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Differential effects of diazepam infused into the amygdala and hippocampus on negative contrast

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Abstract

Behavioral suppression is observed when animals shift from a high to a lower magnitude of reward in comparison to animals that continuously receive the lower magnitude reward. As previously reported, systemic administration of benzodiazepines promotes recovery from this negative contrast. This study aimed to assess where the neural substrate(s) located in the limbic areas for diazepam to induce such recovery effects on negative contrast. With food-deprived rats, the negative contrast procedure was conducted by comparing a group consuming a 32% sucrose solution which was shifted to 4% with a group consuming only 4% sucrose throughout the experiment. Represented mainly by a decreased number of licks, the negative contrast effects were clearly shown in the control groups receiving the vehicle. Systemic injection of diazepam dose-dependently reduced this contrast. Further, this negative contrast effect was significantly attenuated by local infusion of diazepam (30 μg) into the amygdala, but no such effect was confirmed when diazepam was infused into the hippocampus. Together, the present study shows that a reliable anticontrast effect can be induced by diazepam administration peripherally or locally infused into the amygdala. These data indicate that the amygdala is involved in the recovery effects of benzodiazepines on consummatory negative contrast.

Keywords: Reward reduction; Licking; Benzodiazepine; Anxiolytic; Limbic system

1. Introduction

Behavioral suppression is observed when animals shift from a high to a lower magnitude of reward in comparison to animals that continuously receive the lower magnitude of reward. This successive negative contrast effect can be reliably observed from consummatory behavior over four postshift days when 32% sucrose was shifted to 4%. As previously reported, systemic administration of benzodiazepines promotes recovery from this consummatory negative contrast most effectively from the second postshift day (Flaherty, 1999). Among benzodiazepines, chlordiazepoxide, midazolam, and flurazepam have been reliably shown to be effective in reducing this type of contrast in the past (Becker, 1986; Becker and Flaherty, 1983; Flaherty et al., 1986, 1990a,b, 1992). Surprisingly, in consideration that diazepam is one of the widely prescribed benzodiazepines, there is no specific investigation assessing the effects of diazepam on the consummatory negative contrast.

Although a substantial amount is known about the systemic administration of various drug treatments (including benzodiazepines) to reduce consummatory negative contrast, very little is known about the neural substrate underlying this anticontrast effect in the central nervous system (CNS). The anatomical basis of the anticontrast effect can be explored by microinjection of drug directly into certain brain sites. With respect to candidate microinjection sites in the brain for the present study, the amygdala and the hippocampus were chosen on the basis of previous lesion work on negative contrast. Using electrolytic lesions, damage to the lateral amygdala attenuated the consummatory negative contrast, whereas destruction of the medial amygdala eliminated such contrast (Becker et al., 1984). The parabrachial nucleus containing the efferent projection to the central amygdala is also important for mediating the consummatory negative contrast. Electrophysiologically guided bilateral electrolytic

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lesions of the parabrachial nucleus eliminated such contrast in rats (Grigson et al., 1994). Whereas excitotoxic lesioning of the hippocampus produced no significant change in the consummatory negative contrast, it did eliminate a negative contrast in runway behavior after a 12 to 1 downshift in the pellet reward (Flaherty et al., 1998a). The lack of influence of hippocampal lesioning on consummatory negative contrast was also reported by previous work using colchicine as the neurotoxin (Flaherty et al., 1989).

The purpose of this study was twofold. First, we determined the effects of systemic administration of diazepam on the consummatory negative contrast. As previously mentioned, there is a limited body of research concerning the neural mechanisms of contrast effects. It is presumed that the potential anticontrast effects of systemic diazepam are derived from the drug acting on the benzodiazepine receptors in the brain. Although the benzodiazepine receptors are widely distributed in the CNS, they are particularly abundant in structures of the limbic system such as the amygdala and hippocampus (Mehta and Shank, 1995; Niehoff and Kuhar, 1983; Richards and Mohler, 1984; Squires, 1983; Young and Kuhar, 1980; Young et al., 1981). Thus, the second purpose of this study was to assess the possibility of the amygdala and the hippocampus serving as the neural substrate(s) for diazepam in the induction of such recovery effects on the consummatory negative contrast.

