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Extracellular dopamine in the rat prefrontal cortex during reward-, punishment- and novelty-associated behaviour. Effects of diazepam

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Abstract

Variations of extracellular dopamine (DA_{ext}) levels in prefrontal cortex were assessed by in vivo microdialysis. In rats trained in an operant fixed interval (FI_{30s}) schedule of food delivery, acute exposure to contingent foot shocks resulted in a suppression of responding that was reversed by diazepam (4 mg/kg, ip). No changes in cortical DA_{ext} levels occurred during this period in both control and treated rats. By contrast, in control rats, cortical DA_{ext} levels increased (+25–40%) during the nonpunished component of the operant session, and during noncontingent food delivery (+25%). Control rats placed into an unfamiliar brightly lit openfield exhibited a marked increase in cortical DA_{ext} levels (+100%). This effect occurred neither in rats given diazepam at a dose (2 mg/kg) which stimulated motor activity, nor during a second exposure to the openfield. In conclusion, a benzodiazepine-sensitive activation of mesoprefrontal DA neurones is induced by exposure to novel stressful surroundings and by food availability and consumption. The fact that cortical DA_{ext} levels remained unchanged in rats that exerted complete control upon negative stimuli indicates that an activation of the mesoprefrontal DA system is not required for punishment-induced behavioural blockade.

Keywords: Anxiety; Dopamine; Microdialysis; Prefrontal cortex; Punishment; Rat; Reward; Stress

1. Introduction

Mesoprefrontal dopaminergic neurones projecting from the ventral tegmental area to the prefrontal cortex seem to be activated by aversive events. Thus, several kinds of stresses have been found to increase cortical levels of the metabolites of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and/or homovanillic acid (HVA), as well as cortical DA outflow evaluated by the in vivo microdialysis technique, in awake freely behaving rats. This has notably been reported for restraint stress (Imperato et al., 1990), foot shocks of either low intensity (Reinhard et al., 1982; Inoue et al., 1994) or high intensity (Chrapusta et al., 1997), tail-shock or tail-pinch (Abercrombie et al., 1989; Jedema and Moghaddam, 1994; Gresch et al., 1995), swim stress (Jordan et al., 1994; Petty et al., 1997), social defeat (Tidey and Miczek 1996), exposure to a novel environment (Feenstra et al., 1995), handling (Enrico et al., 1998; Feenstra et al., 1998; Inglis and Moghaddam, 1999; Kawahara et al., 1999), conditioned fear (Inoue et al., 1994; Wedzony et al., 1996), and even psychological stress (Kaneyuki et al., 1991). In addition, the mesocortical dopaminergic pathway seems selectively activated by low levels of stress, whereas other pathways are recruited by stresses of higher levels (Lavielle et al., 1978). The prototypical benzodiazepine anxiolytic, diazepam, on acute administration, prevented stress-induced activation of DA turnover or release (Reinhard et al., 1982; Claustre et al., 1986; Kaneyuki et al., 1991; Feenstra et al., 1995; Finlay et al., 1995; Petty et al., 1997, but see Imperato et al., 1990), suggesting the existence of a close relationship between cortical dopaminergic neurotransmission and stress-related disorders.

However, all these results were obtained in situations that involved uncontrollable aversive stimuli, whereas only few studies investigated the consequences of controllable negative events on the activity of mesocortical DA neurones (Miyauchi et al., 1988; Heinsbroek et al., 1991). Yet, controllability appears to be a crucial characteristic of the negative stimuli used in experimental procedures of anxiety.

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For instance, in Geller and Seifter-like operant conflict paradigms (Geller et al., 1962; Cook and Davidson, 1973), rats trained to press a lever for food reward, exhibit intense operant responding during nonpunished periods (purported to be 'non-anxiogenic'), whereas an almost complete behavioural suppression occurs, due to response-dependent foot-shock delivery associated with food, during punished periods (purported to be 'anxiogenic'). A drug-induced increase of punished responding is considered as indicative of an anxiolytic effect.

The present microdialysis study aimed at examining whether variations of cortical DA_{ext} levels occur in the course of a benzodiazepine-sensitive operant conflict, in an attempt to correlate biochemical and behavioural data. To this end, an operant conflict schedule of reinforcement was modified to allow concomitant analysis of rats' behaviour and collection of 15-min microdialysis samples for DA_{ext} measurements during the punished and nonpunished periods. In order to determine the possible nonspecific effects of response rate, food consumption, and shocks on cortical DA_{ext}, rats were also given noncontingent food pellets, or noncontingent foot shocks. In addition, the effect of novelty on cortical DA_{ext} levels was investigated in rats subjected to an unfamiliar brightly lit openfield. Finally, the behavioural and neurochemical effects of diazepam were studied under these experimental conditions.

