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Relationship Between Memory and Fear: Developmental and Pharmacological Studies

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PLETNIKOV, M. V., Z. I. STOROZHEVA AND V. V. SHERSTNEV. *Relationship between memory and fear: Developmental and pharmacological studies.* PHARMACOL BIOCHEM BEHAV **54**(1) 93-98, 1996.—Habituation of the acoustic startle response (ASR) and freezing responses were assessed simultaneously in rats of different ages. Results showed that until 30 days of age rats were not able to express long-term habituation of the ASR, whereas irrespective of age, all rats exhibited the increased freezing responses as a result of fear conditioning. In addition, the interaction between fear and memory was studied using the same behavioral model in adult rats treated intraperitoneally with diazepam at doses 1.2 and 2.5 mg/kg. Diazepam administration did not result in a significant change in initial startle responsiveness but elicited a profound suppression of startle amplitude over trials. Furthermore, animals given diazepam showed more initial freezing than the vehicle-treated controls, while fear-conditioned freezing was decreased by diazepam. The pattern of results is discussed in relation to developmental and pharmacological dissociations between the different behavioral components of responses to aversive and stressful cues.

Startle Habituation Freezing Ontogeny Diazepam Rat

THE ACOUSTIC startle response (ASR) in rats is a frequently used model system for investigating behavioral plasticity (6,21). Repeated presentations of the auditory stimulus have been shown to induce habituation of the ASR. Recent data indicate that short-term habituation of the ASR is observed in rats regardless of age, while long-term habituation of this reflex was found to be exhibited only by adult animals (25,26). One of the plausible reasons of this form of infantile amnesia concerns the immaturity of the relevant brain structures involved in acquisition and retention of habituation of the ASR (16,26). However, the fact that the preweanling rats are unable to express long-term habituation of the ASR can also be accounted for by an excitatory influence of long-term senitization on the startle amplitude (20,31). Accordingly, long-term sensitization could be considered to be the result of fear conditioning that occurs when the contextual cues of the startle chamber, as the CS, are associated with the startle stimulus, as the US. Thus, in preweanling rats conditioned fear may inflate the startle amplitude, suppressing long-term habituation. If so, testing another response (e.g., freezing) of rat pups to aversive stimulation may reflect in more detail the past experience of developing subjects as well as providing additional data about the interaction of fear and habituation processes underlying a complex whole-body defensive response.

Monitoring a variety of defensive behavioral indices in a single animal whithin a session has other advantages, too. Recent findings clearly indicate that the response of an animal to threat or aversive stimulation represents a complex behavioral pattern consisting of somewhat independent, though interactive, components (e.g., motor, emotional, cognitive, etc.) (3,14). This suggestion is in disagreement with the more traditional psychological concept about a global fear response (22) and places significant constraints on general neurobiological theories of fear (13). The sophisticated picture of responses to threat points to the importance of simultaneous characterization of the individual variables of the defense profile. Besides the major theoretical advantage, this procedure would aid the search for more selective and effective clinical compounds for treating various fear-linked psychopathologies (3,10,14).

In this context, the aim of the present experiments was to study habituation of the acoustic startle response in rats of the different ages with simultaneous assessment of their freezing

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responses. Freezing has been shown to be one of the most frequently expressed fear-related behaviors in rats (4,9). In addition, the relationship between fear and habituation of the ASR was analyzed on adult rats that were treated with diazepam. This drug has been shown to reduce the fear state associated with the acoustic startle stimulus (31). Thus, diazepam-induced attenuation of fear accompanying the startle response would allow us to shed more light on the interaction between memory and fear processes in the behaving animal.

METHOD

Subjects

The adult and young subjects were experimentally naive male rats of the Wistar strain reared in our Behavioral Department vivarium. The adult rats were 90 days of age and weighed between 250 and 300 g. The young ones were 30 days of age, and weaned on 27th day after birth, and weighed between 60 and 90 g at testing. All animals were kept on 14 L : 10 D cycle. Lights were on at 0900 h. All the experiments were conducted from 1000–1300 h. Rats were housed two a cage and had ad lib access to food and water.