2. Methods

2.1. Subjects

The subjects were male Wistar rats weighing 200 ± 25 g on arrival. With the permission of the Institutional Laboratory Animal Committee, they were purchased from the Breeding Center of Experimental Animals at National Taiwan University Hospital, Taipei, Taiwan. Each rat was housed individually in a vivarium with a 12:12-h light–dark cycle (lights on at 0700 h). All experimental sessions were conducted between 0900 and 1500. The temperature of the colony was maintained at 23 ± 1 °C throughout the experiment. Rats were provided with Purina rat chow (5001) and tap water ad libitum during the 14-day adaptation to the animal colony. Subsequently, all subjects were maintained on a restricted feeding regimen that provided a limited amount of food pellets (~5–15 g per day) to maintain their body weight at about 85% of free-feeding. The body weight of the subjects were 310 ± 28 g at the start of the contrast experiment. An interval of 30 to 60 min separated the end of experimental sessions and a daily period of food intake. Treatment of rats complied in all respects with the Chinese Psychological Association's ethical standards for the use of animals in research (Chinese Psychological Association, 1996).

2.2. Drug

Diazepam HCl (Sigma) was dissolved in vehicle containing 40% propylene glycol and 10% ethyl alcohol mixed in saline.

2.3. Surgery and microinjection

Under sodium pentobarbital (40 mg/kg ip) anesthesia, each rat underwent a standard stereotaxic operation for bilateral implantation of the stainless steel cannula. Atropine sulfate (0.25 mg/kg) was also given to reduce mucous secretions. The rat was placed in the stereotaxic instrument (David Kopf Instruments). After the scalp was incised, the scalp muscle was reflected from the skull. Bilateral burr holes were drilled in the cranium to permit lowering of the 23-gauge guide cannulae to the specific stereotaxic coordinates. Two jewelry screws were fixed on the front and posterior skull to serve as anchors. This entire assembly was secured onto the skull with dental cement. The tips of the guide cannulae terminated 1.5 mm above the acute injection site. Stainless steel stylets were inserted into the guide cannulae to maintain the patency of the guides until the microinjections were conducted. At the end of surgery, penicillin (50,000 IU) was intramuscularly administered to reduce the likelihood of postoperative infection. Subjects were allowed 7 days to recover from surgery.

At the time of the licking test, the stylets were replaced by 28-gauge injection needles each connected with PE20 tubing to a 2-μl Hamilton microsyringe. For each infusion site, drug or vehicle solution was administered in a volume of 0.5 μl over 1 min. At the end of infusion, the injector needles were left in place for one additional minute to enhance diffusion from the infusion site. As determined by Paxinos and Watson (1986), the coordinates for the final infusion sites in the central amygdala and dorsal hippocampus were AP = − 2.3 mm, L = ± 4.2 mm, D = − 8.0 mm, and AP = − 3.3 mm, L = ± 2.2 mm, and D = − 3.2 mm, respectively. The AP and L coordinates were determined relative to the bregma, and D as depth was determined relative to the dura surface.

2.4. Apparatus

A likometer apparatus (DiLog Instruments and System) was used to measure licking. A stainless steel tube similar to that used in home cages was mounted in the front of the licking test cage. The tube could be reached with about 1 mm of tongue extension into an opening. This water source was a 25-ml burette, whose lower end was connected to a stainless steel spout fixed at 5 cm above the cage floor. The drinking tube was connected to a circuit that passed a current of no more that 60 nA through the rat each time its tongue made contact with the tube. The commercial software, Quick Lick, controlled the collection and analysis of raw data. The dependent variable adopted for measuring

the licking performance was the number of licks. This variable represents the total number of licks or the number of times the tongue made contact with the tube for each session.

2.5. Procedure

In the first part of the experiment, we determined the dose effects of systemic administration of diazepam on the consummatory negative contrast. After adaptation to food deprivation, 36 subjects were randomly assigned to two groups ($n = 18$ per group). One group of subjects was exposed to and trained with a 4% sucrose solution, while the other group was exposed to and trained with a 32% sucrose solution. The subjects were given 5 min of daily access to their respective sucrose solution in the test cage for 10 days. By the end of this phase, most of the rats had reached an asymptotic level of consumption of the sucrose solutions. Both groups were then given 4% sucrose solutions on the following 2 days as the test phase for negative contrast. The licking performance was measured for 5 min daily in these two postshift sessions. During the phase of negative contrast, each group was further divided into three subgroups ($n = 6$ each) for receiving the dose treatments of diazepam (0, 1, and 5 mg/kg, respectively). With a constant volume of 1 ml/kg, the injection was conducted via an intraperitoneal route 30 min before commencement of the behavioral session.

