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Effects of ddC and AZT on Locomotion and Acoustic Startle I: Acute Effects in Female Rats[1]

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MORSE, D. E., H. DAVIS, E. POPKE, K. BROWN, V. O'DONOGHUE AND N. GRUNBERG. *Effects of ddC and AZT on locomotion and acoustic startle. I: Acute effects in female rats.* PHARMACOL BIOCHEM BEHAV **56**(2) 221–228, 1997.—Several synthetic nucleoside analogues, including AZT(RETROVIR), ddC (HIVID), ddI (VIDEX), and d4T (ZERIT), are currently being used in the treatment of HIV infection. Unfortunately, in clinical use the appearance of severe and sometimes debilitating peripheral neuropathy and pain has been associated with the long-term use of several of these drugs (i.e., ddC, ddI and d4T), although not with AZT. To date, standard pre-clinical animal toxicity studies have failed to reveal any adverse neurologic effects of these compounds. However, previously reported preliminary findings suggest that ddC may alter several neuro-behavioral parameters (including locomotor activity, acoustic startle responding, and aggression) in rats and mice following presentation in the animals' drinking water for 7 days. The current series of experiments examined effects of acutely administered ddC and AZT on spontaneous locomotor activity and acoustic startle responses (with and without pre-pulse) in female Sprague–Dawley rats. Following intragastric administration, ddC reduced locomotion at all but the highest dose, whereas AZT had no significant effect on locomotor activity. Acutely administered ddC had no effect on ASR, whereas AZT increased ASR at the highest stimulus intensity. These data support the use of behavioral testing in the development of the antiviral nucleoside analogues, as behavioral testing may be more effective in identifying the neurologically active agents than is standard toxicity testing. **Published by Elsevier Science Inc., 1997**

AZT ddC Rat Locomotion Acoustic startle

IN 1994 it was estimated that the cumulative number of Acquired Immunodeficiency Syndrome (AIDS) cases in the United States exceeded 400,000 (14) and that AIDS had become the leading cause of death in adults aged 25–44 (34). Furthermore, also in 1994, the World Health Organization estimated that there were 4 million cumulative AIDS cases worldwide and projected that the Human Immunodeficiency Virus (HIV) and its associated opportunistic infections will have killed 40–100 million people by the year 2000 (20,33).

The search for pharmaceutical agents to inhibit the progression of HIV infection to AIDS, primarily by slowing the proliferation of HIV, has been intense. One class of pharmaceuticals which continues to figure prominently in this effort is the nucleoside analogues. Although these drugs (including AZT [azidothymidine], ddC [dideoxycytidine], ddI [dideoxyinosine], and d4T [dideoxydehydrothymidine]) appear to slow the progression of the disease, their long-term use is often associated with debilitating and sometimes life-threatening toxicity.

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Dideoxycytidine, ddI, and d4T, in particular, have all been associated with the development of intense and often debilitating pain in the distal extremities, possibly as a result of the development of peripheral neuropathies (8,9,17,30).

To date, standard pre-clinical toxicology studies conducted in animals have not been able to identify the behavioral and neurotoxic effects of these medications prior to the discovery of these serious adverse effects in clinical trials with human patients (31). Only recently have studies begun to examine the electrophysiological and histopathologic effects of ddC in rabbits (5,6,18,19), the electrophysiologic effects of ddC administration in primates (7), and the histopathologic/morphometric effects of ddI administration on peripheral nerve structure and functioning in rats (36). However, there are no published studies which have carefully quantified the behavioral responses of animals to the nucleoside analogues. Behavioral studies with animals could provide a valuable "non-invasive" preclinical screening tool to examine the neurotoxic potential of the anti-HIV nucleoside analogues. If these studies were able to predict behavioral and neurotoxic effects in humans, then the results may help to focus drug development on compounds with activity against HIV that also show reduced neuro- and behavioral toxicity. The purpose of the present series of four experiments was to develop a behavioral paradigm with rats that could be used to detect the adverse effects of anti-HIV nucleoside analogues.

Specifically, the series of studies examined the effects of acutely administered ddC and AZT on spontaneous locomotor activity, the acoustic startle reflex (ASR), and pre-pulse inhibition (PPI) of the ASR in rats. Dideoxycytidine and AZT were studied because these are two of the most commonly prescribed treatments for HIV infection, and because ddC has been associated with a significant increase in the incidence of painful peripheral "neuropathy" with chronic use, whereas AZT has not been so associated. Spontaneous locomotion was examined because it is one of the most common dependent variables in behavioral toxicity research and has proven to be sensitive and reliable (39,40). The acoustic startle paradigm (ASR and PPI) was used as an animal model to study time-dependent sensory-gating deficits (12,13,37) and the underlying processes that may reflect attention (1,2), and has been recommended as a valuable tool in behavioral and neurotoxicity assessment (40). The acoustic startle response (ASR) consists of a series of muscular contractions that occur in response to auditory stimuli of rapid onset or rise time, while pre-pulse inhibition (PPI) refers to the attenuation of the ASR response when the startle stimulus is preceded by a non-startling tone. Based on the findings of Morse et al. (31), we hypothesized that acutely administered ddC would decrease spontaneous locomotor activity and would alter ASR/PPI responsivity in a dose-dependent fashion. In contrast, we hypothesized that AZT administration would neither affect locomotor activity or the ASR/PPI response in drug naive female rats.