2. Materials and methods

2.1. Animals

The experiments were carried out on male Wistar AF rats (C.E.R.J., Le Genest, France) weighing 100 ± 10 g at the beginning of the training and 350–400 g at the time of the experiments. They were housed eight per cage (until surgery) under standard laboratory conditions (12-h light–dark cycle, lights on at 0700 h; room temperature 21 ± 1°C) with free access to food and water in their home cage (except where otherwise specified). The procedures were conducted in conformity with the institutional guidelines on animals and their cares, in compliance with international and national laws and policies (European Communities Council directives no. 86/609/EEC, November 24, 1986, and French Council directives no. 87–848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions no. 0299 to M.H., and no. 0597 to M.H.T.).

2.2. Surgical procedure

Rats were cannulated under chloral hydrate (400 mg/kg, ip) anaesthesia. A vertical stainless guide cannula (15 mm long; 0.9 mm o.d.; 0.6 mm i.d.) was implanted in vertical position into the centre of the prelimbic region of the left medial prefrontal cortex, so that the tip of the dialysis membrane reached the following coordinates, according to the rat brain atlas of Paxinos and Watson (1986): AP + 2.7 mm anterior to bregma; ML 0.6 mm lateral; DV − 4.0 mm below the surface of the dura. A dummy stainless steel stick was left in the guide until the microdialysis experiment. The guide was secured to the skull with cyanoacrylate glue and dental acrylic cement (GC Unifast).

From the surgery onwards, rats were housed singly in plastic cylinder cages (26 cm in diameter; 33 cm high), with free access to food and water during 6 days, before returning to the usual restricted food regimen (see Sec. 2.5.1.2.).

2.3. Microdialysis device

Probe construction has been described in details elsewhere (Beaufour et al., 2001). Briefly, concentric dialysis probes were made in the laboratory with polyacrylonitrile fibers (Hospal ref. AN69HF, France), allowing a 2-mm dialysis contact with the cerebral tissue. The in vitro recovery of probes for DA, determined at random from different batches, was on average 15%. The in vivo values of DA_{ext} have not been corrected for this recovery.

On the test day, the probe cannula was connected to an inlet polyethylene tubing. The outlet line was made of a silica glass capillary tubing (TSP 75/150, Phymep, dead volume of 3 μl). Probes were perfused with a buffered Ringer solution (140 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, 1.0 mM $MgCl_2$, 2.0 mM sodium phosphate buffer, pH = 7.4) at a flow rate of 1.0 μl/min. The solution was delivered to the probe via a liquid swivel mounted on a counterbalanced arm fixed above the experimental device. Dialysate fractions (15 μl/15 min) were collected at the level of the liquid swivel, in vials containing a 6-μl mixture of 0.1 N perchloric acid and 0.33 mM L-cysteine to prevent DA oxidation.

2.4. High performance liquid chromatography (HPLC) analysis

DA contents of the dialysate samples were determined without prior purification using HPLC coupled with coulometric detection. The apparatus consisted of a C_{18} reverse phase column (3-μm size particle; 4.6 × 150 mm^2, LC-18-DB, Supelco) kept at 29°C and perfused at a flow rate of 1 ml/min with a mobile phase containing 30 mM sodium phosphate buffer, 0.48 mM *l*-octane-sulfonate, 0.5 mM EDTA, 5–7% methanol, 4% acetonitrile, pH = 3.5. DA elution was monitored using a coulometric detector (Coulochem II; 5014 B cell; Eurosep) with electrodes set at − 150 and + 250 mV.

Twenty microliters of the dialysate samples were automatically injected into the HPLC column every 20 min by a refrigerated (4°C) sample injector (Autosampler Midas, type 830, Spark). DA (elution time: 5–7 min) was quantified by reference to authentic standards. The minimal amount of DA that could be reliably quantified, i.e., that yielded a 3:1 signal/noise ratio, was 3 fmol/dialysate sample.

2.5. Combined behavioural and microdialysis procedure

The operant device, training, and experimental schedules of reinforcement have been described in details elsewhere (Beaufour et al., 2001). Only a brief description of those specific points is made herein.

2.5.1. Operant procedure

2.5.1.1. Operant apparatus. The initial training was conducted in standard ventilated, sound attenuated operant chambers ($24 \times 22 \times 21$ cm^3) (Campden Instruments, UK), fitted with two levers, an electrified grid floor and an automatic magazine delivering food pellets (45 mg, Campden Instruments), and supplied with one light (24 V, 3 W) located above the right lever.

After surgery, additional training sessions and the microdialysis session were conducted in modified operant chambers ($25 \times 22 \times 27$ cm^3), custom-made with Campden pieces of material, supplied with retractable levers. The food tray was modified to facilitate the access to the pellets and only the right lever was inserted into the box at the beginning of every session conducted in these chambers. This was done in order to reduce the risk of damaging the dialysis probe protruding from the head of the animals on the test session. Holes in the ceiling of the chambers and their sound-insulated cubicle allowed the passage of the microdialysis tubing.

2.5.1.2. Operant sessions. One week prior to the beginning of the training, rats were placed on a daily schedule of food restriction (13 g of standard chow/day/rat) that was maintained until the end of the experiments (except where otherwise specified).