The second part of the experiment investigated the effects of diazepam locally infused into the amygdala or the hippocampus on consummatory negative contrast. For subjects with surgical preparation for an intra-amygdala or intrahippocampus microinjection, the licking experiment was continuously conducted for 14 days following the postsurgery recovery. In the first 10 days, as aforementioned, the subjects were randomly assigned to two groups ($n = 20$ each), which received the daily 5-min access to either the 4% or 32% sucrose solution in the test cage. Both groups were then given the 4% sucrose solution on the following 4 days as the test phase of the negative contrast. During the postshift sessions, each group was further divided into two subgroups ($n = 10$ each) for receiving a microinjection of diazepam (30 μg) or vehicle, respectively. The licking test was conducted for 5 min immediately after the completion of the microinjection procedure.

2.6. Histology

After the behavioral testing, subjects were given an overdose of sodium pentobarbital and perfused intracardially with normal saline followed by 10% formalin. The brain was removed and placed in a sucrose/formalin mixture for at least 24 h. The brain was sectioned at 40 μm with a freezing microtome. The mounted slices were stained with cresyl violet for verifying the locations of the cannula tips. Behavioral data from two subjects with intra-amygdala treatment were excluded, because their injection sites fell beyond the boundary of the target or were not symmetrical. All cannula placements for intra-hippocampus treatment were located within the intended site of infusion.