To obtain preweanling animals, female rats of the Wistar strain weighing 150 g were purchased from the Breeding Laboratory in Krukowo, Moscow District, and were allowed to acclimate to animal cages for about 2 weeks before mating with resident male breeders. All the rat pups were born in the vivarium. Approximately 24 h after birth, all litters were reduced to eight animals each; if possible, four males and four females were retained. Throughout all the experiments, with the exception of test periods, pups were with their own dams. To partly reduce distress occasioned by the separation from the mother, the preweanling rats were placed in the startle chamber containing shavings from the familiar cage and the nest. The preweanling subjects were 18 days old at the start of experiments.

Apparatus

The startle apparatus has previously been described in detail (27). Animals were tested in a Plexiglas startle chamber (14 × 10 × 16 cm) enclosed within a separate, dimly illuminated Plexiglas box (23 × 20 × 25 cm). The startle chamber was situated on a flexible platform. Four sensing elements were set into this plat form and detected any change of pressure induced by the startle chamber. The voltage resulting from a tension amplifier was sent to an ink recorder (Nikhon Koden, Japan), and was digitized and integrated by a microcomputer system. Startle amplitude was measured as the highest voltage peak within 100 ms from stimulus onset.

The experimental box contained, in a separate compartment, a sound generation system (100Y-101, Russia). A tweeter (1GD-400R) was mounted and centered 10 cm from the short side wall of the startle chamber. Continuous white background noise (72 dB) helped mask extraneous sudden auditory stimuli. Test stimuli were 110 dB white noises of 500 ms duration with 10 ms rise/fall time and were superimposed on the background noise.

Training Protocol

All animals (each group $n = 7$) regardless of age were given a training procedure. On the first day, the animals were placed in the startle chamber 5 min before the first stimulus presentation. During this 5-min period, freezing behavior was scored by a trained observer using a simple system of the ink recorder that allowed the total amount of freezing to be marked in relative units. Freezing was defined as the absence of visible movements, including the vibrissae, except for respiration. Scoring began 20 s after the rat was placed in the chamber. Freezing assessement was followed by presentation of 10 startle stimuli on a 20-s interstimulus interval.

On the second test day, the animals were replaced in the starle chamber, and freezing was assessed for a 5-min period, followed by presentation of 10 startle stimuli at the same interstimulus interval.

Drugs

Adult rats (90 days of age) were randomly allocated to three experimental groups ($n = 17$). One group received diazepam at a dose of 1.2 mg/kg; another was treated with 2.5 mg/kg of diazepam. The third group was given an injection of 100% dimethyl sulfoxide (DMSO), which was the vehicle for all diazepam groups. Injections were given intraperitoneally in a volume of 1 ml/rat on the first day 30 min before placing a rat in the startle chamber.

Speed Treadmill Experiment

In order to shed more light on the specificity of effects of diazepam, an additional series of experiments on a separate group of adult rats ($n = 7$) was conducted using a speed treadmill model. An apparatus consisted of a central drum (diameter 60 mm) divided into sections for individual animals. The drum surface is of knurled Perspex providing for a nonslip surface for a rat. The drum made 15 revolutions per minute. An animal was given an intraperitoneal injection of diazepam in the dose 2.5 mg/kg. The injection was made 30 min before the start of the experiment. Measurement of the time spent on the rotating drum was performed until the animal stopped running and fell.

Statistics

Behavioral data were evaluated by using one- or two-way analyses of variance (ANOVA). Further analyses were made by Duncan's test to determine the source of detected differ-

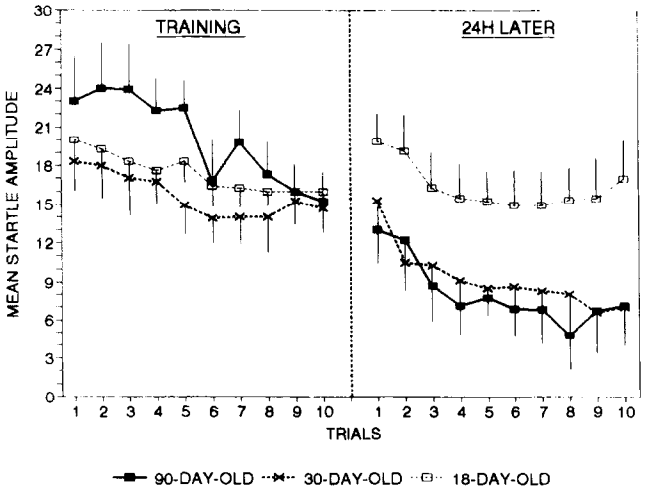

FIG. 1. Mean startle responses (in relative units) and SEM for the 18-, 30-, and 90-day-old Wistar rats during the training session (training) and during the 24-h test (24 h later).

ences in ANOVAs. The criterion of significance was set at $p < 0.05$.

