# Significance of Dopamine Transmission in the Rat Medial Prefrontal Cortex for Conditioned Fear

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**Previous studies have demonstrated activation of dopamine transmission in the medial prefrontal cortex (mPFC) by conditioned fear stimuli. Therefore, the present study investigated the functional significance of mPFC dopamine for a conditioned fear response to a tone. We examined the effects of inhibition or stimulation of mPFC dopamine transmission by local microinfusion of the D1/D2-receptor antagonist *cis*-flupenthixol or the indirect dopamine receptor agonist D-amphetamine, respectively, in a classical fear-conditioning paradigm in Wistar rats. Rats received tone–shock pairings and were later exposed to the tone alone. Freezing was used as measure of conditioned fear. Presence of the drugs in the mPFC during the tone–shock pairings did not affect freezing during subsequent presentation of the tone alone. However, when *cis*-flupenthixol and D-amphetamine were present in the mPFC during presentation of the tone alone, freezing to the tone was reduced. We demonstrated that the decreased freezing could be explained neither by state dependency nor infusion-induced alterations in activity. Our data indicate that mPFC dopamine transmission is important for the retrieval/expression, but not the formation, of conditioned fear. The reduction of conditioned fear by prefrontal infusion of both *cis*-flupenthixol and D-amphetamine may reflect normal expression of conditioned fear requires an optimal level of mPFC dopamine activity.**

## Introduction

In rats and humans, the medial prefrontal cortex (mPFC) is characterized by similar connections and has been suggested to play a pivotal role in emotional responses (Nauta, 1971; Davidson and Irwin, 1999; Bechara *et al.*, 2000; Öngür and Price, 2000; Simpson *et al.*, 2000). Research on emotion in laboratory rodents has focused on fear, the neural substrates of which can readily be studied in classical fear-conditioning experiments (LeDoux, 2000). In classical fear conditioning, an emotionally neutral conditioned stimulus (CS), such as a tone, is paired with an aversive unconditioned stimulus (US), usually a foot shock, so that subsequent presentation of the CS elicits conditioned fear responses.

Conditioned fear is accompanied by marked alterations in mPFC neurotransmission. In particular, neurochemical studies in rats have consistently suggested activation of mPFC dopamine transmission by presentation of a CS previously paired with foot shocks [(Goldstein *et al.*, 1994, 1996; Inoue *et al.*, 1994; Yoshioka *et al.*, 1996; Morrow *et al.*, 1999b,c; Feenstra *et al.*, 1999, 2001; Feenstra, 2000), but see Wilkinson *et al.* (Wilkinson *et al.*, 1998)]. This activation was proposed to be necessary for the normal expression (Morrow *et al.*, 1999c) or extinction of fear responses to a CS (Morrow *et al.*, 1999b; Feenstra *et al.*, 2001). Moreover, specific patterns of electrical activity (Garcia *et al.*, 1999; Baeg *et al.*, 2001) and increased extracellular norepin- ephrine (Feenstra *et al.*, 1999, 2001) were evoked in the mPFC by fear CS.

The functional significance of the reported alterations in mPFC neurotransmission for a normal conditioned fear response is not clear. Currently, a popular view is that processes in the mPFC are important for inhibitory control of conditioned fear (Maren, 2001). This view is based on the findings that lesions to the dorsal (mainly anterior cingulate) mPFC enhanced acquisition of conditioned fear (Morgan and LeDoux, 1995; Vouimba *et al.*, 2000) and lesions to the ventral mPFC impaired fear extinction (Morgan *et al.*, 1993; Quirk *et al.*, 2000). Importantly, destruction of the most caudal and ventral infralimbic mPFC was demonstrated to be critical for the extinction deficit (Quirk *et al.*, 2000). Based on the finding that 6-hydroxydopamine lesions of the mPFC, depleting the local catecholamine, in particular dopamine, innervation, reduced extinction of very strong, but not moderate, conditioned fear, mPFC dopamine was proposed to be important for fear extinction under certain conditions (Morrow *et al.*, 1999b). There is also strong evidence that some subregions of the mPFC are important for the normal expression of conditioned fear. Thus, in line with Nauta's (Nauta, 1971) earlier proposition that frontal cortex lesions result in an 'interoceptive agnosia', reflected by an absence of normal emotional responses, lesions destroying the central mPFC, including prelimbic and infralimbic parts, but sparing the most caudal and ventral infralimbic portions, reduced the expression of conditioned fear (Frysztak and Neafsey, 1991, 1994; Lacroix *et al.*, 2000b; McLaughlin *et al.*, 2001). Finally, there are several studies (Holson, 1986; Rosen *et al.*, 1992; Gewirtz *et al.*, 1997) in which mPFC lesions, destroying considerable parts of the prelimbic and infralimbic subregions, did not affect conditioned fear responses. This may be related to compensatory processes induced by permanent lesions (Bast *et al.*, 2001a). Altogether, the specific functional significance of prefrontal processes accompanying conditioned fear, the best documented of which is activation of dopamine transmission, awaits clarification.

In contrast to permanent lesions, local microinfusions of neuroactive substances allow the role of different transmitter systems in the mPFC during different stages of a fear-conditioning experiment to be addressed specifically. In order to clarify the functional significance of alterations in mPFC dopamine transmission for aversively conditioned emotional responses, the present study examined the effects of specifically manipulating mPFC dopamine transmission by local microinfusion of the dopamine D1/D2-receptor antagonist *cis*-flupenthixol or the indirect dopamine agonist D-amphetamine in a classical fear-conditioning paradigm in Wistar rats. Freezing, an immobile posture in response to an inescapable aversive CS (Fanselow, 1984), was used as measure of conditioned fear.

## Materials and Methods

### *Animals*

One hundred male adult Wistar rats (Zur:Wist[HanIbm]; Research Unit Schwerzenbach, Schwerzenbach, Switzerland), ~2 months old and

weighing ~250–300 g at the time of surgery, were used for the experiments of the present study (experiment I, 24 rats; experiment II, 39; experiment III, 37). The animals were housed under a reversed light–dark cycle (lights on 19:00–07:00) in a temperature (21 ± 1°C) and humidity (55 ± 5%) controlled room and were provided with food and water *ad libitum*. All rats received bilateral implantation of guide cannulae aiming at the mPFC. Before surgery, rats were housed in groups of four per cage; after surgery, they were individually caged. Beginning 3 days before surgery and thereafter until the beginning of the behavioral experiments, all rats were handled daily. All experimental procedures were carried out in the dark phase of the cycle. Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) and Swiss regulations for animal experimentation were followed.