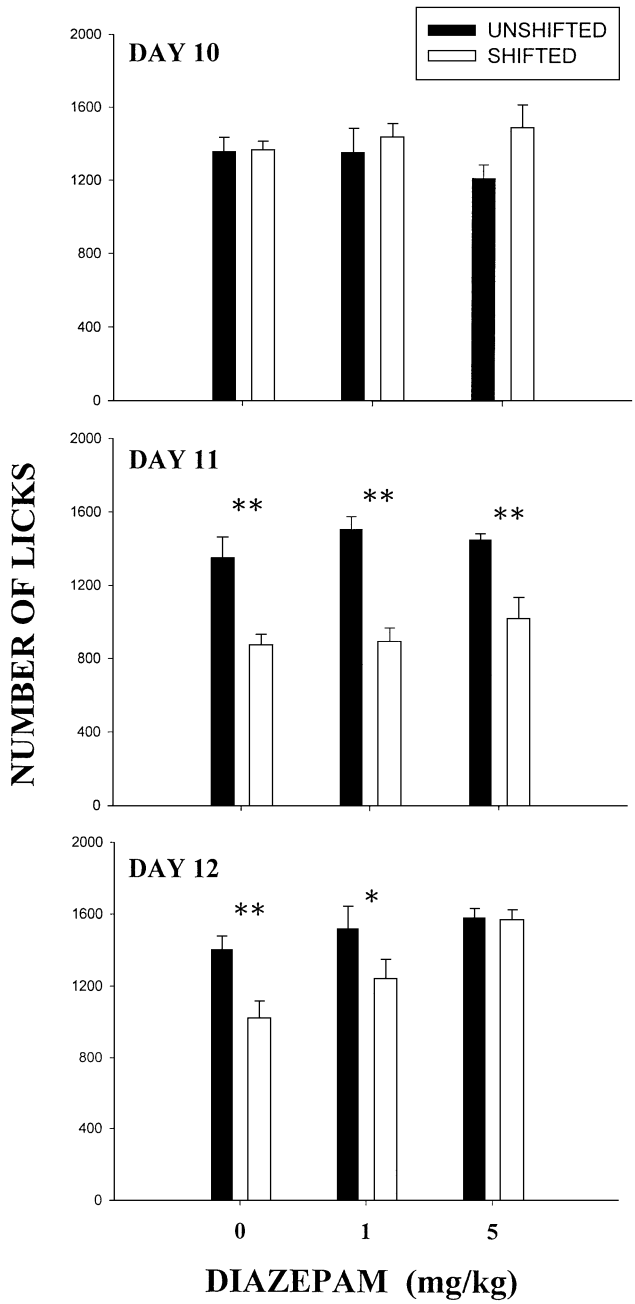

Fig. 1. Dose effects of diazepam on the number of licks (mean ± 1 S.E.M.) for the last day of the preshift phase (Day 10, top panel) and for 2 days of the postshift period (Day 11, middle panel, and Day 12, bottom panel). The shifted group received 32% sucrose for the first 10 days and was then given 4% sucrose for the remainder of the experiment (Day 11 and Day 12), whereas the unshifted group was provided with 4% sucrose throughout the experimental sessions. $*P < .05$, $**P < .01$; significant difference between shifted and unshifted groups for the indicated dose treatment.

2.7. Statistical analyses

All data were assessed using a two-way analysis of variance (ANOVA) with planned comparisons (Statistica, version 5.5). For the first part of the experiment, both systemic diazepam dosing and sucrose concentration were the between-subjects factors. For the second part of the experiment, sucrose concentration was a between-subjects factor, and the experimental day was a within-subjects factor. A probability level of $P < .05$ was taken as significant in all tests.

3. Results

Fig. 1 shows the dose effects of systemic administration of diazepam for the number of licks on the consummatory negative contrast. On the last day of the preshift phase (Day 10) as shown on the top panel of Fig. 1, no significant difference was revealed between the shifted and unshifted groups designed for receiving diazepam treatment on the following two postshift days. When the sucrose concentration was shifted from 32% to 4% on Day 11, as shown on the middle panel of Fig. 1, the shifted group had significant fewer licks compared to the unshifted group under each dose treatment of diazepam: $F(1,10) = 15.04$, $P < .01$ for the vehicle; $F(1,10) = 37.04$, $P < .001$ for the dose of 1 mg/kg; and $F(1,10) = 12.77$, $P < .01$ for the dose of 5 mg/kg. For the data presented in the middle panel of Fig. 1, an independent design of two-way ANOVA significantly confirmed the main effect of the sucrose concentration shift, $F(1,30) = 58.11$, $P < .001$. Neither the main effect of drug dosage nor the dose-by-shift interaction reached a significant level. Data collected on Day 12 as the second day of the postshift phase are presented in the bottom panel of Fig. 1. Under the vehicle control condition, the shifted group continued to produce significantly fewer licks than the unshifted group, $F(1,10) = 10.12$, $P < .01$. Such a significant difference between shifted and unshifted groups also appeared in subjects given 1 mg/kg diazepam, $F(1,10) = 5.84$, $P < .05$. However, no significant difference was revealed between the shifted and unshifted groups under the administration of 5 mg/kg diazepam ($P > .05$). For the data presented in the bottom panel of Fig. 1, an independent design of the two-

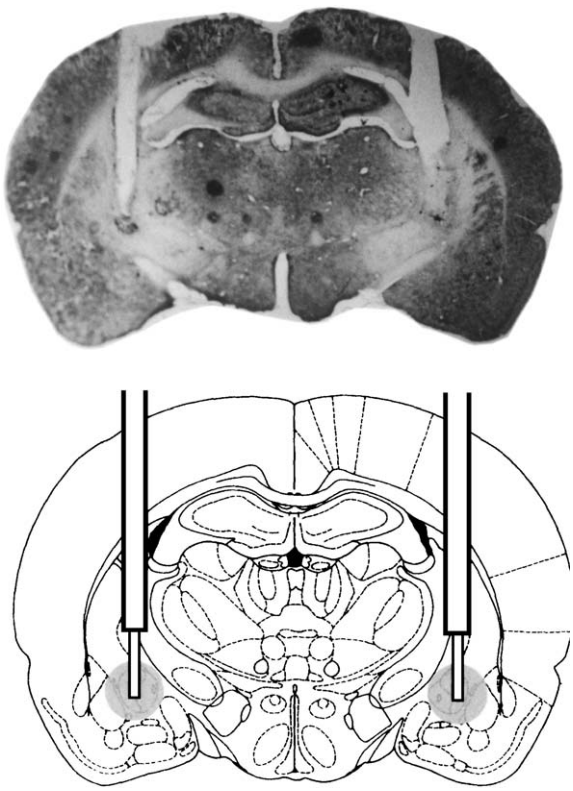

Fig. 2. Representative photomicrographs and schematic representations of coronal sections showing that the microinjections conducted in the amygdala were mainly located in the central nucleus. The photomicrograph (top) shows typical placement of the guide cannula. At the bottom, the line drawing adapted from Fig. 27 of Paxinos and Watson (1986), the circular gray area surrounding the central nucleus of the amygdala is where cannula placement was judged to be accurate.