RESULTS

Habituation of the ASR

The animals of all three ages showed a marked startle response to the auditory stimulus. Successive presentations of the stimulus gave rise to a decrease of the startle amplitude irrespective of age. These data are depicted in the left panel of Fig. 1. Thus, there were no age differences in within-training session habituation of the ASR. Analysis of the data confirmed our conclusions. A 3 (age) × 10 (trial) ANOVA yielded a significant effect of trial, $F(9, 180) = 2.67$, $p < 0.05$. The effect of age and the age × trial interaction were not significant.

On the second series of stimulus presentations, that occurred 24 h later, the auditory stimulus elicited similar high ASR in the 18-day-old rats, indicating that no long-term habituation occurred. On the contrary, the 30- and 90-day-old rats responded much less on the second day than they did on the first, suggesting that they remembered their prior exposure to the stimulus. The startle data on the second day are presented in the right panel of Fig. 1.

To assess long-term habituation of the ASR, separate one-way ANOVAs were made at each age, comparing the startle amplitude on the initial training trial with that on the first test trial. The only reliable effect was for the 90 day olds, $F(1, 12) = 12.7$, $p < 0.01$. The same tests for the two younger groups both yielded no significant differences (Fs < 1.0).

In addition, 2 (day) × 10 (trial) ANOVAs were conducted on each of the younger groups of rats in order to assess a possible savings score by comparing rates of habituation during each of the 2 days. Two-way ANOVA on the data for the preweanling group failed to reveal any significant effect for day or for trial. Neither was a day × trial interaction (all Fs < 1.0). On the other hand, the same test for the 30-day-old animals resulted in a significant effect of trial, $F(9, 120) = 2.33$, $p < 0.05$; and a significant effect of day, $F(1, 120) = 9.42$, $p < 0.01$. However, the day × trial interaction was not reliable, $F < 1.0$.

In summary, there were no age differences in rate of habituation of the ASR during the training session. There were, however, profound ontogenetic differences in long-term habituation of the ASR.

Freezing Behavior in Rats of the Different Ages

The amount of time rats of different ages remained immobile is shown in Fig. 2. No visible age differences in the level of basal freezing were found, although the adult rats had tendency to crouch a little less. An ANOVA on the data of the first day revealed no group effect, $F < 1.0$.

The amount of conditioned freezing is depicted in the right panel of Fig. 2. The results indicate that all rats, regardless of age, displayed much more freezing when tested just before the 24-h test session. A 3 (age) × 2 (day) ANOVA resulted in a significant effect of day, $F(1, 36) = 35.5$, $p < 0.01$, but no effects of age or of an age × day interaction (Fs < 1.0). Post hoc tests comfirmed that, irrespective of age, rats spent more time immobile before the 24-h test session than they did during the prestimulus baseline period ($p < 0.01$).

The results of the freezing test suggest that both the preweanling and the older rats displayed fear-conditioned state over 24 h. In contrast to long-term habituation of the ASR that reflects memory of the startle motor responsiveness, increased freezing is thought to be connected with past fear-related experience.

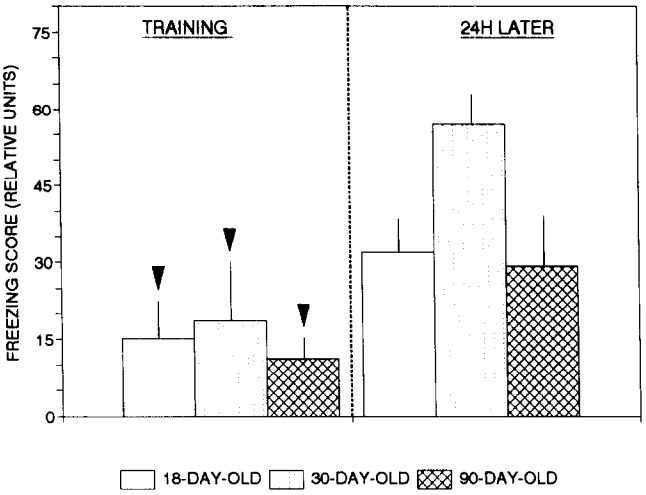

FIG. 2. Mean and SEM time spent immobile by the 18-, 30-, and 90-day-old rats during the 5-min prestimulus period immediately before the training session (training) and during the 5-min period immediately before the 24-h test session (24 h later). Arrowhead—$p < 0.01$ compared with the corresponding data on the second day.