EXPERIMENT 1

The purpose of this experiment was to examine the effects of acute intragastric (IG) ddC administration on spontaneous locomotor activity in female Sprague–Dawley rats. Spontaneous locomotor activity is a simple but sensitive behavioral measure, which is frequently used to detect effects of pharmacological agents that may be behaviorally/neurologically active or have neurotoxic effects (39). As stated previously, because ddC has been reported to produce neurotoxic effects in a significant proportion of patients (8,9,17), and based on a previous report (31), we hypothesized that acutely administered ddC would decrease spontaneous locomotor activity in a dose-related fashion.

METHOD

Subjects

The subjects were 64 female Sprague–Dawley rats (Charles River) weighing 150-175 g at the start of the experiment. Before administration of drug or deionized water (control), subjects were housed in same-sex groups of two or three in standard polypropylene shoebox cages (35.6 cm × 15.2 cm × 20.3 cm) fitted with a stainless steel grid cage top, and equipped with individual sipper-tube water bottles. The housing room was maintained at 23°C and 50% relative humidity on a 12: 12 h light dark cycle (lights off at 1000 h). Subjects had continuous access to pelleted laboratory chow (Agway Prolab 3200) and mains water.

Drug Administration

Subjects were randomly assigned to one of eight treatment groups: 0, 62.5, 125.0, 250.0, 500.0. 750.0, 1000.0, 1250.0 mg ddC/ kg body weight. The dosages were selected in order to span the range from zero to maximum solubility in water. Dideoxycytidine (Sigma Chemical, lot # 18F3706) was dissolved in deionized water and the concentrations adjusted to maintain a constant dose volume of 20 ml/kg body weight. Drug solutions or water were administered by IG intubation using an 18 gauge, 3 inch long curved animal feeding needle with a 2.25 mm ball, attached to a 6 ml plastic syringe.

Locomotor Activity Testing

Locomotor activity was measured using an Omnitech Electronics Digiscan infrared photocell system (Test box model RXYZCM (16 TAO); Omnitech Electronics, Columbus, Ohio), located in a dedicated room within the animal facility. This room was constructed of cinder block walls, acoustic tile ceiling, and steel doors (2) that kept sound to a minimum. Ambient room light was 26.5 FC, at the test apparatus, during the light portion of the cycle and the ambient room noise was 54 dB. Animals were placed singly in a 40 × 40 × 30 cm clear Plexiglas arena, with a thin layer of animal bedding and approximately 25 g of pellet chow. A water bottle was suspended in a Plexiglas cylinder from the lid of the arena. A photocell array measured horizontal locomotor activity using 15 pairs of infrared photocells located every 2.5 cm from side-to-side and 15 pairs of infrared photocells located from front-to-back in a plane 2 cm above the floor of the arena. A second side-to-side array of 15 pairs of additional photocells were located 10.5 cm above the arena floor to measure vertical activity. Data were automatically gathered and transmitted to a personal computer via an Omnitech Model DCM-8-BBU analyzer. Animals were monitored continuously with data recorded as cumulative activity over 15 min time-periods. The rats were not able to see the experimenter during the testing and vice-versa. The animals were not disturbed during the activity monitoring period.

Procedure

All animals were acclimated to handling for 5 min per day, three of the five days prior to testing, including the day before testing. On the day of testing, the appropriate dosage of ddC or water was administered by IG intubation, immediately prior

to the beginning of the dark portion of the subjects' circadian light-dark cycle. Subjects were placed individually into locomotor monitoring arenas, data collection began immediately, and subjects were left undisturbed for the 48 hour monitoring period. Eight subjects (including one or two water controls and at least three different treatment groups) were monitored simultaneously in eight different arenas.

Data Analysis

All subjects were included in the data analyses. Data from the 15 min cumulative intervals were combined into one-hour blocks for each subject. A repeated-measures ANOVA was performed with treatment group as the between-subjects factor and time as the repeated-measure.

Subsequent one-way ANOVAs tested for dose effects at each hourly time point. Post hoc comparisons were made using two-tailed Dunnett's *t*-tests, with $\alpha = 0.05$. Linear regression was performed for each time point.