***Implantation of Guide Cannulae for Intracerebral Infusion***

Rats were anesthetized with 1 ml of Nembutal (sodium pentobarbital, 50 mg/ml; Abbott Labs, North Chicago, IL) per kg body wt and their heads were placed in stereotaxic frames (Kopf Instruments, Tujunga, CA). After application of a local anesthetic (lidocaine), the scalp was incised to expose the skull, and bregma and lambda were aligned in the same horizontal plane. A pair of guide cannulae (9 mm, 26 gauge, stainless steel) in a Perspex holder (custom made) were implanted through small holes (1.5 mm diameter) drilled on each side of the skull. The tips of the guide cannulae aimed at the following coordinates (in mm): 3.0 anterior and ±0.5 lateral to bregma and 3.1 ventral to skull surface. The guide cannulae were fixed with dental cement for which three small stainless screws, previously screwed into the skull, served as anchors. Stainless steel stylets (34 gauge) which extended 0.5 mm beyond the tips of the guide cannulae were placed inside the guide cannulae to prevent occlusion. After surgery, the experimenters gave the rats daily health checks and gentle handling, and replaced missing stylets. The behavioral experiments commenced 5 days after surgery.

***Intracerebral Microinfusion and Drugs***

For microinfusions into the mPFC, rats were manually restrained and the stylets removed from the guide cannulae. Infusion cannulae (34 gauge, stainless steel, custom made) were then inserted into the guide cannulae. The infusion cannulae were fixed to 26 gauge cuffs at one end to prevent them from slipping through the guide cannulae and connected to 10 µl Hamilton microsyringes mounted on a microinfusion pump (KD Scientific or WPI sp200i). The tips of the infusion cannulae protruded into the mPFC 1.5 mm beyond the tip of the guide cannulae, thus aiming at a final coordinate of 4.6 mm below the skull surface. The rats were bilaterally infused with *cis*-flupenthixol dihydrochloride (FLU, 20 or 25 µg, $C_{23}H_{25}F_3N_2OS \times 2HCl$; Sigma, Switzerland) or D-amphetamine sulfate [AMPH, 13.6 µg (10 µg free base), $C_{18}H_{26}N_2 \times H_2SO_4$; Sigma, Switzerland] in 0.5 µl vehicle (0.9% saline) or with 0.5 µl vehicle (VEH) only. Drug solutions were freshly prepared on the day of infusion. The infusion speed was 0.5 µl/min. After infusion, the infusion cannulae were left in the brain for 60 s to allow for absorption of the infusion bolus by the brain tissue and then replaced by the stylets. Rats were then immediately subjected to the behavioral testing.

AMPH and FLU were chosen for this first examination of the functional significance of mPFC dopamine transmission for conditioned fear because these compounds affect both D1- and D2-receptor-mediated processes. Furthermore, we could draw on the experience from previous experiments performed in our laboratory (Lacroix *et al.*, 2000a) in which these drugs had been infused into the mPFC. While the indirect dopamine agonist AMPH may also stimulate the release of other monoamines (Azzaro and Rutledge, 1973), the direct nonselective dopamine agonist apomorphine also affects transmission by other monoamines; for example, apomorphine has similar affinities to dopamine and alpha1-adrenoceptors (Baldessarini *et al.*, 1994). AMPH was infused into the mPFC at a dose of 13.6 µg (corresponding to 10 µg of the free base)/0.5 µl/side in the present experiments because this dose produced a behavioral effect in a previous study from our laboratory (Lacroix *et al.*, 2000a). With regard to FLU, 12 µg/0.5 µl/side infused into the mPFC did not yield any behavioral effect in the aforementioned study (Lacroix *et al.*, 2000a) and so a dose of 20 µg/0.5 µl/side was used in the first fear-conditioning experiment (experiment I). Since this dose yielded a behavioral effect without any adverse side effect (e.g. disrupted motor coordination, brain damage), a slightly higher dose of 25 µg/0.5 µl/side was used in the final fear conditioning experiment (experiment III) in order to obtain the behavioral effect reliably.

Two experiments were conducted to control for the low pH (2–3) of the FLU solutions. Subjects were tested for the expression of conditioned fear and for activity following infusion of acidified (adjusted to pH 2 by concentrated hydrochloric acid, $n = 5$), as detailed below (experiment I, activity experiment). No significant behavioral effects were observed relative to rats infused into the mPFC with normal saline (pH 6–7, $n = 5$, data not shown). Therefore, subsequent comparisons were made between rats infused into the mPFC with FLU solutions (pH 2–3) and subjects receiving mPFC infusion of normal saline (pH 6–7).

***Apparatus for Behavioral Testing***

Eight operant test boxes, four shock boxes and four no-shock boxes (Habitest; Coulborn Instruments, Allentown, PA) were used for the fear-conditioning experiments (experiments I–III). Shock boxes were used for conditioning and context-test sessions, while the no-shock boxes were used to assess fear to the tone CS in an environment distinct from that during conditioning. Shock boxes were fitted with a parallel grid shock floor (16 parallel bars, E10-10RF; Coulborn Instruments), through which scrambled shocks could be delivered, and placed in light- and sound-attenuating chambers measuring 55 × 40 × 55 cm (wide × long × high). These chambers had two side walls of aluminium and rear and front walls of clear Perspex. A white waste tray was situated below the grid floor. The four no-shock boxes were fitted with a lattice grid (E10-18NS; Coulborn Instruments) and placed in light- and sound-attenuating chambers measuring 72 × 45 × 45 cm. They had three black walls and a front wall of clear Perspex. A brown waste tray was situated below the lattice grid. The four shock and the four no-shock boxes were placed in two different rooms. Presentation of the tone CS and delivery of electric foot shock were controlled by a PC with dedicated software (S. Frank, Psychology Department, University of Tel Aviv, Israel) connected to a Coulborn Universal Environment Interface (E91-12) with Coulborn Universal Environment Port (L91-12). The tone CS [85 dB(A)] was produced by a 2.9 kHz tone module (E12-02) fixed at one wall of the operant chamber. Shocks were delivered with a Coulborn Precision Animal Shocker (E13-12), which generated bipolar rectangular 10 ms current pulses with a frequency of 10 Hz. Background noise was provided by a ventilation fan affixed to the light- and sound-attenuating chambers during all sessions. A monochrome minivideo camera with a wide angle (100°) 2.5 mm lens (VPC-465B; CES AG, Zurich, Switzerland) was attached to the center of the ceiling of each operant chamber. Four infrared (875 nm) light-emitting diodes (HSDL-4220; Hewlett Packard) positioned in the ceiling of each operant chamber provided light sufficient for camera function. Throughout all sessions, images from the test boxes were provided by these cameras, integrated into a four-quarter single image (100 000 pixels) by a multiplexer (DX216CE; Sony) and recorded by a video-recorder (SVT1000; Sony).