The Effect of Diazepam on Habituation of the ASR in Adult Rats

Presentation of the auditory stimulus gave rise to reliable decrement of the startle amplitude in all groups. The mean startle amplitude across trials on the first day is depicted in the left panel of Fig. 3. The statistical analysis yielded no group differences between the mean startle amplitudes for the first trial, $F(2, 48) = 0.76$, $p = 0.41$, indicating that there were no reliable differences in the initial startle responses among the

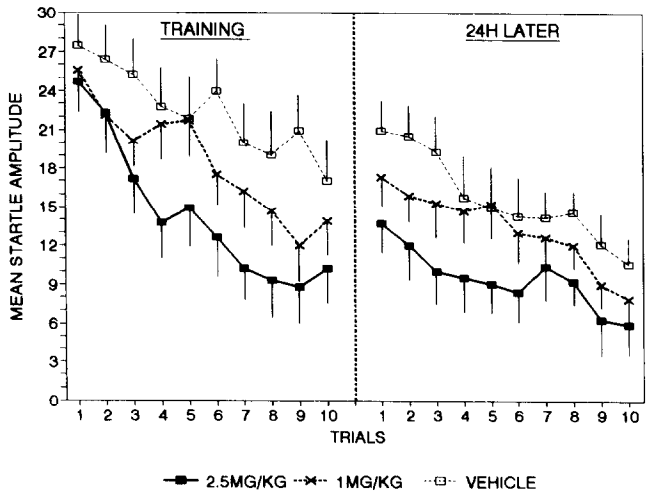

FIG. 3. Mean startle responses (in relative units) and SEM for the diazepam groups and the vehicle group on the ten trials of the training session (training) and the 24-h test session (24 h later).

three groups. A 3 (group) × 10 (trial) ANOVA of the change in the startle amplitude over the training session resulted in a significant effect of trial, $F(9, 480) = 6.3$, $p < 0.05$, and a reliable effect of group, $F(2, 480) = 11.2$, $p < 0.01$, but no effect of interaction of group × trial ($F < 1.0$). Because group differences only appeared as a result of presentations of the acoustic stimulus, the findings suggest that diazepam affected the development of response decrement rather than simply depressed motor responsiveness.

When the rats were reexposed to the auditory stimulus 24 h later, all groups showed profound long-term habituation of the ASR. To assess retention of habituation, separate one-way ANOVAs were made at each group, comparing the startle amplitudes on the initial training trial with that on the initial test trial. Significant effects were found for the control group, $F(1, 32) = 7.7$, as well as for the diazepam-treated groups, $F(1, 32) = 9.2$ and $F(1, 32) = 11.3$, for 0.1 and 2.5 mg/kg, respectively, all $p < 0.05$. The within-session results for the second day are presented in the right panel of Fig. 3. A 3 × 10 (group × trial) ANOVA on the startle response during the test session yielded the significant trial effect, $F(9, 480) = 8.4$, $p < 0.05$, and a reliable group effect, $F(2, 480) = 10.7$, $p < 0.01$, without any effect of the interaction of group × trial, $F < 1.0$, $p > 0.1$.

The pattern of within-session data on the second day was similar to that on the first day, and suggests that the drugged rats displayed more profound habituation of the ASR on both days.