RESULTS

Figure 1a presents the effects of ddC administration on horizontal locomotor activity for each treatment group at 1, 2, and 3 h after dosing. The activity of all groups was greater at 1 hour than at subsequent hours [$F(2, 112) = 178.12$, $p < 0.01$], a result that likely reflects a stimulatory effect of the novel test environment on early session locomotor activity. In addition, at 1, 2, and 3 h post administration, the 62.5 mg/kg ddC group displayed significantly less activity than did water controls ($p < 0.05$). At 3 h post administration, there was a significant overall main effect for dose [$F(7, 56) = 2.67$, $p < 0.05$], and the 62.5, 125.0, 250.0, 500.0 and 1000.0 mg/kg ddC groups displayed significantly less horizontal activity than did controls ($p < 0.05$). Figure 2 presents the horizontal activity of representative dose groups of ddC treated animals as measured during the 48 hour observation period. The results for vertical activity (see Figure 1b) were similar to horizontal activity with a significant difference between hour 1 and all subsequent hours [$F(2, 112) = 259.64$, $p < 0.01$]. However, there was no significant main effect for dose. Linear regression revealed no significant dose-response effects.

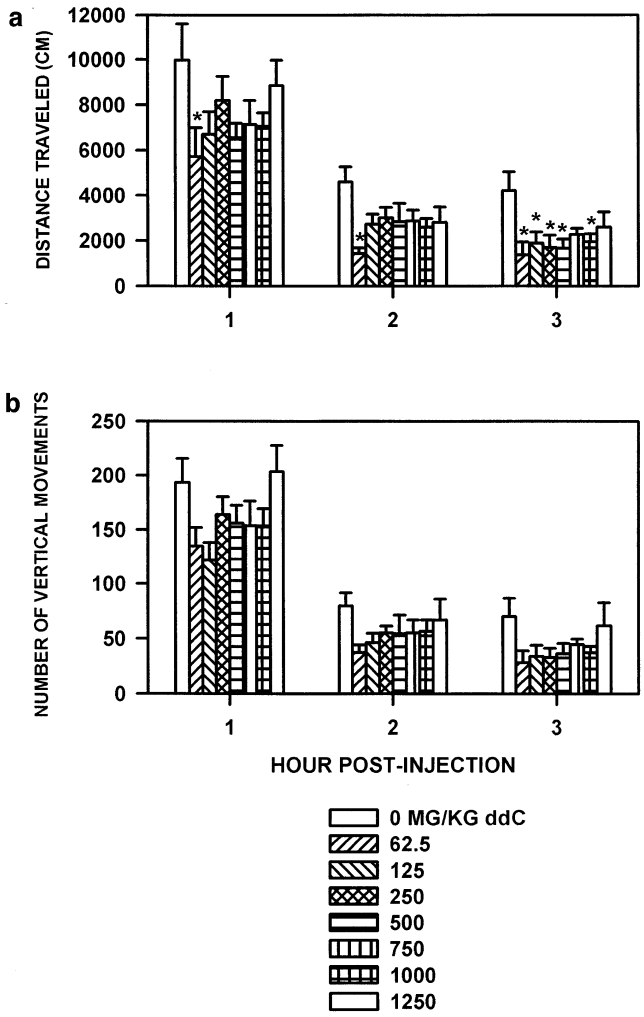

FIG. 1. Effects of ddC on horizontal (panel a) and vertical (panel b) activity for 3 h following drug administration (*= $p < 0.05$).

EXPERIMENT 2

The purpose of this experiment was to examine the effects of the acute IG administration of AZT on spontaneous locomotor activity in female Sprague–Dawley rats. AZT is the most commonly prescribed medication for HIV infection and, to date, has not been reported to increase the incidence of peripheral neuropathy. The experiment was designed to be comparable to Experiment 1 but, to use AZT instead of ddC.

METHOD

Subjects

The subjects were 56 female Sprague–Dawley rats (Charles River) weighing 150–175 g at the start of the experiment. The housing and animal care conditions were identical to those described in Experiment 1.

Drug Administration

Subjects were randomly assigned to one of seven treatment groups: 0, 7.8, 15.6, 31.2, 62.5, 125.0, 250.0 mg AZT/ kg body weight. As in the previous experiment, AZT drug doses were selected to span the range of the compounds' solubility in water. AZT (acquired from the NIAID; lot # AZT (30) 3-92) was dissolved in deionized water and the concentrations adjusted to maintain a constant dose volume of 20 ml/kg body weight. Drug solutions or water were administered by IG intubation as described in Experiment 1.

Procedures

The procedures for handling, locomotor testing, and statistical analysis were identical to those described for Experiment 1.

