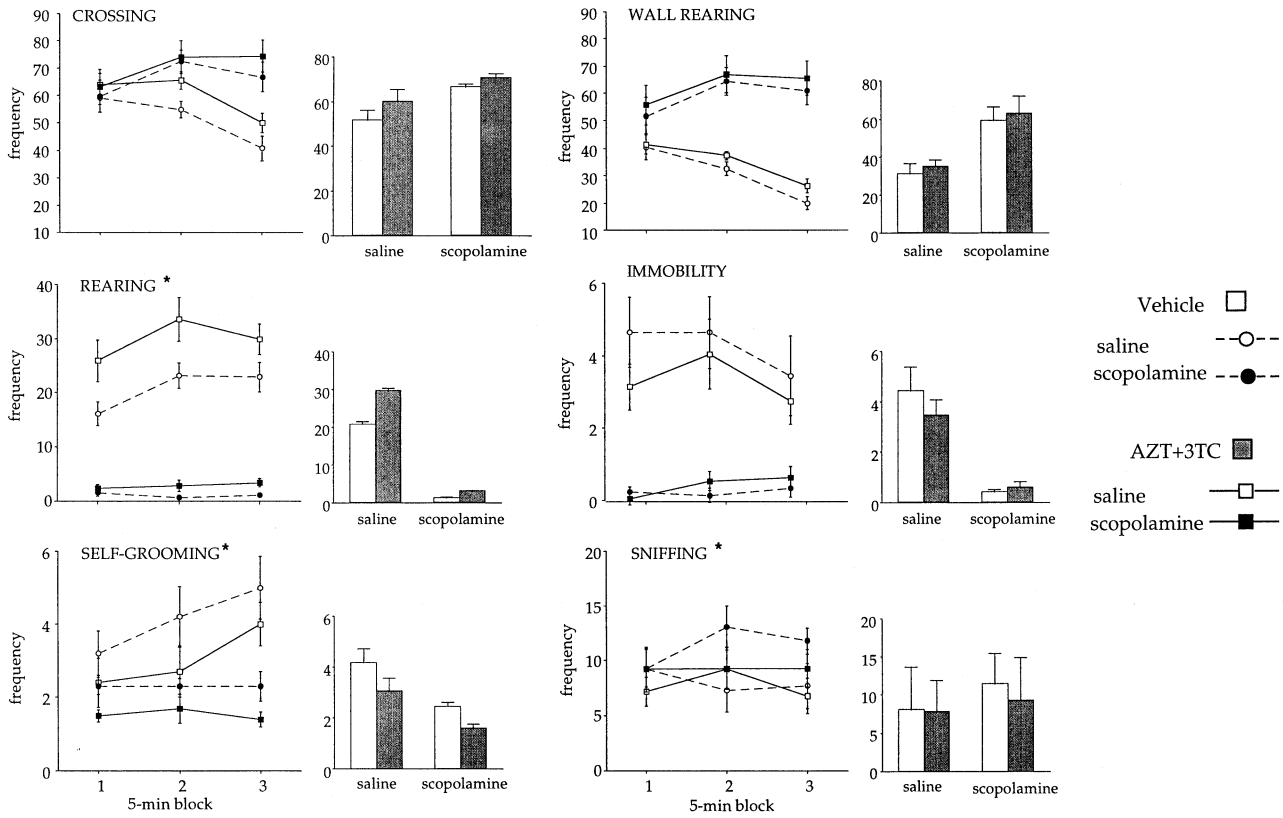

Fig. 1. Frequencies (mean ± S.E.M.) of different behavioral items over the 5-min blocks of a 15-min open field test by mice prenatally treated with vehicle or AZT + 3TC solution, and receiving an intraperitoneal injection of either saline or scopolamine (2 mg/kg) solution 15 min prior to the beginning of the test. Bars on the right represent the mean frequencies (± S.E.M.) during the entire test. * Significant effect ($P < .05$) of the prenatal treatment, either as a main factor or as a two- or three-way interaction.

Neither a main effect of sex nor any interaction of sex with the other variables was found on any of the behavioral items considered.