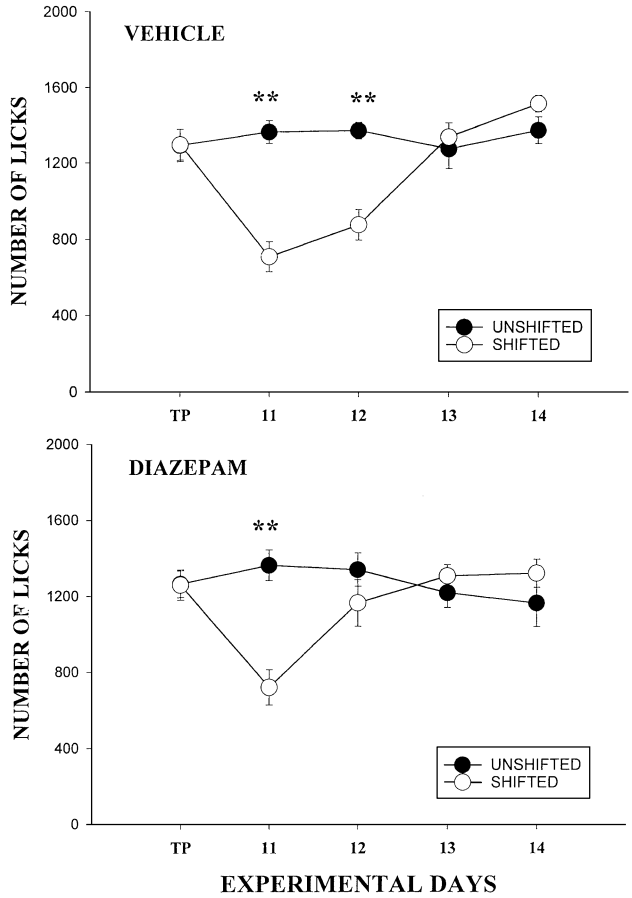

Fig. 3. The number of licks (mean ± 1 S.E.M.) on the terminal preshift day and on the four postshift days for the shifted (32–4%) and unshifted (4–4%) groups. Intra-amygdala microinjections of vehicle (top panel) and diazepam (30 μg, bottom panel) were conducted over four postshift days. ** $P < .01$; significant difference between shifted and unshifted groups on the indicated day.

way ANOVA significantly confirmed the main effects of the drug dose and sucrose concentration shift, $F(2,30) = 10.04$ and $F(1,30) = 13.41$, respectively (both $P < .001$). The dose by shift interaction was not significant.

Fig. 2 presents photographic and schematic illustrations showing the region of the amygdala, mainly located in the central nucleus, in which all correct microinjection sites for intra-amygdala treatments were included. The effects of intra-amygdala diazepam on the number of licks are presented in Fig. 3. Analysis of the number of licks with intra-amygdala vehicle control (Fig. 3, top) from the terminal preshift period through Day 14 revealed a significant negative contrast by showing a fewer number of licks in the shifted group on Days 11 and 12, $F(1,18) = 43.62$, $P < .001$ and $F(1,18) = 28.86$, $P < .01$, respectively. The curve of the shifted group overlaps that of the unshifted group on Days 13 and 14. In intra-amygdala vehicle subjects, negative contrast reliably appeared during the first two postshift sessions. As shown in the bottom panel of Fig. 3, intra-amygdala infusion of diazepam attenuated this contrast effect on Day 12. Such a case was not true for the first postshift test as statistical analysis revealed that the number of licks for the shifted and unshifted groups significantly differed on Day 11, $F(1,18) = 27.48$, $P < .001$.