RESULTS

Figures 3a and 3b present the effects of AZT on horizontal and vertical activity, respectively, of each treatment group of rats for 1, 2, and 3 h after drug administration. The horizontal and vertical activity of all groups was greater at 1 hour than at all subsequent hours (Horizontal activity: $F(2, 108) = 59.90$, $p < 0.01$; Vertical activity: $F(2, 108) = 67.62$, $p < 0.01$], likely reflecting a stimulatory effect of the novel test environment on early locomotor activity. Within each hour, there was no

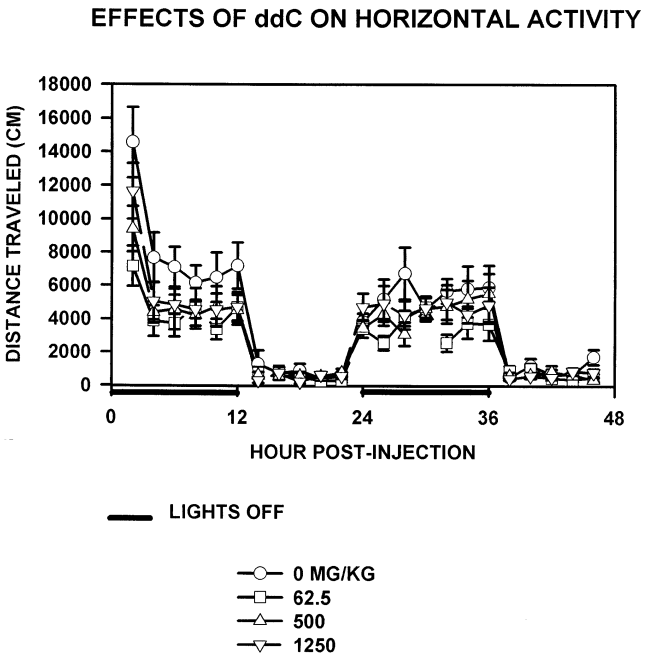

FIG. 2. Effects of representative doses of ddC on horizontal locomotor activity for 48 h after drug administration.

statistically significant main effect for drug on horizontal or vertical activity ($p > 0.05$). Figure 4 presents the effects of representative doses of AZT on horizontal activity for the entire 48 hour assessment period. Linear regression revealed a significant dose-response decrease in horizontal activity at hour 2 ($r = .29$, B= $-.28$, $p < 0.05$).

EXPERIMENT 3

The purpose of this experiment was to examine the effects of the acute IG administration of ddC on the acoustic startle reflex (ASR) amplitude and pre-pulse inhibition (PPI) of ASR in female Sprague–Dawley rats. As discussed previously, both the ASR and PPI responses are thought to measure reactivity, sensory-gating, and/or information processing which may reflect underlying attentional processes. ASR is a sensitive index of reactivity that may reflect processes that underlie attention (1,2). Many studies have reported that ASR is affected by drugs that alter neurochemical transmission (15,16,21,26). PPI refers to the attenuation in the startle response that occurs when the startle stimulus is briefly preceded by a non-startling tone. PPI has been used as a behavioral model to study time-dependent information processing deficits and may reflect processes that underlie sensory-gating (12,13,37).

METHOD

Subjects

The subjects were 28 female Sprague–Dawley rats (Charles River) weighing 200–250 g at the start of the experiment. Animal care was similar to that described for Experiment 1, except that the animals were individually housed for 2 wk prior to testing.

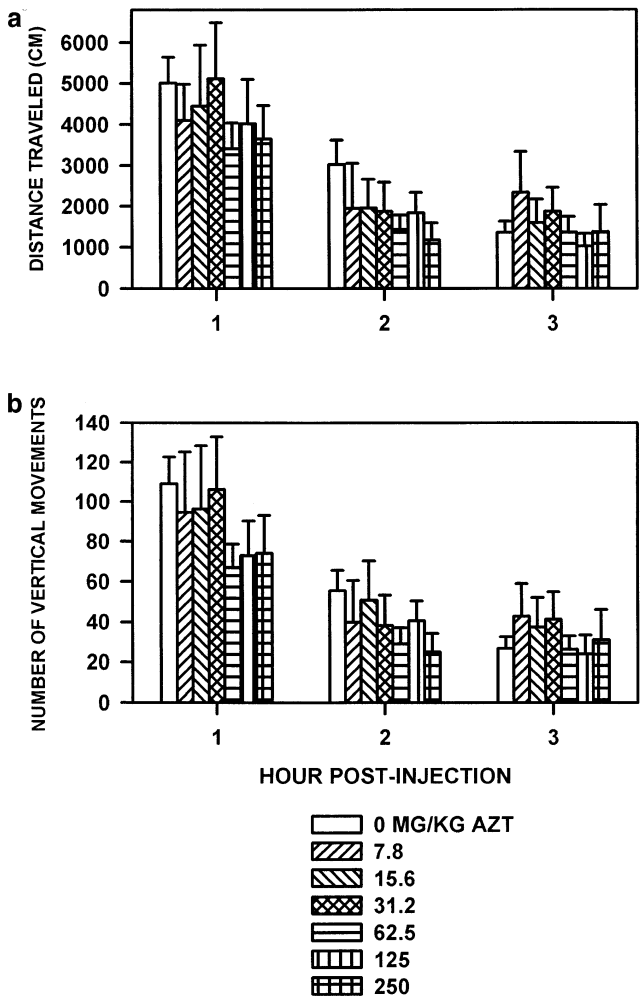

FIG. 3. Effects of AZT on horizontal (panel a) and vertical (panel b) activity for 3 h following drug administration.