Training sessions. Rats were subjected 5 days a week to 45-min operant sessions, during which they were trained to press the right lever according to a fixed interval (FI) schedule of food delivery that was progressively lengthened from FI_{1s} to FI_{30s}. The light located above the right lever provided the sole illumination of the chamber throughout the sessions. The numbers of right lever presses and pellets earned by each rat were recorded every 5 min. Presses on the left lever had no consequence and were not recorded. About 15 sessions were necessary to ensure stable responding under the FI_{30s} schedule. The FI_{30s} schedule of food reinforcement was chosen in order to restrict the number of food pellets that the rat could earn, and therefore avoid satiation-induced reduction of responding in the course of the session.

Test session. On the day of the microdialysis study, rats were subjected to a single 45-min operant test session during which the schedule of reinforcement was modified. The operant session consisted of an initial 30-min nonpunished period, during which rats were rewarded, as during training, by food pellets delivered according to a FI_{30s} schedule, followed by a 15-min punished period, during which presses were both rewarded by a food pellet and punished by a mild electric foot shock (0.5 mA, 45 ms) according to a FI_{30s} schedule of reinforcement. This punished period was not signalled by a particular stimulus.

2.5.1.3. Experimental design. After the postsurgery recovery period, rats were given six to nine additional 45-min FI_{30s} training sessions in the modified operant chambers. In order to be familiarized to the entire procedure required for the microdialysis study, rats were left in the darkened chamber for at least 2 h before the beginning of the operant sessions (signalled by the insertion of the right lever and the illumination of the right light). Animals were also allowed to stay in the operant chamber overnight before at least one of these training sessions and before the microdialysis test session. For all these periods, they were equipped with a harness attached by a thin stranded wire to the counterbalanced arm fixed outside the insulating cubicle. Rats were also habituated to the injection procedure by receiving an intraperitoneal administration of saline (0.9% NaCl) immediately before the beginning of the last four training sessions.

On the test morning, rats were gently hand-restrained to insert the microdialysis probe into the guide cannula and were returned to the chamber. Three and a quarter hours after the beginning of the perfusion, three 15-min dialysis samples were collected (Sample nos. 14–16) to determine DA_{ext} basal levels. Rats were given diazepam, or its vehicle, 3 min prior to the 16th sample collection (this timing took into account the 3-μl dead volume from the probe outlet to the collection point), and were returned to the operant chamber. The active lever was immediately inserted and the right light turned on, indicating the beginning of the operant test session that consisted of two 15-min nonpunished periods (Sample nos. 17–18) and one 15-min punished period (Sample no. 19). The operant session ended by lever retraction and light off. After a 30-min rest period, corresponding to dialysate Sample nos. 20–21, rats were given a series of 15 noncontingent, electric foot shocks (0.5 mA, 45 ms), delivered randomly every 60 ± 40 s (Sample no. 22). After an additional 30-min rest period (Sample nos. 23–24), the rats were removed from the operant chamber. The noncontingent shock period was introduced in the microdialysis session to control for possible effects on cortical DA_{ext} of shocks per se. For this reason, they were applied at intensity and duration identical with those used in the conflict period, and their number, identical for all rats, was chosen as approximately the mean number of controllable shocks received by diazepam-treated animals. The order of presentation of the operant component (thus of the contingent shock period) and the noncontingent shock period was not counterbalanced across individual rats, to avoid the deleterious influence of the latter on overall lever pressing.

2.5.2. Noncontingent food pellets delivery

In a control experiment, food-restricted rats were trained in the FI_{30s} schedule of lever pressing for food reward, as described above. After guide cannula implantation and postsurgery additional training, they were subjected to a single microdialysis test session. As distinct from the usual procedure, after saline injection, the right lever was not inserted into the operant chamber when the right light stimulus was turned on, signalling the beginning of the session. Noncontingent food pellets were delivered by the experimenter, at a rhythm of one pellet every 30 ± 10 s during 45 min, corresponding to 15-min dialysis Sample nos. 17–19. At the end of this period, the right light was turned off, rats were given a 30-min rest period (Sample nos. 20–21), and then the right light was again turned on for 15 min, without lever insertion and without free pellets delivery (Sample no. 22). The session ended after an additional 30-min rest period (Sample nos. 23–24).

2.5.3. Openfield test

Independent groups of drug- and test-naive, nonfood-restricted, rats ($\sim$250 g) were implanted with a guide cannula, as described above. They were housed singly for a 6–10-day postsurgery period, during which they were habituated to the injection procedure and to the harness. After a night spent in a transparent acrylic microdialysis cage ($30 \times 30 \times 38$ cm^3), rats were subjected to a single microdialysis session, in an ambient dim light (7 lx). Dialysate sample collection started 3:15 h after dialysis probe insertion; three 15-min baseline samples (Sample nos. 14–16) were collected before the administration of diazepam or its vehicle. Thirty minutes later, rats were gently moved to an unfamiliar openfield for a 15-min period (Sample no. 19) and then back to the microdialysis cage for 45 min. At the end of this period, rats were moved again to the openfield for a second 15-min period (Sample no. 23), and back to the microdialysis cage for an additional 30-min rest period (Sample nos. 24–25). The openfield ($48 \times 48 \times 40$ cm^3) was made of wood painted in white and brightly lighted (175 lx); the floor was divided into 12 sectors by black lines. Rat behaviour was monitored by means of a video camera. The number of lines crossed every 3 min and the total time spent in the central square of the openfield were recorded.