Effects of Diazepam on Freezing Behavior in Adult Rats

The amount of freezing before the training session is shown in the left panel of Fig. 4. The rats given IP injections of diazepam in both the doses displayed much more freezing behavior than the vehicle-injected rats did. One-way ANOVA on the freezing response confirmed this outcome, yielding a significant group effect, $F(2, 48) = 9.4$, $p < 0.01$. Post hoc tests detected that freezing was significantly lower in the controls than in the 1.2 mg/kg or 2.5 mg/kg diazepam groups ($p < 0.01$). On the second day, the control group exhibited the general pattern of responding that was observed in the intact rats (see Fig. 2). Separate ANOVA on the freezing data of the vehicle group resulted in a significant day effect, $F(1, 32) = 6.1$, $p < 0.05$.

On the contrary, on the second day, diazepam-treated rats froze less than they did on the first day. To determine the source of this difference, fear-conditioned freezing of each of the diazepam groups was compared with that of the control group. Post hoc test revealed a reliable difference in the amount of freezing between the 2.5 mg/kg diazepam group and the control one. The difference in the level of freezing between the 1.2 mg/kg diazepam group and the controls was not reliable ($p > 0.1$).

So, the increased initial freezing was revealed as a result of diazepam administration, while under the same conditions fear-conditioned freezing did not have the tendency to rise, as was observed in the control rats.

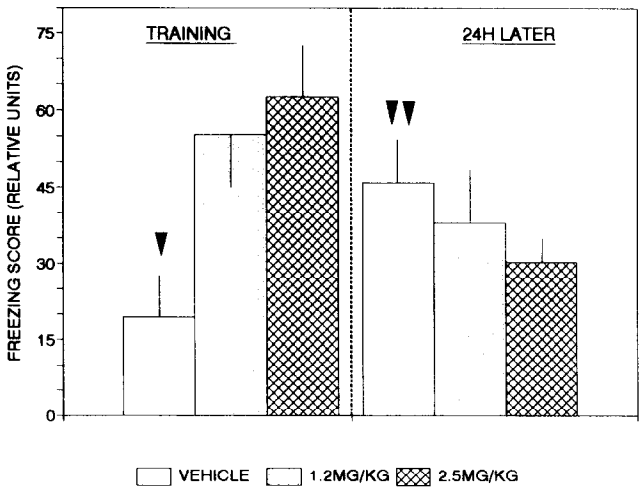

FIG. 4. Mean and SEM time spent immobile by the diazepam groups and the control group during the 5-min prestimulus period immediately before the training session (training) and during the 5-min period immediately before the 24-h test session (24 h later). One arrowhead—$p < 0.05$ compared with each of the diazepam groups on the first day. Two arrowheads—$p < 0.05$ compared with the 2.5 mg/kg diazepam group on the second day.

TABLE 1

THE EFFECTS OF DIAZEPAM (2.5 mg/kg) ON THE TIME SPENT ON THE ROTATING DRUM

Groups	Mean (s)	SEM
Control ($n = 7$)	22.31	4.26
Diazepam ($n = 7$)	23.73	3.44

Effects of Diazepam on Motor Coordination and Fatigue Resistance of Adult Rats

Table 1 shows that diazepam (2.5 mg/kg) did not affect the time spent by rats on the rotating drum. A one-way ANOVA did not revealed any differences between the groups, $F(1, 12) = 0.044$, $p > 0.5$.

DISCUSSION

Results replicate and extend our previous findings about habituation of the ASR in rats of different ages (26). Irrespective of age, rats demonstrated a clear similarity in rates of habituation during the training session, while long-term habituation of the ASR was observed only in the 30- and 90-day-old rats.

However, the 18-day-old subjects, like the young and adult rats, were able to express fear-conditioned freezing when tested 24 h after the training session. The data indicating a developmental dissociation between long-term habituation of the ASR and conditioned fear state allow us to propose, as did Leaton et al. (20,31), that conditioned fear may have masked habituation process in the developing rats. This age-related interaction between fear and habituation of the ASR follows a commonly reported direct linkage of fear state to the startle amplitude that is most clearly reflected in the fear-potentiated startle paradigm (6,7).

On the other hand, the present results show that fear-conditioned freezing to the startle chamber was exhibited by the 30- and 90-day-olds just before the 24-h test session, indicating that increase of the freezing response was observed simultaneously with profound habituation of the ASR. The similar clear discordance between freezing and the startle amplitude has been shown by investigating fear-potentiated startle in developing rats (15). The present data allow us to extend Campbell's group conclusion across all the ages tested, and to

suggest that the augmentation of the startle amplitude is not necessarily associated with expression of the high level of freezing.