Drug Administration

Subjects were assigned to one of four dose treatment groups: 0, 125.0, 500.0, or 1000.0 mg ddC/kg body weight. Group assignment was based on initial body weights and designed to insure the comparability of group mean weights. The drug source, route and techniques for drug administration were identical to Experiment 1.

Startle and Pre-Pulse Testing

ASR responses were measured using a four-station acoustic startle test system (Coulbourn Instruments, Allentown, PA) based on published reports (1–3). Specifically, animals were enclosed in $8 \times 8 \times 16$ cm open air cages that restrict locomotion but do not restrain the animal.

Cages were placed on one of four platforms in a sound-attenuating test chamber. Background noise within the test chamber was produced by a ventilating fan and was measured at 56 dB (SPL). Startle-eliciting acoustic stimuli consisted of 20 ms noise bursts of 98 dB, 112 dB or 122 dB. Pre-pulse stimuli consisted of a 20 ms, 1 kHz pure tone of 68 dB (12 dB above background).

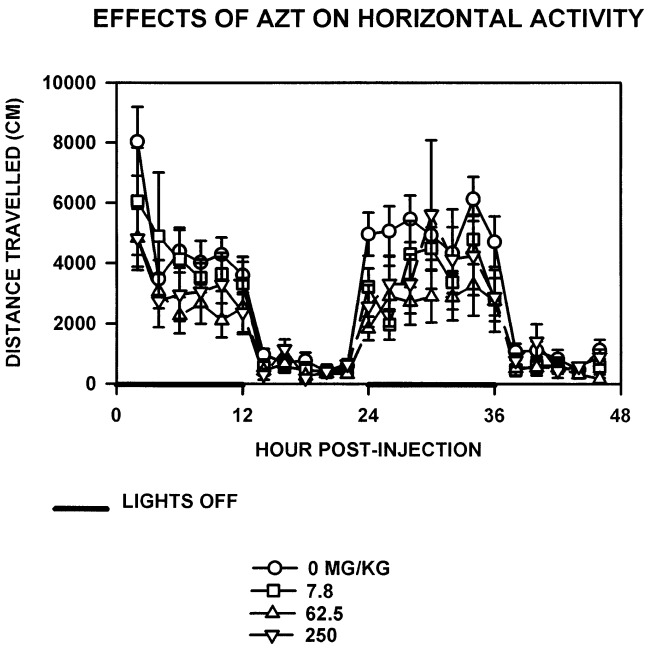

FIG. 4. Effects of representative doses of AZT on horizontal locomotor activity for 48 h after drug administration.

The onset of the pre-pulse stimuli preceded the onset of the startle-eliciting stimuli by 100 msec. Trials with no stimuli and trials with pre-pulse tone alone also were presented. The subject's movements in response to stimulus presentation were measured as voltage change by a strain gauge system incorporated in each platform, and subsequently converted to grams of body weight change (following analog to digital conversion). Responses were recorded by an interfaced microcomputer as the maximum response occurring within 200 msec of the onset of the startle-eliciting stimulus. Each baseline and test session consisted of a 3-min quiet adaptation period followed by random presentation of the various stimuli: no stimulus, pre-pulse alone, 98 dB alone, 112 dB alone, 122 dB alone, pre-pulse and 98 dB, pre-pulse and 112 dB, pre-pulse and 122 dB. Each subject underwent 8 repetitions of each trial type, with a random inter-trial interval range of 10–30s. Orientation sessions and test sessions for each animal were separated by at least two days to minimize effects of habituation on PPI (38).

Procedure

Each animal received three exposures to the startle apparatus. The first exposure consisted of a single test session designed to acclimate the animals to the acoustic startle apparatus. This acclimation procedure has been used to minimize the effect that the stress of a novel environment may have otherwise had on ASR and PPI during subsequent testing (2,3,4,35). During this first session, animals were exposed to the startle stimuli but no data were recorded. The second exposure consisted of a baseline session in which the animals received an IG infusion of water 2 h before startle testing. The purpose of this second exposure was to acclimate the subjects to the IG drug administration procedure. For the third exposure, the animals received IG drug or water infusions, were returned to their home cages for 2 h, and were subsequently placed in the ASR chamber for testing.