Sixteen cubicles (25 × 40 × 40 cm), each contained within an individual sound-attenuating wooden cabinet, were used for the activity experiment examining the effects of FLU and AMPH infusion into the mPFC on general activity. The cubicles had three walls of clear plastic and one side wall of wooden panels. The floor of each cubicle was a black removable pan holding a thin layer of dark, absorbent, autoclaved earth. The ceiling was open. A fan mounted on the wall of each cabinet provided ventilation. A monochrome minivideo camera with a wide angle (100°) 2.5 mm lens (VPC-465B; CES AG, Zurich, Switzerland), centered ~49 cm above the compartment floor, was mounted in the ceiling of each cabinet. Six infrared (875 nm) light-emitting diodes (HSDL-4220; Hewlett Packard) positioned in the ceiling of each cabinet provided light sufficient for camera function. Images provided from each of the 16 cubicles by these cameras were integrated into a sixteen-part single image (400 000 pixels) by a 16-channel multiplexer (YS-DX216CE; Sony) and the image was recorded by a video-recorder (SVT1000; Sony).

*Automated Measurement of Activity and Freezing*

The video images were transferred to a computer (fear-conditioning experiments, 7600/120 Power Macintosh; control experiment, Dell OptiPlex GXpro with a Pentium Pro Processor) equipped with an analysis program (Image, http://rsb.info.nih.gov/nih-image) and a macroprogram

(P. Schmid, Behavioral Neurobiology Laboratory, Swiss Federal Institute of Technology Zurich). The percentage of changed pixels between two adjacent 1 s images from one of the test boxes was used as a measure of activity (Richmond *et al.*, 1998). Freezing is commonly identified as cessation of any movement except for respiratory movements (Anagnostaras *et al.*, 1999). If the percentage of changed pixels between two adjacent 1 s images was <0.05%, this corresponded well to such immobility and the behavior of the rat was scored as 'freezing' for the respective second. Validation and principle of the automated analysis of freezing behavior have been described in detail in previous publications (Richmond *et al.*, 1998; Pryce *et al.*, 1999).

### *Fear-conditioning Experiments*

Three fear-conditioning experiments (experiments I–III) were conducted testing the effects of FLU or AMPH infusion into the mPFC on the formation, retrieval/expression and extinction of fear conditioning to a tone. In all three experiments there were three types of sessions: conditioning, context-test and tone-test sessions. Conditioning sessions consisted of two (experiment I) or six (experiments II and III) tone–shock pairings (30 s tone coterminating with a 1 s, 0.5 mA foot shock) preceded and followed by 2 min (experiment I) or 1.5 min blocks (experiments II and III) in the shock boxes. Context-test sessions consisted of 8 min during which the rats were placed in the shock boxes without receiving any stimulation. Context-test sessions were conducted to assess long-term conditioned fear (i.e. conditioned fear persisting beyond the conditioning session) to the context in which the foot shocks were experienced, as well as for extinction of fear to the context, in order to obtain a pure measure of fear to the tone during the subsequent tone test. Tone tests, to assess long-term conditioned fear to the tone, were conducted in the no-shock boxes – i.e. an environment distinct from the conditioning context – and consisted of a continuous 8 min tone presentation, preceded by 2 min without stimulation. During all sessions, freezing was assessed by the automated analysis system as a measure of conditioned fear. In those experiments involving drug infusions before conditioning (experiments II and III), the effects of the drug infusions on unconditioned behaviors were assessed by observing some unconditioned behaviors in the different infusion groups during the conditioning session. The video images taken during conditioning were observed in order to determine whether all infusion groups reacted to the shock by similar vigorous twitching and jumping – marked components of the unconditioned immediate shock response (Anagnostaras *et al.*, 1999). Activity before the first tone or shock was assessed using the percentage of changed pixels between adjacent 1 s video images given by the automated system. The different infusion groups, as well as conditioning and test procedures, in experiments I, II and III are described in detail below and summarized in Table 1.

#### *Experiment I*

In experiment I, rats received bilateral infusions of VEH ($n$ = 8), AMPH (13.6 μg, $n$ = 8), or FLU (20 μg, $n$ = 8) into the mPFC immediately before the tone-test session. The three infusion groups were matched for freezing levels during conditioning and the context test. The conditioning session consisted of two tone–shock pairings. Using a low number of pairings, the aim was to achieve a relatively low level of conditioned freezing to the tone in order to facilitate detection of an extinction deficit. Context-test sessions were conducted 1 day after conditioning, tone-test sessions 2 days after conditioning.

#### *Experiments II and III*

Experiment I indicated that infusions of FLU as well as AMPH before tone test resulted in reduced conditioned freezing to the tone, suggesting that both treatments interfere with the retrieval/expression of conditioned fear. Experiments II and III were aimed at confirming this suggestion. In particular, we tested whether the reduced conditioned freezing during tone test might merely have reflected state dependency, i.e. that associations formed in an altered brain-state may subsequently be retrieved only in a similar brain-state (Overton, 1964), rather than an impairment of the specific processes underlying retrieval/expression of conditioned fear. For that purpose, FLU (experiment II) and AMPH (experiment III) were infused not only immediately before the tone test, but also before conditioning sessions. Thus, the results of experiments II and III are also relevant regarding the role of mPFC dopamine transmission in the formation of conditioned fear. In both experiments all rats received a first infusion of either VEH or drug immediately before conditioning and a second infusion immediately before the tone test. In experiment II, all rats received either bilateral infusions of VEH ($n$ = 19) or FLU (25 μg; $n$ = 20) into the mPFC before conditioning. Immediately before the tone test all rats received a second infusion of VEH or FLU. Half of the rats received the same infusion as before conditioning and the other half received a different infusion, resulting in four groups differing with respect to the combinations of infusions (before conditioning–before tone test): VEH–VEH ($n$ = 9); VEH–FLU ($n$ = 10); FLU–VEH ($n$ = 10); and FLU–FLU ($n$ = 10). In experiment III, all rats received either bilateral infusions of VEH ($n$ = 18) or AMPH (13.6 μg; $n$ = 19) into the mPFC before conditioning. Immediately before the tone test, all rats received a second infusion of VEH or AMPH. Half of the rats received the same infusion as before conditioning and the other half received a different infusion, resulting in four groups differing with respects to the combinations of infusions (before conditioning–before tone test): VEH–VEH ($n$ = 8); VEH–AMPH ($n$ = 10); AMPH–VEH ($n$ = 9); and AMPH–AMPH ($n$ = 10). In both experiment II and III, the groups that received the same infusion before conditioning, but different infusions before the tone test, were matched for freezing levels during the conditioning and context-test sessions.