Interestingly, the developmental dissociation between habituation of the ASR and fear follows a growing body of the most recent data about behavioral, neurophysiological, and pharmacological dissociations of different defensive responses (5,12,24). In a broader context, these findings are in concordance with the contemporary view of an integrated picture of behavior as a sophisticated hierarchical relationship of the different functional systems with specific underlying neurochemical mechanisms (2,18,19).

In addition to the theoretical consideration, studying the interplay of memory and fear seems to be also useful from the practical perspective. A large body of evidence has conclusively shown that specificity and efficacy of most of anti-anxiety drugs depend on a variety of interactive factors modulating fear/anxiety state in animal (1,14,28). Among them, memory of interaction with an aversive, stressful environment was shown to be the major factor (11). Our findings about the effects of diazepam on habituation and fear tested over days can be also considered in respect to this problem.

The lack of action of diazepam on the initial startle amplitude and the time spent on the rotating drum suggests that neither motor coordination nor muscle strength were affected. Furthemore, differences in the startle responsiveness emerged between diazepam and vehicle groups only after repeated stimulation. All the outcomes raise the possibility that habituation of the ASR was facilitated because of anxiolytic properties of the drug (10,23). These findings and conclusions are similar to those of Leaton's group, who employed the slightly modified paradigm of habituation of the ASR (31). However, in addition to the well-known anxiolytic effects of diazepam, unexpected action of this drug was observed in the present research. The increased basal freezing behavior following diazepam injections in contrast to vehicle seems to be quite a paradoxical phenomenon, although in the literature there are data indicating that benzodiazepine anxiolytic drugs are able to increase freezing response. De Boer et al. (8) have found that chlodiazepoxide increased freezing in rats in the shock-prod avoidance model. The authors, however, suggested that, in this paradigm, freezing cannot be an indicator of changes in fear and anxiety because the drug did reduce hormonal responses, which can be considered more appropriate fear indicators. Different reasons for diazepam-enhanced freezing have been proposed by Treit et al. (30) and Tsuda et al. (29). They explained the paradoxical effects of diazepam as a result of modulation of defensive repertoire in a rat by environmental constraints. Obviously, a final answer will come from future experiments.

The data indicate that on the second day the vehicle-injected animals showed a common pattern of freezing; that is, the basal freezing is normally much less than the conditioned one when tested across days (see Figs. 2 and 4). A quite different dynamic of freezing was observed in the rats given diazepam. On the second day, these animals exhibited less freezing in comparison both with their responding on the first day and with freezing of the control rats during testing. The observation of a decrease of the freezing response over days in the drugged rats prompts us to speculate that diazepam attenuated fear conditioning process. In this connection, the present data are consistent with those of a great body of investigations describing anxiolytic properties of diazepam in the fear-conditioned behavioral paradigm (23). The anxiolytic effect of diazepam on fear-conditioned freezing is also reminiscent of data on the anxiolytic-like properties of 7-OH-DPAT that was revealed to decrease similar freezing under the same conditions (25).

On the other hand, attenuation of fear-conditioned freezing might be due to the well-known amnestic effects of benzodiazepines (17). However, if this were the case, this amnestic action should have been also observed in relation to long-term habituation of the ASR, but more profound habituation of the ASR was revealed in the diazepam-treated rats.

The present results about the different responses to the drug (i.e., diazepam) among the different components of defensive behavior (habituation vs. fear, the initial freezing response vs. fear-conditioned freezing) are in concordance with the findings about the discrepant pattern of anxiolytic drugs responses within and between animal models documented by a number of investigators (10,14). In addition, our data support the contemporary viewpoint that no simple behavioral index, no matter how reliable and sensitive to a drug it may be, should be taken into account as a sufficient one for predicting clinical efficacy and specificity of this compound (3). In a similar vein, for future screening new clinical compounds with more selectivity and efficacy, it is useful to employ animal models of fear that pinpoint the impact of the different interactive variables of responses to threat.

In conclusion, our findings show the developmental and pharmacological dissociations between the different symptoms of the whole-body defensive response of a rat. These dissociations suggest age-dependent interaction of fear and memory processes as well as complex relationship between motor, emotional, and cognitive components resulting in their different responses to the drug.

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