Treatment of Data and Statistical Analysis

Startle amplitudes were determined for each animal by subtracting the response on the no-stimulus control trials from the response recorded during each of the other trial types (startle trials with and without pre-pulse). These data were then analyzed by 3-way ANOVA's, dose × pre-pulse × stimulus, with subsequent 2-way ANOVA's , dose × pre-pulse, for each stimulus intensity. Dose was a between subject factor, while stimulus intensity and pre-pulse were within-subject factors. In addition, linear regression analyses were performed for each stimulus intensity with and without pre-pulse. Finally, percent of pre-pulse inhibition (%PPI) was calculated using the formula 100–(100 × (amplitude on pre-pulse + pulse trials)/(amplitude on pulse alone trials)). %PPI was analyzed using a mixed effects ANOVA, with dose as the between-subject factor and stimulus intensity as the within subject factor. All tests were 2-tailed and used an of 0.05 or less to determine significance.

RESULTS

Figures 5a, 5b, and 5c present the effects of ddC on ASR responses to startle stimuli of 98, 112, and 122 dB, respectively. with and without the presentation of pre-pulse stimuli. Overall, there was no main effect for ddC [$F(3, 24) = 0.68$, $p > 0.05$] and no significant interactions of ddC dosage with the presence or absence of pre-pulse or with stimulus intensity. Presentation of pre-pulse startle significantly reduced ASR responses overall [$F(1, 24) = 165.08$, $p < 0.01$].

Subsequent 2-way ANOVAs indicated that the effect of pre-pulse was significant at 98 dB [$F(1, 24) = 13.42$, $p < 0.01$], 112 dB [$F(1, 24) = 135.73$, $p < 0.01$], and 122 dB [$F(1, 24) = 101.13$, $p < 0.01$]. Stimulus intensity significantly increased ASR responses [$F(2, 48) = 156.86$, $p < 0.01$].

There was also a significant stimulus × pre-pulse interaction with greater effects of pre-pulse at higher stimulus intensities [$F(2, 48) = 63.09$, $p < 0.01$]. None of the linear regression analyses yielded significant effects. There were no significant effects of ddC on %PPI.

EXPERIMENT 4

The purpose of this experiment was to examine the effects of the acute intragastric administration of AZT on the acoustic startle reflex amplitude (ASR) and pre-pulse inhibition (PPI) of ASR in female Sprague–Dawley rats. This experiment was designed to be comparable to Experiment 3 but, to use AZT instead of ddC.

Subjects

The subjects were 48 female Sprague–Dawley rats (Charles River) weighing 175–208 g at the start of the experiment. Animal care was similar to that described for Experiment 1, except that the animals were individually housed for 8 days prior to testing.

Drug Administration

Subjects were randomly assigned to one of four drug treatment groups: 0, 100.0, 200.0, or 400.0 mg AZT/kg body weight.