Latency to approach the novel object was not significantly affected by prenatal treatment (see Table 1), though AZT + 3TC-treated mice tended to contact the novel object faster than vehicle-treated controls. A main effect of scopolamine was found, scopolamine-injected animals approaching and contacting the object significantly faster than saline controls ($P < .05$), and this effect was more marked in AZT + 3TC- than in vehicle-treated animals. Time spent in contact with the object was not affected by the cholinergic

Table 1
Duration(s) of selected behavioral items during a 20-min open-field test

		Prenatal treatment	
Behavioral items	Postnatal treatment	Vehicle	AZT + 3TC
Wall rearing *	saline	56.41 (± 5.5)	72.70 (± 3.54)
	scopolamine	73.15 (± 7.45)	74.48 (± 9.17)
Rearing	saline	42.55 (± 3.34)	56.85 (± 25.91)
	scopolamine	1.67 (± 0.45)	2.41 (± 0.54)
Immobility *	saline	25.91 (± 7.21)	15.74 (± 3.28)
	scopolamine	1.70 (± 0.47)	5.85 (± 2.94)
Self-grooming *	saline	38.07 (± 8.17)	31.33 (± 6.17)
	scopolamine	11.37 (± 0.75)	8.57 (± 1.34)
Sniffing	saline	12.39 (± 3.07)	13.11 (± 1.57)
	scopolamine	14.07 (± 1.55)	13.20 (± 1.93)
Latency to contact object *	saline	80.89 (± 21.77)	57.5 (± 16.71)
	scopolamine	40.61 (± 14.7)	21.94 (± 5.63)

Data are means (± S.E.M.). Total duration was averaged over the three 5-min blocks. $N = 9$ in each treatment group. No main effect of prenatal treatment was found.

* Indicates significant effect of scopolamine ($P < .01$).

antagonist. The interaction between prenatal treatment and scopolamine challenge was not significant. Neither a main effect of sex was found, nor any interaction of sex with prenatal treatment or scopolamine challenge.

4. Discussion

Our findings clearly show that prenatal exposure to the combination of two widely used nucleoside analog antiretrovirals, namely AZT and 3TC, fails to influence locomotor behavior and responsiveness to the cholinergic antagonist scopolamine in adult mice of the CD-1 strain, when tested in a conventional open field test. Mice exposed prenatally to AZT + 3TC combination showed a habituation profile in locomotor activity comparable to controls; moreover, scopolamine affected this same habituation response in both vehicle- and AZT + 3TC-treated mice. We are well aware of the limitations of the use of a single dose in a behavioral study like the present one: specifically, it is possible that the administration of low or intermediate doses of scopolamine would have revealed significant group differences in combination- and vehicle-treated animals. However, as far as the 2 mg/kg dose of scopolamine is concerned, the present results are suggestive of a normal development of those neural circuitries modulating habituation and response inhibition (mainly cholinergic), which mediate the behavioral effects of muscarinic receptor blockade [5,17,37].

Whereas responding to the muscarinic antagonist was not altered by the prenatal treatment, mice exposed to AZT + 3TC combination show alterations in two behavioral items, namely rearing and self-grooming. Rearing frequency was indeed markedly enhanced in AZT + 3TC-treated animals receiving saline. Unlike locomotion, rearing behavior is considered to reflect not only exploratory activity, but also emotionality levels associated to alertness when exploring a novel environment. Self-grooming frequency was decreased in AZT + 3TC-treated mice in comparison to vehicle controls. Self grooming is considered to be a specific rodent behavioral response to stressful situations, as an increase in grooming behavior is normally observed in response to situations associated to a novel set of stimuli [27,48]. In accordance with our present results, a still in progress experiment carried out in our laboratory shows that adult mice prenatally treated with 160 mg/kg AZT (same dose as in the present study) displayed higher rearing frequency and reduced self-grooming behavior in a modified open-field with objects. Since in a previous study we found that prenatal exposure to 500 mg/kg 3TC did not affect these same behavioral items at adulthood [11], it is likely that the effects we observed are attributable to AZT rather than to 3TC.