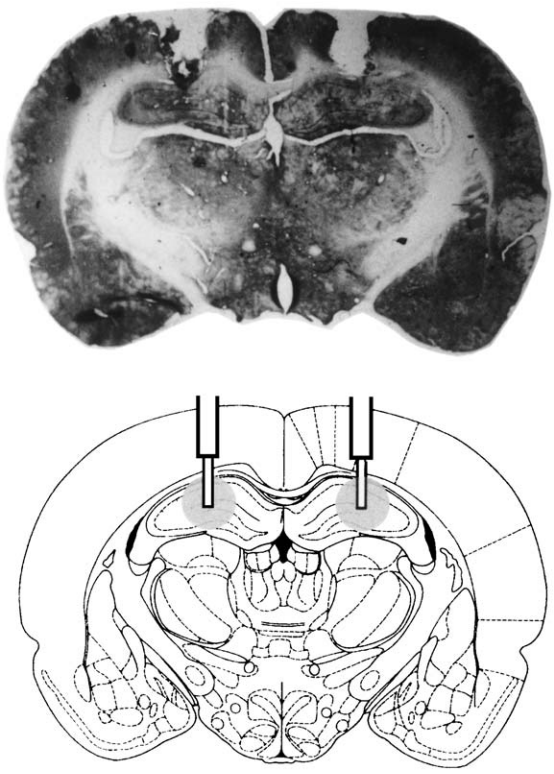

Fig. 4. Representative photomicrographs and schematic representations of coronal sections show the microinjections conducted in the dorsal hippocampus. The photomicrograph (top) shows typical placement of the guide cannula. At the bottom, the line drawing adapted from Fig. 31 of Paxinos and Watson (1986), the circular gray area surrounding the dorsal hippocampus is where cannula placement was judged to be accurate.

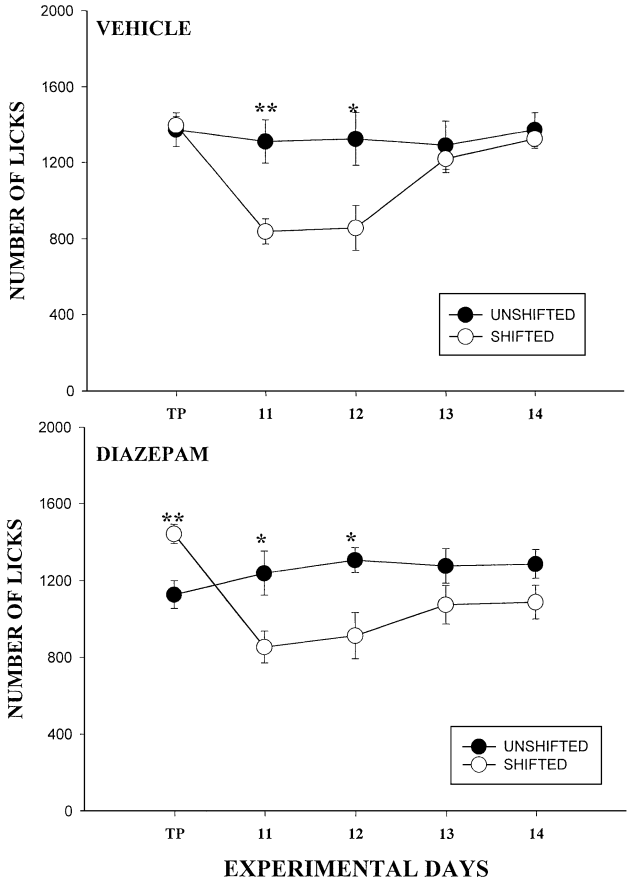

Fig. 5. The number of licks (mean ± 1 S.E.M.) on the terminal preshift day and on the four postshift days for the shifted (32–4%) and unshifted (4–4%) groups. Intrahippocampus microinjections of vehicle (top panel) and diazepam (30 μg, bottom panel) were conducted over the four postshift days. $*P < .05$, $**P < .01$; significant difference between shifted and unshifted groups on the indicated day.

Fig. 4 presents photographic and schematic illustrations showing the region of the dorsal hippocampus in which all correct microinjection sites for intrahippocampus treatments were included. The licking effects of intrahippocampus diazepam on the number of licks are presented in Fig. 5. As shown in the top panel of Fig. 5, the negative contrast effects significantly appeared on the first two postshift days under the intrahippocampus vehicle condition. Between-group differences in the number of licks were significant on Days 11 and 12, $F(1,18) = 12.77$, $P < .01$ and $F(1,18) = 6.55$, $P < .05$, respectively. Such a significant difference was not the case for either Day 13 or 14. From the bottom panel of Fig. 5, intrahippocampus diazepam treatment did not produce a significant anticontrast effect. Between-group differences in the number of licks remained at a significant level on Days 11 and 12, $F(1,18) = 7.36$ and $F(1,18) = 8.26$, respectively, both $P < .05$. A significant difference between the shifted and unshifted groups was confirmed on the terminal preshift day in the bottom panel of Fig. 5, $F(1,18) = 12.81$, $P < .01$.