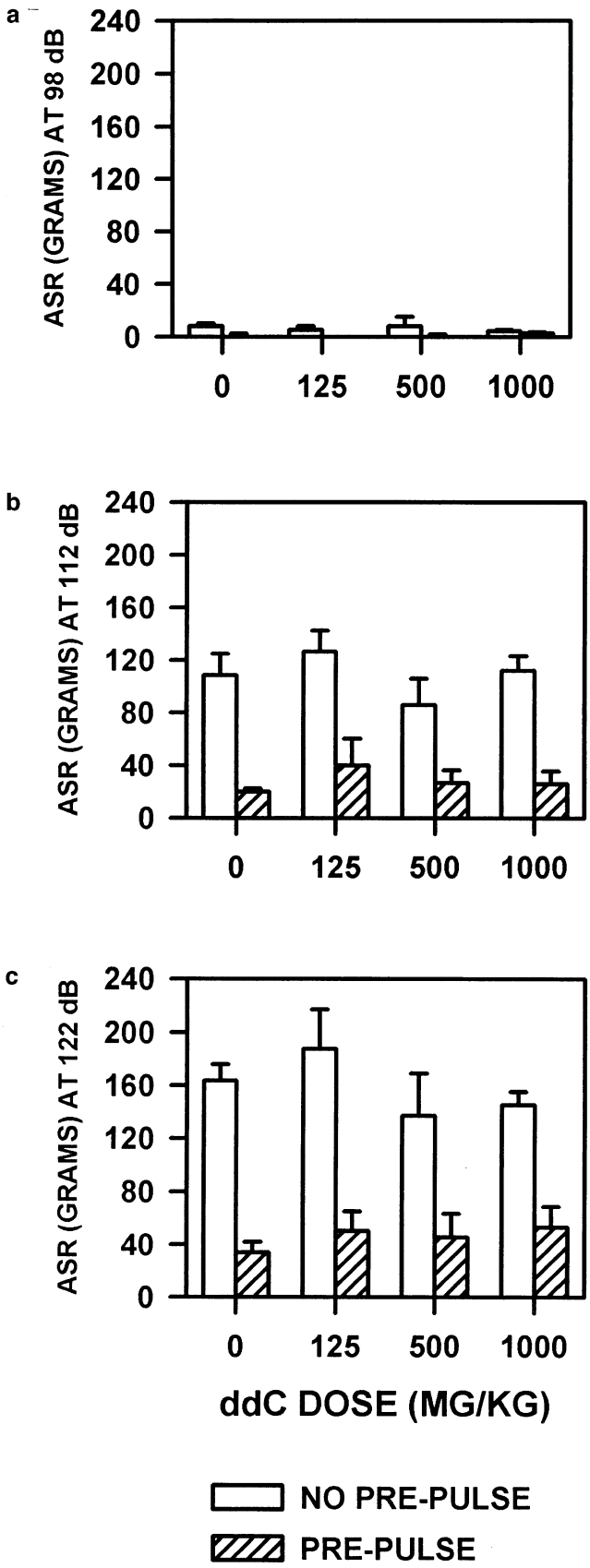

FIG. 5. Effects of ddC on the acoustic startle response, with and without pre-pulse, at 98 dB (panel a), 112 dB (panel b), and 122 dB (panel c).

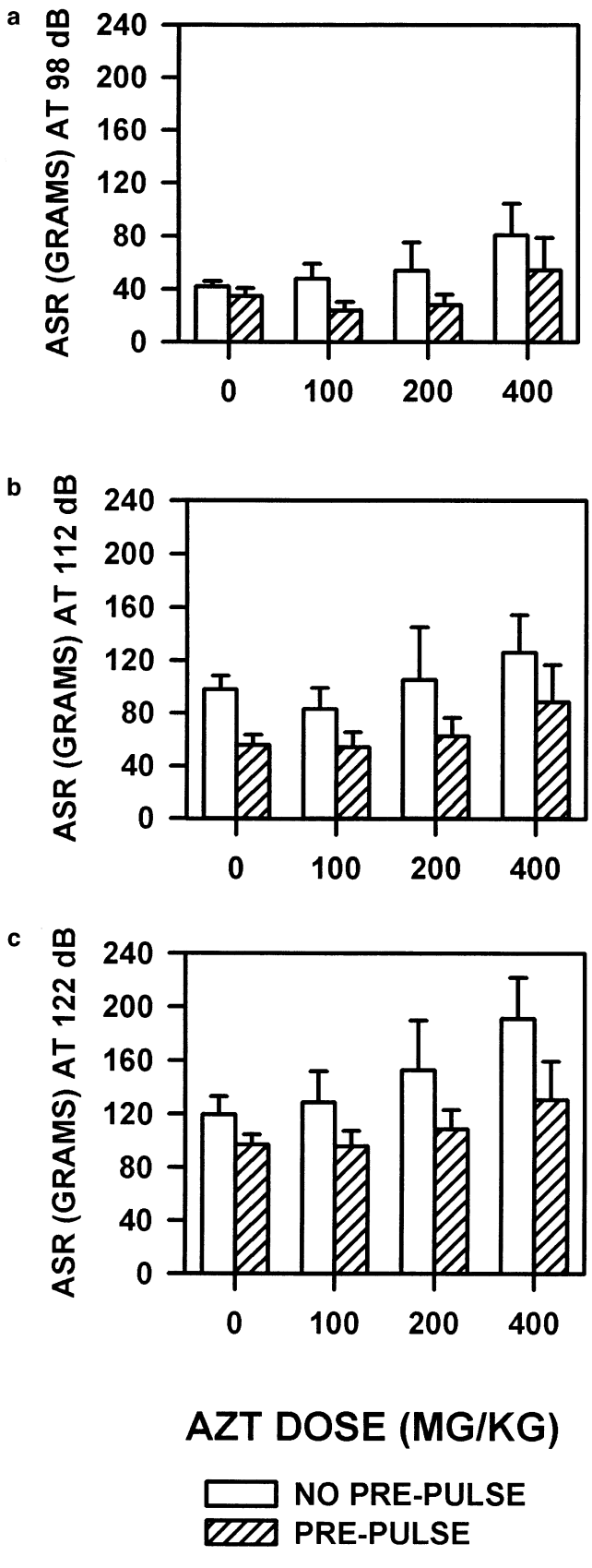

FIG. 6. Effects of AZT on the acoustic startle response, with and without pre-pulse, at 98 dB (panel a), 112 dB (panel b), and 122 dB (panel c).

As in the previous experiments, these doses were selected to span the range of solubility of AZT in deionized water. The drug source, route and techniques for drug administration were identical to Experiment 2.

Procedure

All procedures for handling, ASR testing, and statistical analysis were identical to those described for Experiment 3, except that AZT was administered instead of ddC.

Treatment of Data and Statistical Analysis

Data were analyzed as described for Experiment 3. Data for 5 subjects (from different treatment groups) were lost as a result of equipment malfunction.