For conditioning, rats were put in the shock boxes for a total of 13.5 min and received six tone–shock pairings. A higher number of pairings than in experiment I was used in order to facilitate the detection of infusion-induced reductions in conditioned freezing. Six days after conditioning, a context test was conducted to minimize residual contextual freezing during the subsequent tone test. The tone test was conducted 7 days after conditioning. The time span between conditioning and test sessions was chosen so that repeated infusions were 1 week apart in order to allow for a recovery of the brain tissue from disturbances that may result from one intracerebral infusion before applying the next one.

**Table 1**
Summary of the procedures in the three fear-conditioning experiments (experiment I-III)

| Experiment | Infusion | Conditioning (shock box) | Context test (shock box, 8 min) | Tone test (no-shock box, 10 min, tone on after 2 min) |
|---|---|---|---|---|
| Experiment I | Immediately before tone test: VEH, FLU (20 μg/side), AMPH (13.6 μg/side) | Two tone–shock pairings each preceded and followed by 2 min blocks (7 min) | One day after conditioning | Two days after conditioning |
| Experiment II | Immediately before conditioning and tone test: VEH, FLU (25 μg/side) | Six tone–shock pairings each preceded and followed by 1.5 min blocks (13.5 min) | Six days after conditioning | Seven days after conditioning |
| Experiment III | Immediately before conditioning and tone test: VEH, AMPH (13.6 μg/side) | As in experiment II | As in experiment II | As in experiment II |

AMPH, D-amphetamine sulfate; FLU, *cis*-flupenthixol dihydrochloride; VEH, vehicle. Tone: 30 s, 2.9 kHz, 85 dB(A). Shock: 1 s, 0.5 mA foot shock, concurrent with the last second of the tone. Conditioned fear, in the form of freezing, during conditioning and testing was automatically measured in the form of freezing.

### *Activity Experiment*

Twenty-three rats used in experiment I were subjected to an additional experiment, 2 weeks after termination of experiment I, in order to test whether FLU or AMPH in the mPFC induced alterations in general activity that might have influenced the freezing response (Bast *et al.*, 2001b). (In experiments II and III, unconditioned activity was monitored before the first shock during the conditioning sessions, i.e. during the first 1.5 min

following the infusions, but possible infusion-induced alterations in unconditioned general activity after these first 1.5 min could not be assessed.) On day 1, basal activity was measured for 30 min. The animals were then allocated to one of three infusion groups, counterbalanced with respect to the infusions received during experiment I and matched according to their baseline activity. On day 2, rats received infusions of VEH ($n$ = 9), FLU (20 μg/side, $n$ = 7), or AMPH (13.6 μg/side, $n$ = 7) immediately before their activity was monitored for 2 h. On day 3, activity was measured for 30 min without a preceding infusion.

### *Histology*

After completion of the behavioral experiments, the cannulated rats were deeply anesthetized with an overdose of 2.5 ml/kg Nembutal (sodium pentobarbital, 50 mg/ml, i.p.) and transcardially perfused with 0.9% NaCl solution to rinse out the blood, followed by 250 ml of 4% formalin (4°C) to fix the brain tissue. After extraction from the skull, the brains were post-fixed in 4% formalin solution and subsequently cut into 40 μm coronal sections on a freezing microtome. For the verification of the infusion sites, every fifth section through the mPFC was mounted on a gelatine-treated slide and stained with cresyl violet. After staining, the sections were dehydrated and coverslipped. Subsequently, they were examined with a light microscope under ×10 magnification. It was verified that the tips of the infusion cannulae were placed in the mPFC and their approximate locations were drawn onto plates from the atlas of Paxinos and Watson (Paxinos and Watson, 1998). Moreover, brain damage around the infusion sites was compared between the infusion groups.

### *Data Analysis*

Statistical analysis was conducted using the Statview software system. From the freezing scores obtained for each second ('freezing' or 'not freezing'), the percentage of time spent freezing in a given time block was calculated. By averaging the percentage of pixels changed between adjacent 1 s video images in a given time block, a relative activity measure for this time block was calculated. Data were first subjected to analysis of variance (ANOVA), using the different infusions as between-subjects factor and the different time blocks of testing as repeated-measures factors. *Post hoc* comparisons were conducted using Fisher's protected least significant difference test. Level of significance was set at $P < 0.05$. Freezing during the context-test sessions is not reported, since it was negligible in all three experiments (on average <15%), reflecting that the conditioning procedures used in the present study did not result in considerable conditioned fear to the conditioning context. Data during the first four 1 min blocks of tone presentation served as a reliable measure of tone freezing that was not confounded by within-session habituation (Corcoran and Maren, 2001).

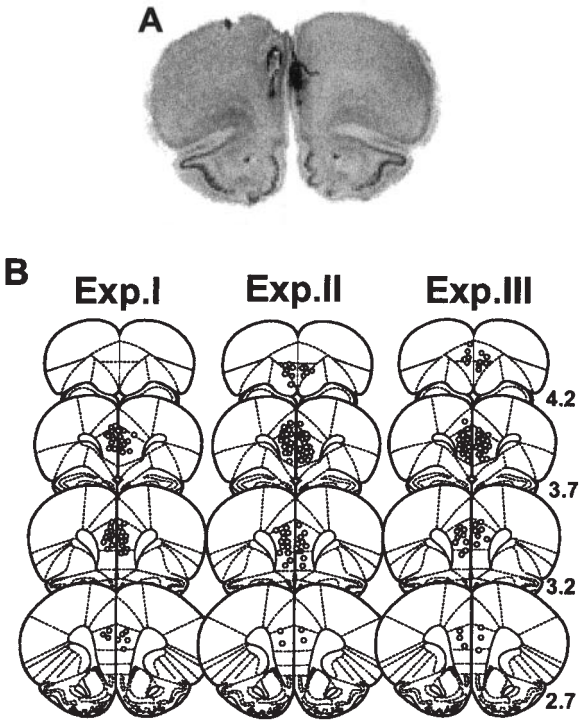


**Figure 1.** Infusion sites in the medial prefrontal cortex. (*A*) Photomicrograph of a coronal brain section with the tracks of the guide cannulae and beneath them the infusion sites visible in both hemispheres. (*B*) Approximate location of the tips of the infusion cannulae depicted on plates of coronal sections through the rat brain (Paxinos and Watson, 1998); numbers on the right indicate distance from bregma in millimeters.