2.6. Histological analysis

Immediately after the dialysis experiment, animals were deeply anaesthetised and sacrificed. Brains were removed and stored at -20°C. They were sectioned in a cryostat and stained with Cresyl violet for verification of probe location and absence of bleeding.

2.7. Drugs

Chloral hydrate (Sigma, France) was dissolved in saline (0.9% NaCl). Diazepam (Hoffmann-La Roche, Switzerland) was suspended in acacia gum, and the final volume adjusted with saline. Drug or vehicle was injected intraperitoneally in a volume of 5 ml/kg body weight.

2.8. Statistical analyses

2.8.1. Behavioural data

Results from the operant procedure were expressed as mean ($\pm$S.E.M.) total number of lever presses performed during the first two 15-min nonpunished periods, and total number of lever presses emitted and shocks received during the subsequent 15-min punished period. Responses of control and treated rats were compared independently during these three periods, using the two-tailed, unpaired Student's *t* test. In the openfield experiment, the mean ($\pm$S.E.M.) number of lines crossed and the time spent in the central square by control and treated rats were compared using the two-tailed, unpaired Student's *t* test.

2.8.2. Neurochemical data

Baseline levels of DA_{ext} were calculated for each rat from the mean of the three dialysate samples (Nos. 14–16) collected prior to vehicle or drug administration. The results were expressed as mean ($\pm$S.E.M.) of the individual percent changes from this basal value. The DA_{ext} levels (%) in dialysis samples collected after the injection were compared to baseline levels using the two-tailed, paired Student's *t* test. The DA_{ext} levels (%) in control and treated rats during each period of interest were compared independently by two-tailed, unpaired Student's *t* test.

Correlations between individual operant data and DA_{ext} levels (%) were calculated using the Bravais–Pearson's *r* coefficient of correlation.

3. Results

3.1. Operant behaviour

During the two 15-min nonpunished periods, control rats emitted 80–100 presses/15 min, and therefore the number of pellets obtained was near the maximum possible (23–27 pellets/15 min). The response-contingent delivery of electric foot shocks concomitant with food pellets during the subsequent period resulted in a progressive reduction of lever pressing (circa 20–25 presses/15 min) and rats received, on the average, 6–7 shocks + pellets/15 min (Fig. 1A).

During the punished period, rats given diazepam (4 mg/kg) emitted more responses than their vehicle-injected counterparts (22.3 ± 7.2 vs. 120.7 ± 41.3 lever presses, $t_{12} = 2.35$, $P < .05$) and received more shocks (6.7 ± 1.6 vs. 17.4 ± 3.6, $t_{12} = 2.69$, $P < .05$).

During the two initial nonpunished periods, diazepam-treated rats also made more lever presses than controls (100.3 ± 16.3 vs. 176.4 ± 27.0, $t_{12} = 2.41$, $P < .05$, and

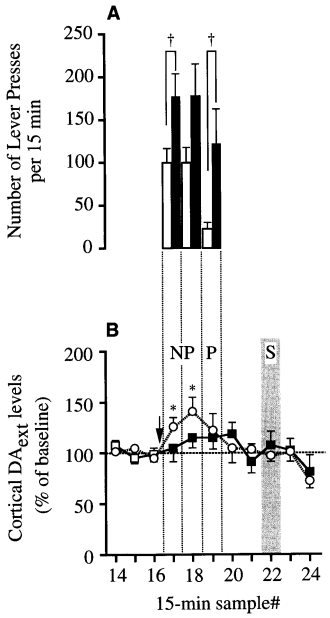

Fig. 1. Effects of diazepam on (A) the number of lever presses for food in 15-min periods during nonpunished and punished components of the operant conflict procedure, and (B) extracellular DA levels measured in prefrontal cortex, in dialysate samples collected before injection (baseline Sample nos. 14–16), during the operant session (Sample nos. 17–19), during noncontingent foot-shock delivery (Sample no. 22) and during resting periods (Sample nos. 20–21, nos. 23–24). During the operant session, rats were rewarded by food pellets according to a FI_{30s} schedule of reinforcement during the first two 15-min nonpunished periods (NP) and received both one pellet and one electric foot shock (0.5 mA, 45 ms) according to the same FI_{30s} schedule during the subsequent, nonsignalled, 15-min punished period (P). Rats were never subjected to the punishment paradigm before the dialysis experiment. During noncontingent shock delivery (S), rats were given 15 electric foot shocks (0.5 mA, 45 ms) delivered randomly every 60 ± 40 s. Extracellular DA was measured in 15-min dialysate samples and expressed as percentage of the mean baseline value in Sample nos. 14–16, collected before the injection and the beginning of the operant session (mean basal levels of DA, 100% = 9.37 ± 0.72 fmol/20 μl). Diazepam (4 mg/kg, $n = 7$, closed symbols) or vehicle ($n = 7$, open symbols) was injected intraperitoneally at the beginning of the operant test session (↓). Results are expressed as mean ± S.E.M. *$P < .05$ vs. baseline levels (paired Student's t test), †$P < .05$ vs. controls during the same period (unpaired Student's t test).