RESULTS

Figures 6a, 6b, and 6c present the effects of AZT on ASR to the 98, 112, and 122 dB with and without pre-pulse, respectively. As in the previous experiment there was no main effect for AZT on the amplitude of the ASR [$F(3, 39) = 1.06$, n.s] and no significant interaction with the presence or absence of pre-pulse or with stimulus intensity. Presentation of pre-pulse significantly reduced the ASR response overall [$F(1, 39) = 26.08$, $p < 0.01$]. Subsequent 2-way ANOVAs revealed that this effect was significant at 98 dB [$F(1, 39) = 15.98$, $p < 0.01$], 112 dB [$F(1,39) = 19.09$, $p < 0.01$], and 122 dB [$F(1, 39 = 24.62$, $p < 0.01$]. Stimulus intensity significantly increased ASR response [$F(2, 78) = 122.56$, $p < 0.01$]. There was also a significant stimulus $\times$ pre-pulse intensity with greater effects of pre-pulse at higher stimulus intensities [$F(2, 78) = 5.01$, $p < 0.01$]. The linear regression analyses revealed a significant increase with dose in response to the 122 dB stimulus without pre-pulse ($r = +0.31$, $B = 0.19$, $p < 0.05$). There were no significant effects of AZT on %PPI.

DISCUSSION

The purpose of this series of experiments was to determine whether "non-invasive" behavioral testing with rats could be used to examine and differentiate between the effects of clinically identified neurotoxic and non-neurotoxic anti-HIV nucleoside analogues. Two prototypical nucleoside analogues were studied in this series of experiments; ddC, which has been associated with significant adverse neurologic effects in humans and, AZT, which has not been linked with increased rates of neuropathy in patients. Locomotion and acoustic startle reflex amplitudes (ASR), with and without pre-pulse (PPI) attenuation, were used as dependent variables. These measures were selected because they have previously been shown to be useful, sensitive, and reliable behavioral measures to test the effects of a variety of pharmacological agents. As discussed earlier, the long-term administration of ddC to HIV-infected patients has been widely recognized as being associated with an increased incidence of painful "peripheral" neuropathy, whereas the chronic use of AZT has not been associated with significantly increased rates of neuropathy.

The results of the four experiments reveal distinctly different behavioral effects of ddC and AZT. ddC produced a dose and time dependent decrement in locomotor activity for all but the highest dosage group with maximal behavioral effects at 3 h after drug administration. In contrast, AZT had no effects on locomotion. These results corroborate the previous report (31), in that "non-exploratory" spontaneous locomotor activity late in the test session was profoundly suppressed in the ddC treated animals. In the current study, the time-dependent decrement in locomotor activity (i.e., beginning 1–2 h following drug administration) likely reflects the pharmacokinetics of ddC absorption (22,23), but may be confounded by adaptation of the test animals to the "novel" test environment. However, the corresponding changes in spontaneous locomotor activity following both acute and continuous drug exposure (31), suggest that the behavioral changes are dependent on circulating drug, but, are not dependent on peak plasma drug concentration [Cmax]. Environmental factors may also play a role, as early "exploratory" responses to the "novel" test environment were similar for the ddC and control treated animals, while late session "non-exploratory" locomotor activity was significantly reduced among the ddC treated animals. The U-shaped dose-response curve of ddC on locomotion may reflect two underlying linear functions, but at this point we have no mechanistic explanation for this phenomenon. In addition, the exact characteristics of the changes in locomotor activity (e.g., general movement vs. stereotypy) merit further investigation. In contrast to the locomotor effects, the acute administration of ddC had no effect on ASR amplitude, with or without the presentation of a pre-pulse stimulus. AZT administration, on the other hand, increased ASR responses without pre-pulse to the 122 dB stimulus. It is noteworthy that all of the animals, regardless of treatment by drug or dosage, appeared healthy and indistinguishable from control animals. These results disconfirm our initial hypotheses and warrant further investigation. The results of these experiments indicate that these behavioral measures can be used to distinguish between two important anti-HIV drugs. As such, it may be possible to screen anti-HIV nucleoside analogues preclinically with these simple behavioral measures in female rats, to predict potential untoward neurobehavioral side effects in humans. It is important to note, however, that this first series of experiments examined acute administration of the drugs in female rats only. In human patients of both sexes, these medications are given repeatedly for up to many months or years. Therefore, future studies should examine the effects of acute and repeat/chronic administration of these drugs on multiple behavioral measures in both male and female test animals. In summary, the present findings indicate that spontaneous locomotor activity and ASR responses can be used with rats to produce identifiable and distinctive patterns of results that distinguish between two anti-HIV drugs, which have demonstrated differential neurotoxicity in humans. If these findings replicate with other anti-HIV nucleoside analogues, then a behavioral toxicologic approach could be used in the preclinical screening for adverse neurologic effects of this class of compounds and as an aid to early drug development.

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