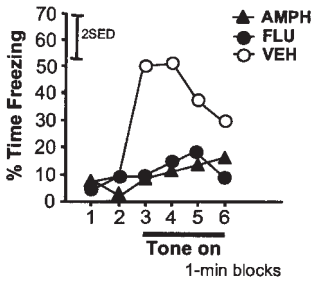


**Figure 2.** Freezing during tone-test sessions of experiment I. Infusions of VEH, FLU and AMPH were given immediately before the tone test. Mean percentage of time spent freezing during the tone test is depicted for the two 1 min blocks preceding the tone, as well as for the first four 1 min blocks of tone presentation. Bars represent two standard errors of the differences of means (2 SED), derived from the appropriate mean square of the ANOVA according to the published formula (Cochran and Cox, 1950). The 2 SED provide an estimate of population variance for between-groups comparisons and are used in line graphs for the sake of clarity.

## Results

### *Histology*

In all rats used in experiments I–III, the tips of the infusion cannulae were located within or around the borders of the mPFC (Fig. 1). Therefore, behavioral data of all rats were included in the analysis. Damage was restricted to the area immediately surrounding the guide and infusion cannulae and did not differ between the infusion groups.

### *Experiment I: FLU and AMPH in the mPFC during Tone Test*

In experiment I, in which rats received only one infusion of FLU, AMPH, or VEH immediately before the tone test, the three groups did not differ in their freezing levels during conditioning and context test, reflecting successful matching (data not shown). Freezing exhibited by the three different infusion groups during the tone test of experiment I is depicted in Figure 2.

Conditioned freezing to the tone previously paired with foot shocks was lower in the AMPH and FLU groups than in the VEH rats. During the two 1 min blocks before tone onset, rats exhibited virtually no conditioned fear, as evidenced by low levels of immobility (<15%). ANOVA of freezing levels during the first four 1 min blocks of the tone presentation revealed a significant effect of infusion [$F(2,22) = 5.19$, $P < 0.02$]. *Post hoc* comparisons indicated that average freezing levels were lower in the AMPH ($P < 0.01$) and FLU ($P < 0.02$) rats than in the VEH group. AMPH and FLU rats did not differ ($P > 0.96$). Thus, the results of experiment I indicated that both AMPH

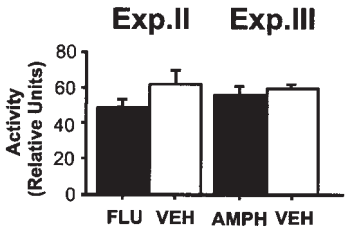


**Figure 3.** Effects of the different infusions on unconditioned activity during the 1.5 min before the first tone–shock pairing in experiments II and III. Infusions of VEH, FLU and AMPH were applied immediately before behavioral testing. Presented values are means + SEM.

and FLU in the mPFC reduced expression of conditioned fear. To corroborate this suggestion, we conducted experiments II and III.

### *Experiments II and III: FLU and AMPH in the mPFC during Conditioning and Tone Test*

*Unconditioned Behaviors during Conditioning Session*

Figure 3 depicts unconditioned activity during the 1.5 min block preceding the first tone–shock pairing for the different infusion groups in experiments II and III.

FLU (experiment II) and AMPH (experiment III) rats did not differ significantly from VEH rats in the unconditioned activity exhibited throughout the 1.5 min block preceding the first tone–shock pairing at the beginning of the conditioning session. ANOVA of average activity throughout the 1.5 min preceding the first tone–shock pairing did not yield significant differences between FLU and VEH rats in experiment II [$F(1,37) = 1.75$, $P > 0.19$] or AMPH and VEH rats in experiment III [$F(1,35) = 0.65$, $P > 0.43$].

Inspection of the video images from the conditioning sessions of experiments II and III revealed that all infusion groups exhibited similar vigorous twitching and marked jumps as immediate response to the foot shock, indicating that FLU or AMPH infusions did not affect shock sensitivity.

*Conditioned Freezing during Conditioning and Tone Test*

The freezing data for conditioning and tone test of experiment II, in which rats received infusions of either FLU or VEH before conditioning, as well as before the tone test, are depicted in Figure 4.

During the conditioning session, the development of conditioned freezing in response to the inescapable foot shocks did not differ between rats that received FLU or VEH infusion before conditioning. ANOVA of average freezing levels during the seven 1.5 min blocks preceding and following the tone–shock pairings, as well as of the six 30 s blocks of tone presentations, did not yield an effect of the infusion received before conditioning [$F(1,37) = 0.84$, $P > 0.36$; $F(1,37) = 2.29$, $P > 0.13$] or an interaction of infusion × time block [$F(6,222) = 0.47$, $P > 0.83$; $F(5,185) = 1.84$, $P > 0.10$]. There was only a significant effect of 1.5 min block [$F(6,222) = 56.31$, $P < 0.0001$] and 30 s tone presentation [$F(5,185) = 44.53$, $P < 0.0001$], reflecting the gradual increase of freezing with repeated shock administration.

During the tone test, the two groups that received FLU immediately before the tone test (VEH–FLU, FLU–FLU) exhibited less conditioned freezing to the tone than the groups that received VEH immediately before the tone test (VEH–VEH, FLU–VEH), while the infusion received before conditioning had

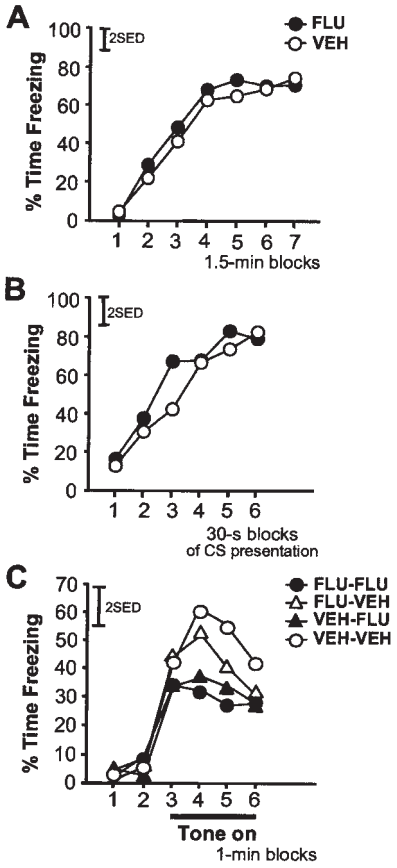


**Figure 4.** Freezing during conditioning and tone-test sessions of experiment II. A first infusion of VEH or FLU was given immediately before conditioning, resulting in two infusion groups for conditioning. A second infusion of VEH or FLU was given immediately before the tone test, resulting in four infusion groups (FLU–FLU; FLU–VEH; VEH–FLU; VEH–VEH), differing with respect to the combination of infusions received before conditioning and tone test. Mean percentage of time spent freezing during conditioning is depicted for the seven 1.5 min blocks preceding and following the six tone–shock pairings (*A*) as well as for the six 30 s tone presentations of the pairings (*B*). Mean percentage of time spent freezing during the tone test is depicted for the two 1 min blocks preceding the tone, as well as for the first four 1 min blocks of tone presentation (*C*). Bars represent two standard errors of the differences of means (2 SED).