98.7 ± 17.9 vs. 176.9 ± 36.9, $t_{12} = 1.91$, $P = .08$, respectively). This unexpected effect, not observed in experiments devoted to the pharmacological validation of the present schedule of reinforcement (unpublished) and in other microdialysis studies (Beaufour et al., 2001), was due to performances of control rats which were twofold lower in the present study than in the previous ones.

3.2. Extracellular DA levels in the prefrontal cortex during operant conflict paradigm and noncontingent foot-shock delivery

The mean basal levels of cortical DA_{ext} measured in Sample nos. 14–16 collected before the injection and the beginning of the operant session were 9.95 ± 1.37 and 8.78 ± 0.59 fmol/20 μl for the to be vehicle- and diazepam-injected groups of rats, respectively (Fig. 1B).

Compared to baseline, cortical DA_{ext} levels were significantly enhanced in vehicle-injected rats during the first two nonpunished periods (Sample no. 17: +25%, paired $t_6 = 3.35$, $P < .05$; Sample no. 18: +41%, $t_6 = 3.11$, $P < .05$), but not during the subsequent 15-min punished period (Sample no. 19). In rats given diazepam (4 mg/kg), DA_{ext} levels did not differ significantly from baseline, whatever the period considered. In control animals, individual cortical DA_{ext} levels measured in the dialysate Sample no. 18 seemed to be related to the number of lever presses performed during the corresponding nonpunished period; however, probably because of the small number of animals, the correlation failed to reach the critical level of statistical significance ($r = +.72$, $P = .07$). No such relationship was observed during the other periods of the operant session, or in diazepam (4 mg/kg)-treated rats, whatever the period considered (largest $r = +.11$).

Cortical DA_{ext} levels measured in dialysate samples collected during noncontingent delivery of 15 electric foot shocks (Sample no. 22), or during the next rest period (Sample no. 23), did not significantly differ from baseline in rats that had received vehicle or diazepam (4 mg/kg) 75 min before (largest paired $t_6 = 1.08$, ns). There were no between group differences in cortical DA_{ext} levels measured in Samples no. 22 and 23 (both unpaired $t_{12} < 1$, ns).

3.3. Extracellular DA levels in the prefrontal cortex during noncontingent pellets delivery

DA_{ext} levels were significantly enhanced during noncontingent delivery of food. Compared to baseline, this effect was significant during the last 30 min, corresponding to Sample no. 18 (+25%; paired $t_6 = 2.70$, $P < .05$) and Sample no. 19 (+23%; $t_6 = 2.45$, $P = .05$). The presentation of the right light stimulus only did not modify cortical DA_{ext} levels in the corresponding Sample no. 22 ($t_6 = 1.22$, ns) (Fig. 2).

3.4. Behaviour and extracellular DA levels in the prefrontal cortex during exposure to an unfamiliar openfield

In control rats introduced for the first time in a brightly lit openfield, locomotor activity, as measured by the number of lines crossed, rapidly declined over time. Rats given diazepam (2 mg/kg), 30 min before the test, exhibited a significant overall increase of activity (lines crossed in 15 min: 41.9 ± 3.6 vs. 99.4 ± 23, $t_{12} = 2.47$, $P < .05$), which was particularly marked during the first 3-min period (31.9 ± 1.9 vs. 64.0 ± 7.5, $t_{12} = 4.17$, $P < .001$), and also reached statistically significant level during the last period of the session (0 ± 0 vs. 7.7 ± 2.9, $t_{12} = 3.03$, $P < .01$). Diazepam-treated rats spent more time in the central square than control rats (1.0 ± 0.5 vs. 4.9 ± 1.4 s, $t_{12} = 2.59$, $P < .05$) (Fig. 3).

The mean basal levels of cortical DA_{ext} in Sample nos. 14–16 collected before the injection were 3.90 ± 1.24 fmol/20 μl and 3.97 ± 1.04 fmol/20 μl for the to be vehicle- and diazepam-injected groups of rats, respectively.

In control rats, cortical DA_{ext} levels were significantly increased above baseline in Sample no. 19 collected during the first exposure to the openfield (+103%, paired $t_6 = 4.11$, $P < .01$), but not in the next three samples. In diazepam-treated rats, DA_{ext} levels never differed significantly from baseline. Between-group comparisons indicated that DA_{ext} levels (%) were lower in rats given diazepam compared to controls during the first openfield test (203.0 ± 25.1 vs. 120.7 ± 10.2, $t_{12} = 3.04$, $P < .01$) and the next rest periods (Sample no. 20: 152.6 ± 24.2 vs. 83.6 ± 23.2, $t_{12} = 2.06$, $P = .06$; Sample no. 21: 131.3 ± 23.0 vs. 65.4 ± 19.3, $t_{12} = 2.19$, $P < .05$; Sample no. 22: 128.6 ± 14.2 vs. 78.6 ± 8.4, $t_{12} = 3.03$, $P < .01$).