Applewhite-Black et al. [3] have suggested that prenatal exposure to AZT might affect the development of dopaminergic system. Specifically, they reported enhanced sensitivity to the hyperactivating effect of amphetamine in female offspring tested in an automated activity apparatus at the preweaning stage. Our present findings would support a dopaminergic involvement in the behavioral effects of AZT. As a matter of fact, much evidence suggests a dopaminergic regulation for both rearing and grooming behaviors. Open rearing is increased in rats and mice by administration of amphetamine [40], D1 receptor agonists [18] and alteration of rearing behavior has been reported in mice overexpressing [18] or lacking [14] the D1 receptor gene. Novelty-induced grooming is decreased by administration of dopaminergic antagonists, such as haloperidol, as well as by lesions of brain dopaminergic pathways [26,44]. Moreover, grooming, and in particular intense grooming, is used as a reliable behavioral index of D1-like receptor function, since the expression of this behavior is heightened by all D1-like agonists examined [46,47]. In addition, reduction of grooming occurrence following exposure to a novel environment has been observed in transgenic mice lacking the D1A receptor gene [50].

In a previous study from our laboratory, mice prenatally treated with 500 mg/kg 3TC (same dose as the one used in the present experiment) exhibited normal responsiveness to scopolamine as for the hyperkinetic effects of this agent, but they displayed enhanced sniffing behavior following scopolamine administration [11]. This latter finding led us to suggest an imbalance between cholinergic and dopaminergic regulations in 3TC-treated animals, since scopolamine-induced stereotyped sniffing might result from a facilitatory effects of the antimuscarinic agent on dopaminergic neurotransmission [29,30,41]. Such enhancement of a typical scopolamine effect was no more present in AZT + 3TC-exposed animals: on the contrary, scopolamine failed to increase sniffing behavior throughout the duration of the test in combination-treated animals. A possible explanation for this discrepancy might be found when considering that the AZT effects on dopaminergic function had interfered with the 3TC-induced enhancement of scopolamine effects on sniffing. Yet, in the absence of data on sensitivity to scopolamine in animals treated with AZT alone, final conclusions on the specific functional targets of these two antiretroviral drugs cannot be drawn. Furthermore, though a gross disturbance of cholinergic muscarinic neurotransmission can be excluded on the basis of the present findings, however an influence on cholinergic nicotinic subsystem — known to interact with dopaminergic system in the control of arousal levels and motor behavior — cannot be ruled out as yet.

We have recently examined the neurobehavioral effects of prenatal administration of AZT + 3TC combination in preweaning and juvenile mice (Venerosi et al., 2000, submitted for publication). In the absence of effects on maternal reproductive performance, treated pups showed significant reduction of body weight gain (recovered by the juvenile stage), and delayed maturation of placing and grasping reflexes and pole grasping response. On the whole, it appears that the combination of AZT and 3TC

has more marked effects on reflex development and somatic growth than either drug alone [9,12]. While it cannot be excluded that the significant retardation in body growth may have indirectly influenced the maturation of some sensorimotor reflexes in the early developmental phase, we hypothesize that the alteration in rearing and grooming behavior at adulthood reflects specific effects on CNS development. As a matter of fact, enhanced rearing and reduced grooming frequency are also observed in animals prenatally treated with AZT alone (Calamandrei et al., in preparation), that do not present marked weight differences from controls at birth.

In the light of the introduction into clinical practice of antiretroviral combinations to be administered to vulnerable individuals such as pregnant women and neonates, further studies should be carried out in animals to assess the potential long-term outcomes of prenatal multiple antiretroviral therapy, as well as to identify the neuronal populations sensitive to the effects of nucleoside analogs. To date, even though a dopaminergic hypothesis for some of the behavioral effects of these agents (in particular of AZT) appears to be an attractive one, it has to be supported by more focussed experiments assessing the responsiveness to selected dopaminergic agonists and antagonists in animals pre- and postnatally exposed to either AZT, 3TC or their combination.

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