no effect. Rats did not exhibit conditioned fear before tone onset, as indicated by the negligible freezing levels during the two 1 min blocks preceding tone onset (<10%). Freezing strongly increased with tone onset. However, peak levels of freezing were considerably higher in the rats that received VEH before the tone test than in those that received FLU. This was reflected by the results of a three-way ANOVA (infusion before conditioning × infusion before tone test × 1 min block) of freezing during the first four 1 min blocks of tone presentation. Thus, there was an interaction of infusion before tone test × 1 min block [$F(3,105) = 2.79$, $P < 0.05$] along with an effect of 1 min block [$F(3,105) = 8.22$, $P < 0.0001$), but neither a main effect of infusion before conditioning [$F(1,35) = 0.33$, $P > 0.56$] or of infusion before tone test [$F(1,35) = 2.57$, $P > 0.11$], nor interactions of infusion before conditioning × 1 min block [$F(3,105) = 2.18$, $P > 0.09$] or of infusion before conditioning × infusion before tone test × 1 min block [$F(3,105) = 0.73$, $P > 0.53$]. Further analysis, based on the significant interaction of infusion before tone test × 1 min block, revealed that groups receiving FLU before the tone test (VEH–FLU, FLU–FLU) exhibited less freezing during the second and third 1 min blocks

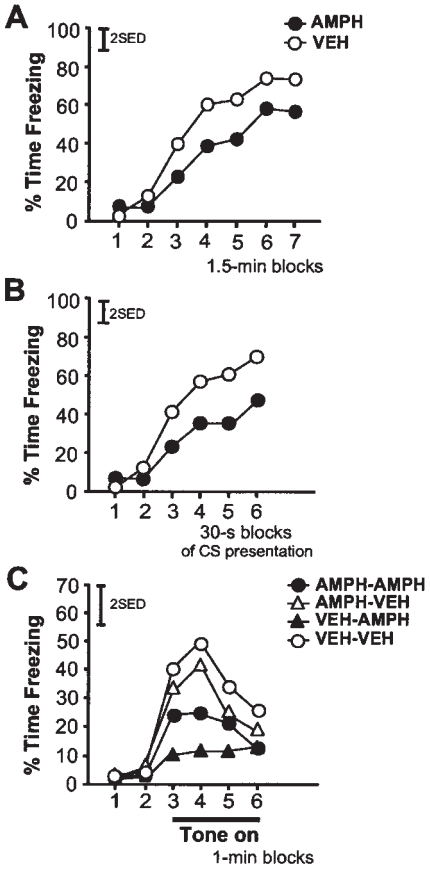


**Figure 5.** Freezing during conditioning and tone-test sessions of experiment III. A first infusion of VEH or AMPH was given immediately before conditioning, resulting in two infusion groups for conditioning. A second infusion of VEH or AMPH was given immediately before the tone test, resulting in four infusion groups (AMPH–AMPH; AMPH–VEH; VEH–AMPH; VEH–VEH), differing with respect to the combination of infusions received before conditioning and tone test. Mean percentage of time spent freezing during conditioning is depicted for the seven 1.5 min blocks preceding and following the six tone–shock pairings (*A*) as well as for the six 30 s tone presentations of the pairings (*B*). Mean percentage of time spent freezing during the tone test is depicted for the two 1 min blocks preceding the tone, as well as for the first four 1 min blocks of tone presentation (*C*). Bars represent two standard errors of the differences of means (2 SED).

of tone presentation than rats receiving VEH before the tone test (VEH–VEH, FLU–VEH). Thus, separate ANOVAs of freezing levels for each single 1 min block yielded a significant main effect of infusion before tone test for the second 1 min block [$F(1,35) = 5.05$, $P < 0.04$] and a strong tendency towards an effect for the third 1 min block [$F(1,35) = 3.67$, $P = 0.06$], while not indicating differences for the first [$F(1,35) = 0.06$, $P > 0.81$] and the fourth [$F(1,35) = 0.10$, $P > 0.75$) 1 min blocks.

The freezing data for conditioning and tone test of experiment III, in which rats received infusions of either AMPH or VEH before conditioning as well as before the tone test, are depicted in Figure 5.

During conditioning, freezing that developed in response to the foot shocks was lower in rats that received AMPH before conditioning than in those that received VEH. ANOVA of freezing during the seven 1.5 min blocks preceding and following the tone–shock pairings, as well as during the six 30 s blocks of tone presentations, yielded a main effect of infusion before conditioning [$F(1,35) = 6.09$, $P < 0.02$; $F(1,35) = 5.63$, $P < 0.03$] and 1.5 min block [$F(6,210) = 47.96$, $P < 0.0001$] or 30 s block

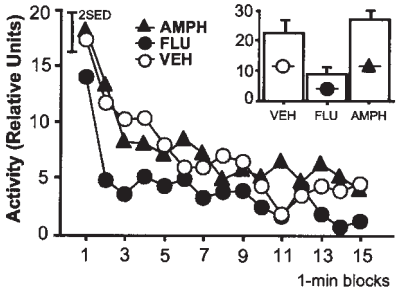


**Figure 6.** General activity after VEH, FLU and AMPH infusion in the activity experiment. Mean activity + SEM is depicted averaged over the complete 2 h (inset bar graph), as well as for each single of the first 15 1 min blocks (main line graph) following infusion. The bar in the main graph represents two standard errors of the differences of means (2 SED).

[$F(5,175) = 37.77$, $P < 0.0001$], respectively. There was also a strong tendency towards an interaction of infusion before conditioning × 30 s block of tone presentation [$F(5,175) = 2.24$, $P = 0.0523$].

During the tone test, the two groups that received AMPH immediately before the tone test (VEH–AMPH, AMPH–AMPH) exhibited less conditioned freezing to the tone than the groups that received VEH immediately before the tone test (VEH–VEH, AMPH–VEH). The infusion received before conditioning had virtually no effect on conditioned freezing during the tone test. Rats did not exhibit conditioned fear before tone onset, as indicated by the negligible freezing levels during the two 1 min blocks preceding tone onset (<5%). In all groups, freezing increased with tone onset. However, freezing reached considerably higher peak levels in the rats that received VEH before the tone test than in those that received AMPH. Three-way ANOVA (infusion before conditioning × infusion before tone test × 1 min block) of freezing during the first four 1 min blocks of tone presentation yielded a main effect of infusion before tone test [$F(1,33) = 4.75$, $P < 0.04$] and a significant main effect of 1 min block [$F(3,99) = 6.00$, $P < 0.001$] without an interaction of infusion before tone test × 1 min block [$F(3,99) = 2.28$, $P > 0.08$]. Although it appeared as if the AMPH–AMPH group exhibited slightly higher levels of freezing than the VEH–AMPH rats, ANOVA did not yield a main effect of infusion before conditioning [$F(1,33) = 0.01$, $P > 0.94$] or any significant interaction involving infusion before conditioning [infusion before conditioning × 1 min block, $F(3,99) = 0.50$, $P > 0.68$; infusion before conditioning × infusion before tone test, $F(1,33) = 1.58$, $P > 0.21$; infusion before conditioning × infusion before tone test × 1 min block, $F(3,99) = 0.75$, $P > 0.52$]. Thus, only the infusion received immediately before the tone test significantly affected levels of freezing to the tone.