When rats were exposed to the openfield for the second time, the activity of controls was clearly reduced compared to the first session, and diazepam (2 mg/kg, 105 min before) no longer stimulated locomotion (lines crossed in 15 min, controls: 13.3 ± 3.0, DZP: 8.6 ± 1.4, $t_{12} = 1.42$, ns). Cortical DA_{ext} levels in the corresponding Sample no. 23 were significantly above baseline in control rats, (+54%, paired $t_6 = 3.40$, $P < .02$), but not in rats given diazepam, resulting in a significant between group difference (153.6 ± 15.8 vs. 84.1 ± 24.7, $t_{12} = 2.37$, $P < .05$). However, in both vehicle- and diazepam-injected rats, DA_{ext} levels in Sample no. 23 did not differ from the corresponding levels measured during the preceding period. Therefore, when DA_{ext} levels in Sample no. 23 were expressed as percentage of DA_{ext} measured in Sample no. 22, vehicle- and diazepam-injected rats no longer differed from each other (140.4 ± 33.3 vs. 103.7 ± 20.4, $t_{12} = 0.94$, ns). During the following resting period (Sample nos. 24–25), DA_{ext} levels in both groups of rats did not significantly differ from baseline.

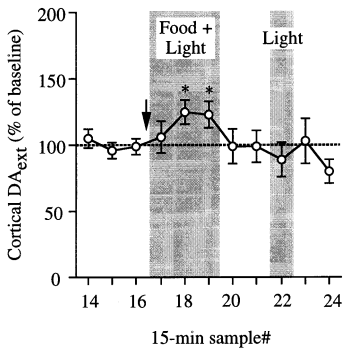

Fig. 2. Cortical DA_{ext} levels during periodic, response-independent, food pellet delivery (Sample nos. 17–19), and during presentation of the right light stimulus usually associated with the operant session (Sample no. 22). Rats, previously trained in the FI_{30s} schedule of lever pressing for food reward, were subjected to a single dialysis session during which the right light stimulus was turned on, but no lever was inserted into the operant chamber. During a 45-min light on period, 90 pellets were delivered by the experimenter (one pellet every 30 ± 10s). Only saline-injected rats ($n = 7$) were subjected to this procedure. ↓: injection time. Mean basal levels of DA, 100% = 10.54 ± 1.63 fmol/20 μl. Results are expressed as mean ± δ S.E.M. $^{*}P < .05$ vs. baseline levels (paired Student's t test).

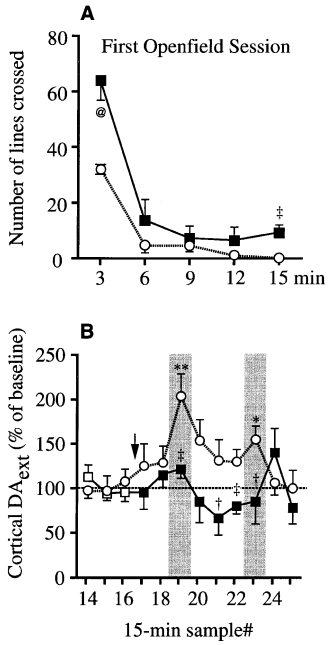

Fig. 3. Effects of diazepam on (A) the number of lines crossed per 3-min bins during the first 15-min exposure to an unfamiliar, brightly lit, openfield, and (B) extracellular DA levels measured in prefrontal cortex, in dialysate samples collected before (baseline Sample nos. 14–16) and after injection (Sample nos. 17–18), during the first (Sample no. 19) and the second (Sample no. 23) exposure to the openfield, and during resting periods (Sample nos. 20–22, nos. 24–25). Mean basal levels of DA, 100% = 3.94 ± 0.78 fmol/20 μl. Diazepam (2 mg/kg, $n = 7$, closed symbols) or vehicle ($n = 7$, open symbols) was injected intraperitoneally 30 min before the first openfield test (↓). Results are expressed as mean ± S.E.M. $^{*}P < .05$, $^{**}P < .01$ vs. baseline levels (paired Student's t test). $^{\dagger}P < .05$, $^{\ddagger}P < .01$, $^{@}P < .001$ vs. controls during the same period (unpaired Student's t test).

4. Discussion

As expected in an operant conflict procedure, the acute delivery of mild, nonpainful, electric foot shocks contingent to lever pressing for food reward resulted in a progressive reduction of responding. This behavioural blockade was released by diazepam, an effect classically considered as reflecting the anxiolytic activity of this benzodiazepine. The concomitant microdialysis study indicated that extracellular DA levels in the medial prefrontal cortex did not change during the punished period, whether or not rats were given diazepam. Therefore, mesocortical DA neurones seem unlikely to play a crucial role in anxiety-related behaviour and in the action of benzodiazepines thereon, at least as they can be approached in operant conflict procedures.