4. Discussion

The present study demonstrated that systemic injection of diazepam alleviated the contrast by increasing the number of licks of shifted subjects, but not of unshifted subjects. This anticontrast effect was more profound with drug administration on the second day of the postshift phase. In addition to the systemic administration of diazepam, microinjection of this drug into the amygdala, but not the hippocampus, reduced the negative contrast of sucrose licking. This anticontrast effect of intra-amygdala diazepam was more conspicuous on the second postshift day.

In terms of systemic administration, diazepam applied in the present study produced a very similar pattern of contrast-reducing effects to that of chlordiazepoxide (Becker and Flaherty, 1983; Flaherty et al., 1980, 1986, 1990a), flurazepam (Flaherty et al., 1992), and midazolam (Becker, 1986; Flaherty et al., 1990b). From these previous studies and the present work, the effective dose(s) for alleviating the consummatory negative contrast were 6, 8, and 10 mg/kg for chlordiazepoxide, 5 mg/kg for diazepam, 20 mg/kg for flurazepam, and 1.0, 1.25, and 2.0 mg/kg for midazolam. Although the potency for reducing the contrast varied for each of these four drugs, it should be of no surprise that they are all categorized as benzodiazepines in terms of pharmacology (Feldman et al., 1997). Consummatory negative contrast reduction by benzodiazepines can be highly related to their ability to facilitate GABA-mediated neurotransmission in producing an anxiolytic effect (Flaherty, 1991, 1999). Further study is needed to reveal the exact mechanisms for addressing how these drugs modulate GABA at specific sites of GABAergic receptors to reduce the consummatory negative contrast. The $\alpha 2$ subunit of $GABA_A$ receptor is recently argued to mediate the anxiolytic effects of diazepam by the use of mice with subtle single point mutations in their $GABA_A$ receptor subunits (Löw et al., 2000; Rudolph et al., 2001).

In addition to alleviating the consummatory negative contrast by diazepam as reported in this work, this compound, given at 1.0, 2.0, or 2.5 mg/kg ip, was effective in eliminating a negative contrast built into a one-way avoidance task (Morales et al., 1992; Torres et al., 1995, 1996). Based on the time spent in a safe compartment acting as an appetitive incentive during one-way avoidance learning, this type of successive negative contrast was manipulated by reducing the magnitude (i.e., 30 to 1 s) of negative reinforcement (Candido et al., 1992). Moreover, flumazenil, a benzodiazepine antagonist, was shown to eliminate the abolition of the avoidance negative contrast of diazepam (Torres et al., 1994). Evidence from this series of studies supports the idea that the GABA system is involved in the anticontrast effect of diazepam. In combination with the current data, a common mechanism underlying the effects of diazepam on the successive negative contrast could exist in both consummatory and avoidance paradigms.

To localize the effects of diazepam in the CNS, the present work found that intra-amygdala, but not intrahippocampus, infusions of diazepam significantly attenuated the consummatory negative contrast. These differential effects were particularly profound on the second postshift day (Day 12). Reduction in the contrast on this specific day of the postshift phase produced by an intra-amygdala infusion of diazepam was similar to that observed from systemic administration of this drug. Thus, the amygdala may be an important location where diazepam induces an anticontrast effect in the brain, at least in comparison to the hippocampus. The evidence from lesion work also supports the role of amygdala involvement in mediating consummatory negative contrast. Lesioning of the amygdala abolished the consummatory negative contrast, and the magnitude of the lesion effects was dependent upon which subareas were destroyed in the amygdaloid complex (Becker et al., 1984). No such anticontrast effect on consummatory negative contrast was seen for lesions applied in limbic areas other than the amygdala, such as the hippocampus or septum (Flaherty et al., 1973, 1989). In addition, given that it contains a great deal of limbic inputs, the nucleus accumbens was assumed to play a modulator role in comparing how the reward value changed. However, lesioning of the nucleus accumbens produced no significant change in consummatory negative contrast (Eagle et al., 1999; Leszczuk and Flaherty, 2000). Although the nucleus accumbens as a target area for amygdaloid efferents was shown not to be involved in processing consummatory negative contrast, the parabrachial nucleus as a source of amygdaloid afferents was demonstrated to have a link with this contrast paradigm. Lesions of the parabrachial nucleus eliminated such contrast (Grigson et al., 1994). Therefore, from these lesioning data, the amygdala is critically important for processing of consummatory negative contrast. This notion is also compatible with the present findings of localization in the amygdala for diazepam to produce its anticontrast effect.