### *Activity Experiment: Effects of FLU or AMPH in the mPFC on General Activity*

The effects of FLU or AMPH infusion into the mPFC on general activity during 2 h following infusion are depicted in Figure 6.

FLU decreased the general activity as compared to VEH infusions, while AMPH did not have any effect. ANOVA of the average activity during the 2 h of testing yielded a significant group effect [$F(2,20) = 14.23$, $P < 0.0001$] and *post hoc* comparisons indicated that activity was lower in the FLU group than in both the AMPH ($P < 0.0015$) and VEH ($P < 0.0001$) groups, which did not differ ($P > 0.2$). A more detailed analysis was done for the activity data collected during the first 15 1 min blocks following infusion, which corresponded to the period of

conditioning and tone-test sessions in the fear conditioning experiments. ANOVA yielded a significant effect of group [$F(2,20) = 8.37$, $P < 0.003$] and 1 min blocks [$F(14,580) = 22.71$, $P < 0.0001$], with the latter reflecting habituation. *Post hoc* comparisons indicated that average activity throughout the 15 min following infusion was lower in the FLU group than in both the AMPH ($P < 0.0015$) and VEH ($P < 0.003$) groups, which did not differ ($P > 0.58$). Although ANOVA did not yield an interaction of group × time block [$F(28,580) = 1.25$, $P > 0.18$], it can be seen from Figure 6 that, consistent with the measurement of unconditioned activity during the 1.5 min preceding the first tone–shock pairing in experiment II (Fig. 3), activity in the FLU group was not considerably lower than in the VEH and AMPH rats during the first 1 min block. Indeed, a separate ANOVA on activity during the first 1 min block did not yield an effect of group [$F(2,20) = 1.40$, $P > 0.27$]. On day 3, groups did not differ in their activity any more (data not shown).

## Discussion

The main finding of the present study is that both the dopamine receptor antagonist *cis*-flupenthixol (experiments I and II) and the dopamine receptor agonist D-amphetamine (experiments I and III) in the mPFC reduced the expression of conditioned fear, measured as freezing, to a tone previously paired with foot shocks. While D-amphetamine also reduced immediate conditioned freezing during conditioning, presence of neither drug during the tone–shock pairings affected conditioned fear during subsequent presentation of the tone alone. These data indicate that dopamine-receptor-mediated processes within the mPFC are important for the retrieval/expression, but not the formation, of conditioned fear.

### *Specificity of the Infusion Effects*

Infusion sites were located in the central mPFC, mainly in the prelimbic and the dorsal infralimbic subdivisions. With the small infusion volume (0.5 µl/side) and fine infusion cannulae (34 gauge) used in the present study, the estimated spread of the infused substances was <1 mm (Myers, 1966; Myers *et al.*, 1971, Routtenberg, 1972). It can therefore be assumed that differences between rats infused with vehicle compared to rats infused with FLU or AMPH reflected a temporary alteration of neuronal activity by blockade of dopamine receptors or increased release of dopamine and possibly other monoamines, respectively, within the central mPFC.

The similar immediate shock responses observed in all infusion groups in the present study, as well as previous findings that neither FLU nor AMPH in the mPFC, at the doses used in the present study, affected the acoustic startle reaction or its prepulse inhibition (Lacroix *et al.*, 2000a; Pezze *et al.*, 2001a; Bast *et al.*, 2002b), indicate that these infusions do not considerably affect sensory processing.

The activity experiment demonstrated that, at the doses used in the present study, FLU in the mPFC decreased activity (Beninger *et al.*, 1990; Pezze *et al.*, 2001a; Bast *et al.*, 2002b), while AMPH did not alter activity. Consistent with the similar unconditioned activity of VEH and FLU rats during the 1.5 min block preceding the first tone–shock pairing in experiment II, the decrease in activity induced by FLU in the control experiment was not evident before the second 1 min block. Infusion-induced hypoactivity cannot, however, account for reduced freezing. Rather, hypoactivity facilitates freezing (Bast *et al.*, 2001b) and may have somewhat concealed reduced conditioned fear in rats infused with FLU.

Finally, the reduced conditioned freezing observed during the tone-test sessions in rats that received drug infusions immediately before the sessions cannot be explained by state dependency, i.e. that information learned in a particular brain state can in some cases only be retrieved with the same brain state prevailing (Overton, 1964). This was demonstrated by the results of experiments II and III, in which the drug–vehicle groups expressed normal freezing, while the drug–drug groups demonstrated a pronounced deficit.

### *Role of the mPFC and Local Dopamine Transmission in Fear Conditioning*

Previous neurochemical studies indicated mPFC dopamine activation by a fear CS [(Goldstein *et al.*, 1994, 1996; Inoue *et al.*, 1994; Yoshioka *et al.*, 1996; Morrow *et al.*, 1999b,c; Feenstra, 2000; Feenstra *et al.*, 2001), but see Wilkinson *et al.* (Wilkinson *et al.*, 1998)] without clarifying the functional relation between dopamine in the mPFC and conditioned fear. The present finding that both the dopamine receptor antagonist FLU and the dopamine receptor agonist AMPH in the mPFC reduced freezing to a tone previously paired with foot shocks suggests that retrieval/expression of conditioned fear depends on normal mPFC dopamine transmission. The fact that the expression of conditioned fear was not completely blocked, but only reduced, by interference with prefrontal dopamine transmission is consistent with the widely held notion that the mPFC has a modulatory, rather than primary, role in the expression of fear (Morrow *et al.*, 1999a). Moreover, AMPH, but not FLU, in the mPFC reduced the expression of conditioned freezing during conditioning. This may be because dopamine receptor-mediated processes important for the retrieval/expression of immediate conditioned fear during conditioning differ from those relevant for the retrieval/expression of long-term conditioned fear assessed in later test sessions. Finally, manipulating mPFC dopamine transmission during the tone–shock pairings did not affect conditioned freezing expressed during a subsequent presentation of the aversive CS, suggesting that mPFC dopamine transmission does not play a role in the formation of a stable fear memory.