However, this conclusion deserves comments with regard to several sets of data. First, controllable shocks (but not uncontrollable ones) have been shown to reduce selectively DA turnover in the frontal cortex (Miyauchi et al., 1988),

and an anxiolytic-like release of punished responding has been described in rats whose mesocortical DA neurones were selectively destroyed (Ravard et al., 1990). Thus, the expression of punishment-induced behavioural suppression appears to require the integrity of DA neurones afferent to the prefrontal cortex, but seems not necessarily associated with their activation.

Second, the possibility to eat food, or even to chew objects, has been claimed to attenuate the effects of stress and to suppress selectively stress-induced increases in DA neurotransmission in the prefrontal cortex (Berridge et al., 1999). Since, in the present study, rats earned some pellets during the punished period, it could be the case that eating would have masked otherwise increased cortical DA_{ext} levels.

Third, uncontrollable shocks also failed to modify DA_{ext} levels in the prefrontal cortex of the same rats. This result contrasts with the enhanced DA_{ext} levels frequently reported in animals given uncontrollable electric foot shocks (Reinhard et al., 1982; Inoue et al., 1994; Chrapusta et al., 1997), or subjected to various kinds of more or less severe aversive events (Imperato et al., 1990; Kaneyuki et al., 1991; Tidey and Miczek, 1996; Wedzony et al., 1996; Enrico et al., 1998; Feenstra et al., 1998; Kawahara et al., 1999). Since several studies suggest that the activation of mesoprefrontal DA neurones is positively related to stress intensity (Inoue et al., 1994; Feenstra et al., 1995; Morrow et al., 1999), it is possible that shocks were not high enough to trigger such an activation, although they were sufficient to suppress operant responding, and enhance striatal 5-HT_{ext} levels (Beaufour et al., 2001). It must be pointed out that rats were given about twice as many uncontrollable shocks as received by vehicle-injected rats during the conflict period, because the experimental design did not aim at controlling the influence of shock density but shock controllability. Indeed, the mesocortical DA response related to stress intensity, having matched the number of uncontrollable shocks to controllable ones (6–7, on average), would not have been more efficient in stimulating cortical DA release than the actual number of uncontrollable shocks (15, for all rats) delivered. Otherwise, it would be necessary to hypothesize a bell-shaped relationship between DA_{ext} levels and stress intensity, a phenomenon that has never been reported. Finally, previous studies showed that prior experience with response contingencies allowing control upon aversive events, as it was the case here during the punished period, can protect the animals from the deleterious consequences of inescapable stresses (Hannum et al., 1976). Such a phenomenon might have also accounted for the absence of variation in cortical DA_{ext} levels.

Fourth, several lesion studies indicated that mesocortical DA neurones projecting to subdivisions of the medial prefrontal cortex could respond differently to stressful stimuli. In particular, DA neurones projecting to the ventral aspect of the medial prefrontal cortex have been suggested to function in coping behaviour, while those projecting to the more dorsal areas are associated with anxiogenic responses (see references in Horger and Roth, 1996). Since the dialysis probe was placed within the centre of the prelimbic region of the medial prefrontal cortex, it might be the case that DA neurones afferent to the ventral and dorsal regions contributed differently, perhaps in opposite manner, to DA_{ext} levels measured in the present study.

During the nonpunished component of the operant test session, diazepam unexpectedly enhanced FI_{30s} responding, in contrast with most studies in which nonpunished responses were either not modified or slightly reduced by benzodiazepines (Cook and Davidson, 1973). Although this pattern of effect was much less marked, or even did not occur, in other microdialysis studies conducted with the same schedule of reinforcement (Beaufour et al., 2001), one can question whether the present procedure was suitable for examining anxiety-related behaviour or was only able to detect drug-induced general behavioural activation. In fact, in FI schedules, rats are reinforced for the first response that occurs after a fixed time interval, whatever the number of responses made before the interval has elapsed. In animals well trained to such FI schedules, a pause generally occurs immediately after the reinforcement, followed by response acceleration to a high terminal rate (Ferster and Skinner, 1957, Chapter 5). Factors such as changes of time perception and/or waiting ability (Thiébot et al., 1985; Ferguson and Paule, 1996; Evenden, 1998a,b), which can affect the within-trial response rates, might be involved in the diazepam-induced increase of nonpunished responding. On the other hand, the so-called 'frustrative non-reward' (Dantzer, 1977; Gray, 1977) could be another factor favouring the development of the post-reinforcement pauses. Indeed, the omission of an expected reward, like other negative reinforcers, induces a benzodiazepine-sensitive suppression of ongoing behaviour (Thiébot et al., 1979). Therefore, it cannot be excluded that, in this particular procedure, the nonpunished component is endowed with a certain degree of 'aversiveness.' If it is the case, the diazepam-induced increase of nonpunished FI_{30s} responding would more likely reflect an anxiolytic-like effect than a general, nonspecific, behavioural activation.