The contrast effect induced in the present task may be relevant to the conflict effect observed in various types of animal models of anxiety, given that both types of behavioral reactions result from disruption of emotionality (i.e., anxiety). Anti-conflict effects after intra-amygdala infusion of diazepam have been shown in several types of animal models of anxiety. Intra-amygdala diazepam treatment reversed the avoidance of brightness by increasing exploration in the light–dark test in mice (Costall et al., 1989). Behavioral suppression in either the Geller or the Vogel conflict tests was attenuated by intra-amygdala diazepam treatment (Nagy et al., 1979; Scheel-Kruger and Petersen, 1982; Shibata et al., 1982, 1989). These data indicate that the anxiolytic effect of diazepam can be attributed to drug action in the amygdala, most likely via activating the GABA/benzodiazepine receptor. This argument is in agreement with the present finding of an anticontrast effect of intra-amygdala diazepam. However, for the present negative contrast task, whether GABA/benzodiazepine receptors in

the amygdala are activated by diazepam awaits further testing.

The lack of an anticontrast effect of diazepam locally infused into the hippocampus on the second day of the postshift phase reflects three interesting issues. First, the hippocampus and the amygdala have different degrees of involvement in consummatory negative contrast. In addition, the role of the amygdala is more critical than that of the hippocampus in modulating this type of contrast. Second, in comparison to the amygdala, the hippocampus might be subservient to the processing of another type of negative contrast. Given that the negative contrast paradigm can be built upon consummatory and runway operant-like behavior, excitotoxic lesioning of the hippocampus indeed left the behavioral response to the consummatory negative contrast intact but eliminated a negative contrast in runway behavior after a 12 to 1 downshift in the pellet reward (Flaherty et al., 1998a). The elimination of contrast after reward reduction in runway behavior was also observed in rats with electrolytic lesion of fimbria–fornix, which is a major input–output pathway of the hippocampus (Salinas and White, 1998). Third, the lack of an effect of intrahippocampus diazepam on the consummatory negative contrast may be due to the location of the microinjection conducted in the present work being limited only to the dorsal part of the hippocampus. Based on the assumption of the heterogeneous function of the hippocampus, further work is needed to determine whether other subareas of the hippocampus are sensitive to diazepam in reducing the consummatory negative contrast.

One might be interested in the capacity of diazepam to ameliorate the effects of successive negative contrast on the second, rather than the first, postshift day. The present study reliably observed this delayed effect of diazepam administered via either intraperitoneal route or intra-amygdala infusion. This outcome is consistent with previous work testing chlordiazepoxide on consummatory negative contrast (Flaherty et al., 1980, 1986, 1990a). Differential effects of benzodiazepines to reverse the successive negative contrast between the first and second postshift days may be attributed to distinct behavioral/physiological processes that occurred within these 2 days. According to a multistage hypothesis (Flaherty, 1999), searching behavior appears as the initial reaction to reward reduction. Then, emotional response is presented in the second stage, which involves stress. This notion is supported by the evidence of elevated level of corticosterone observed from the rat under successive negative contrast on the second, but not the first, postshift day (Flaherty et al., 1985; Mitchell and Flaherty, 1998). Also, the contrast on the second postshift day is more related to the conditioned fear as revealed by a recent factor analytic study (Flaherty et al., 1998b).

In conclusion, the present study shows that a reliable anticontrast effect can be induced by diazepam administered either peripherally or locally infused into the amygdala. These data indicate that the amygdala is involved in the recovery effects of benzodiazepines on consummatory negative contrast.

Acknowledgements

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