As described in the Introduction, the functional relation between neural activity in the mPFC and conditioned fear may depend on the subregion within the mPFC. In several previous studies, lesions centering on the prelimbic and dorsal infralimbic mPFC resulted in decreased conditioned fear, suggesting that processes in these regions may be essential for normal conditioned fear (Frysztak and Neafsey, 1991, 1994; Lacroix *et al.*, 2000b; McLaughlin *et al.*, 2001). Consistent with this suggestion, the results of the present study indicate that dopamine transmission in the prelimbic and dorsal infralimbic mPFC, which was the center of the infusion sites, is critical for the normal expression of conditioned fear. Dopamine transmission in other mPFC subregions may play a different role, given that lesions affecting the anterior cingulate (Morgan and LeDoux, 1995; Vouimba *et al.*, 2000) and the most caudal and ventral infralimbic mPFC (Morgan *et al.*, 1993; Quirk *et al.*, 2000) were reported to have facilitating effects on conditioned fear. This issue may be addressed in future studies.

In some studies, lesions destroying considerable parts of the prelimbic and dorsal infralimbic mPFC did not alter conditioned fear (Holson, 1986; Rosen *et al.*, 1992; Gewirtz *et al.*, 1997; Quirk *et al.*, 2000). Moreover, 6-hydroxydopamine lesions to the mPFC, depleting dopamine (by 86%), norepinephrine (by 51%), which has been implicated in extinction of conditioned responses (Dalley *et al.*, 2001) and serotonin (by 14%) in the rostral cingulate, prelimbic and rostral infralimbic mPFC, did

not affect moderate conditioned freezing to a tone (peak levels ~40–50%), while extinction of strong conditioned freezing (peak levels ~80%) was strongly reduced (Morrow *et al.*, 1999b). The latter suggests that the significance of catecholamine transmission in the mPFC for the extinction of conditioned fear may depend on the intensity of fear (Morrow *et al.*, 1999b; Quirk *et al.*, 2000). The fact that permanent damage to the prelimbic and dorsal infralimbic mPFC, in contrast to our temporary manipulation of local dopamine transmission, sometimes failed to reduce conditioned fear may reflect compensatory processes induced by permanent lesions (Bast *et al.*, 2001a).

### *Interactions of mPFC Dopamine and Fear Circuits*

While efferents to the nucleus accumbens, amygdala, periaqueductal gray, or lateral hypothalamus (Sesack *et al.*, 1989) link the mPFC to structures that have been implicated in the expression of conditioned fear (Fendt and Fanselow, 1999; LeDoux, 2000; Murphy *et al.*, 2000; Pezze *et al.*, 2001b, 2002a), the mPFC may receive information about CS–US associations through afferents from the amygdala (McDonald, 1991) and ventral hippocampus (Jay and Witter, 1991). Both the ventral hippocampus (Bast *et al.*, 2001a,b, 2003; Zhang *et al.*, 2001) and amygdala (Cahill *et al.*, 1999; Fanselow and LeDoux, 1999; LeDoux, 2000) have been implicated in the associative processes underlying formation of conditioned fear and the amygdala may even be the site where the CS–US association is permanently stored [(Fanselow and LeDoux, 1999; Fendt and Fanselow, 1999; LeDoux, 2000), but see Cahill *et al.* (Cahill *et al.*, 1999)]. Interestingly, electrical stimulation of the amygdala (Jackson and Moghaddam, 2001) or the ventral hippocampus (Gurden *et al.*, 2000) has been demonstrated to result in measurable effects on neural processes in the mPFC, including an increase of extracellular dopamine. Thus, mPFC dopamine transmission may contain information about CS–US associations transmitted from the amygdala or the ventral hippocampus and the reduced expression of conditioned fear observed after interference with mPFC dopamine transmission may indicate that the transfer of this information was disrupted.

### *Relation between mPFC Dopamine and Conditioned Fear*

In the present study, both the dopamine receptor antagonist FLU and the agonist AMPH reduced expression of conditioned fear when infused into the mPFC. This may be because normal retrieval/expression of conditioned fear requires time and synapse specific dopamine-receptor stimulation in the mPFC, but this is unlikely in view of the evidence for dopaminergic volume transmission in the mPFC (Bast *et al.*, 2002a). Alternatively, it may reflect an inverted U-shaped function relating the retrieval/expression of conditioned fear to dopamine-receptor stimulation in the mPFC, i.e. that normal retrieval/expression of conditioned fear requires an optimal level of dopamine activity in the mPFC. Similarly, working memory has been suggested to require an optimal level of prefrontal dopamine-receptor stimulation, because PFC infusions of both dopamine-receptor antagonists and agonists impaired working memory (Sawaguchi and Goldman-Rakic, 1991; Williams and Goldman-Rakic, 1995; Zahrt *et al.*, 1997; Arnsten, 1998; Seamans *et al.*, 1998; Goldman-Rakic *et al.*, 2000; Robbins, 2000). These effects have been found with D1-receptor-specific agents and some studies have directly demonstrated that D2-receptor antagonists are without an effect [(Sawaguchi and Goldman-Rakic, 1991; Williams and Goldman-Rakic, 1995; Seamans *et al.*, 1998), but see Arnsten *et al.* and Druzin *et al*. (Arnsten *et al.*, 1995; Druzin *et al.*, 2000)]. Attentional performance also appears to depend on an optimal level of mPFC D1-receptor stimulation (Granon *et al.*, 2000; Robbins, 2000). Electrophysiological findings indicate that behavioral processes involving dopamine-mediated mechanisms in the mPFC may require an optimal level of local dopamine activity, because an optimal level of local D1-receptor stimulation is essential for the 'sharpening' of prefrontal signal transmission (Yang and Seamanns, 1996). A final consideration is that AMPH may not only directly stimulate dopamine, but also norepinephrine and serotonin transmission (Azzaro and Rutledge, 1973) and that this might contribute to the reduction of conditioned fear by AMPH in the mPFC. Thus, future studies should examine the effects of mPFC infusion of specific D1- or D2-receptor antagonists or agonists on conditioned fear in order to clarify whether local D1 and D2 receptors are differently involved in conditioned fear and to substantiate the suggestion that excess prefrontal dopamine receptor stimulation may reduce conditioned fear.

## Conclusion

The present study suggested that inhibition as well as stimulation of dopamine receptors in the (prelimbic and dorsal infralimbic) mPFC reduced conditioned fear to a tone previously paired with a foot shock. Our findings corroborate the view that the mPFC and local dopamine transmission are essential for normal emotional responses. Furthermore, they suggest the retrieval/expression of conditioned fear, as with working memory and attentional performance, may be related to mPFC dopamine transmission by an inverted U-shaped function, indicating an intriguing relation between the substrates of emotion, working memory and attention.

## Notes

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