Extracellular DA measured in the prefrontal cortex of control animals was enhanced by 25–40% in the dialysis samples collected during the two initial nonpunished periods of the operant session, and this effect was prevented by diazepam. A similar FI_{30s} schedule of pressing for food also increased extracellular levels of DA in the rat nucleus accumbens (Cousins et al., 1999). Such increases in DA_{ext} unlikely relate to a potential aversive effect of the FI schedule. Indeed, according to the hypothesis that a 'frustrative non reward' phenomenon would participate in the control of instrumental responding, it could be predicted that the more sensitive were the rats to nonreward, the lower should be their FI_{30s} response rate, and the higher their DA output. This was not the case since, in nontreated rats, a positive relationship was found between responses and

DA_{ext} levels measured in both the prefrontal cortex (present study) and the nucleus accumbens (Cousins et al., 1999). On the other hand, in keeping with previous studies indicating that DA release in cortical areas can be evoked by food intake (Feenstra and Botterblom, 1996; Feenstra et al., 1999), a progressive increase in cortical DA outflow was observed during periodic, response-independent, pellet distribution. This effect reached levels comparable to those measured during the nonpunished periods, and was maintained as long as food was present. Although food-associated stimuli have been found to enhance DA release in the prefrontal cortex (D'Angio and Scatton, 1989; Bassareo and Di Chiara, 1997), the presentation of the food-associated light stimulus did not modify cortical DA_{ext} levels in the present experimental conditions, indicating that consummatory behaviour and the unconditioned stimuli associated with feeding were essential to stimulate cortical DA neurotransmission. Alternatively, the light on stimulus might have acquired poor motivational significance due to its constant association with the operant lever, and therefore might not be an effective predictor of food delivery.

Together, these results suggest that the increase in cortical DA outflow was more likely accounted for by food availability and consumption than by enhanced arousal, pure motor activation, or the putative aversive component of the nonpunished FI_{30s} schedule. The relative reduction of DA release during the punished component of the session would therefore result from the overall smaller number of pellets earned and consumed (although not strictly identical in all rats, due to schedule contingencies) during this period compared to the preceding ones. Diazepam did not alter the quantity of food obtained during nonpunished periods, but counteracted the concomitant increase in cortical DA_{ext} levels. This suggests that the latter effect was not an indirect consequence of food intake per se, but resulted from an independent action of diazepam on mesoprefrontal DA neurones.

It is worth noticing that DA concentrations measured in dialysis samples collected at baseline were higher in rats trained for several weeks in the operant task (9–10 fmol/20 μl) than in test-naive rats to be subjected to the openfield test (4 fmol/20 μl). The observed difference in cortical DA_{ext} levels could be accounted for by stimuli-induced anticipation of food consumption, as suggested by other studies (D'Angio and Scatton, 1989; Phillips et al., 1991; Bassareo and Di Chiara, 1997). However, since rats spent at least 16 h in the operant chamber before collection of baseline dialysis samples, it seems unlikely that contextual cues had retained some ability to activate DA output due to anticipation of feeding. On the other hand, the rats tested in the openfield were fed ad libitum, whereas those trained in the operant procedure were subjected to food restriction. As a matter of fact, food deprivation has been shown to enhance DA utilization in the prefrontal cortex (Carlson et al., 1987). Interestingly, a reduction in both the levels of mRNA encoding the DA transporter (DAT) and the DAT functional activity (DA reuptake) has been described in ventral tegmental area/substantia nigra of food-deprived rats (Patterson et al., 1998). Such an effect could possibly account for higher DA_{ext} levels in terminal fields of dopaminergic neurones, including the prefrontal cortex, as observed in the starving rats of the present study.

In the openfield procedure, a clear-cut increase (+100%) of cortical DA outflow was observed in rats placed for the first time in an unknown, brightly lit enclosure. Such an activation of DA neurotransmission occurred neither in animals given diazepam nor during a second openfield session, when the apparatus was more familiar. On the other hand, diazepam facilitated rat's overall motor activity in this procedure. This effect was particularly marked during the first minutes of the session, when novelty and uncertainty were maximal, indicating that emotional factors limited locomotion and/or exploration. Diazepam also enhanced the time spent in the central square of the openfield, purported more anxiogenic than the periphery. Altogether, these results indicate that cortical DA neurotransmission is activated when animals are faced to unconditioned continuous fear stimuli, i.e., to complex environmental signals of unknown threat to which they have no prepared efficient responses.

In conclusion, moderate electric foot shocks, able to suppress lever pressing for food reward, did not affect the release of DA in the prefrontal cortex, suggesting that mesocortical DA neurones play no crucial role in punishment-induced behavioural suppression and its release by benzodiazepines. This result also indicates that the punished components of conflict procedures cannot be equated to unconditioned stresses. By contrast, as previously reported (Feenstra et al., 1995), cortical DA_{ext} levels were clearly increased during exposure to novel stressful surroundings, giving further support to the idea that mesoprefrontal DA neurones participate in behavioural responses to unconditioned environmental signals of fear, and/or in emotional or cognitive processes necessary to cope with such negative stimuli. In keeping with other studies (D'Angio and Scatton, 1989; Feenstra and Botterblom, 1996; Bassareo and Di Chiara, 1997), mesocortical DA neurones were activated when food was available, and, although the experimental design did not allow a total control over the amounts of food consumed, the present results indicate that this occurred whether or not food delivery was response-dependent.

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