J Pharmacol Sci **114**, 341 – 346 (2010)

Journal of Pharmacological Sciences
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Full Paper

Diazepam-Induced Increases of Synaptic Efficacy in the Hippocampal – Medial Prefrontal Cortex Pathway Are Associated With Its Anxiolytic-like Effect in Rats

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Received July 12, 2010; Accepted September 22, 2010

Abstract. The medial prefrontal cortex (mPFC) has recently been shown to be an important brain region for emotional function as well as cognitive ability. In previous experiments, we studied the population spike amplitude (PSA) in the mPFC induced by stimulation of the CA1/subicular region as an index of synaptic efficacy in the hippocampal–mPFC pathway. In the present study, we investigated the relationship between the anxiolytic effect of diazepam and the changes of synaptic efficacy in this pathway. In contextual fear conditioning tests, diazepam (0.1 mg/kg) was not effective for fear-related freezing behavior. At a dose of 0.5 mg/kg, diazepam decreased freezing behavior 20 min after administration, with no discernible effect 30 min after administration. In electrophysiological experiments, 0.1 mg/kg diazepam had no effect on the PSA in the mPFC. In contrast, 0.5 mg/kg diazepam increased the PSA in the mPFC within 30 min of administration; however, this PSA increase was attenuated over the 30-min period. Based on these results, we propose that the diazepam-induced PSA increase in the mPFC is associated with its anxiolytic-like effect.

Keywords: electrophysiology, hippocampal – medial prefrontal cortex (mPFC) pathway, behavior, anxiety, prefrontal cortex

Introduction

Functional brain imaging techniques, such as positron emission tomography and functional magnetic resonance imaging, are used to investigate the pathologies of psychiatric diseases. Investigations of brain activity in patients with psychiatric disorders (e.g., schizophrenia, depression, and anxiety disorders) have revealed a dysfunction in the frontal cerebral cortex, which has been termed hypofrontality. However, this phenomenon alone cannot explain the etiology of these psychiatric disorders.

When we carry out a pharmacological investigation in rats that focuses on the prefrontal cortex, it is important to consider whether or not the rodent prefrontal cortex is comparable to that in primates. In the past, prefrontal cortex functionality was thought to be unique to primates. However, recent tracer and lesion studies have revealed that rats have a functional prefrontal cortex that includes not only features of the medial and orbital areas that are observed in primates but also includes several features of the primate dorsolateral prefrontal cortex (1).

Previous studies have indicated that the medial prefrontal cortex (mPFC) shares mutual projections with the amygdala complex (2). In addition, previous studies have indicated that the mPFC receives input from the hippocampus in rats (3). Both of these neural circuits are important for emotional regulation. Previous studies have also suggested that the mPFC has inhibitory control over the amygdala during fear-related behavior. For example,

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Published online in J-STAGE
doi: 10.1254/jphs.10182FP

an mPFC lesion (4) in conjunction with inhibition of protein synthesis in the mPFC (5) blocked the extinction of conditioned fear. Furthermore, electrical stimulation of the mPFC inhibited the expression of conditioned fear (6, 7). Previous studies have also reported that the extracellular concentration of dopamine and serotonin (5-hydroxytryptamine: 5-HT) in the mPFC increased during freezing behavior as the expression of conditioned fear (8, 9) and that a selective serotonin reuptake inhibitor (SSRI), which increased 5-HT release in the mPFC, reduced conditioned fear-induced freezing behavior (10). These behavioral and neurochemical studies suggest that the mPFC plays an important role in the inhibitory regulation of fear-related emotions.

In contrast to the amygdala, the mPFC and several hippocampal structures are necessary for the execution of memory-related behaviors. The CA1/subicular region of the ventral hippocampus plays an important role in the acquisition of spatial memory (11). Furthermore, this region is connected to the mPFC (3), and this connection could contribute to the encoding of spatial information (12). Taking these results together, we hypothesized that the connections in the hippocampal–mPFC pathway may be necessary for memory-dependent emotional regulation.

We have studied the population spike amplitude (PSA) in the mPFC that is induced by stimulation of the hippocampus because it is a measure of synaptic efficacy in this pathway (Fig. 1). Electrophysiological experiments in rats showed that stimulation of the CA1/subicular region produces negative-going field potentials in the mPFC. Because the excitatory response of the mPFC was blocked by a selective alpha-amino-3-hydroxyl-5-methylisoxazole-4-propionate (AMPA) receptor antagonist, this negative-going peak reflects the excitatory input from the hippocampus to the mPFC via glutamatergic neurons (3). In the previous experiment, we studied the effect of an SSRI on synaptic efficacy in the mPFC. A single administration of the SSRI fluvoxamine induced an increase of the PSA in the mPFC (13).

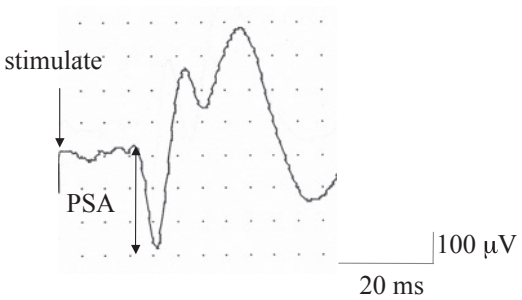


Fig. 1. Typical wave-form of an evoked potential in the mPFC after stimulation of the CA1/subicular region. We assessed the amplitude of the first negative peak in this wave form as the synaptic efficacy in the mPFC and evaluated this as the population spike amplitude (PSA).

Based on these studies, we hypothesized that the synaptic efficacy in the hippocampal–mPFC pathway may be involved in emotional regulation. To test this hypothesis, we investigated the effect of diazepam, an anxiolytic-like agent that is widely used in clinical practice and laboratory research (14), on synaptic efficacy in the hippocampal–mPFC pathway.

Materials and Methods

Animals

Male Wistar rats (280 – 410 g and 10 – 14-week-old) were used for these experiments (Slc:Wistar/ST; Shizuoka Laboratory Animal Center, Hamamatsu). The animals were housed in a 12-h light-dark cycle (lights on from 19:00 – 7:00) at 21 ± 2°C and were given free access to food and water. All experiments in the present study were conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals of Hokkaido University.

Drugs

Diazepam (0.1, 0.5, or 1.0 mg/kg; Wako Pure Chemical Industries, Ltd., Osaka) or flumazenil (10 or 30 mg/kg; Tocris, Hung Road, Bristol, UK) suspended in 1% arabic gum (Nacalai Tesque, Kyoto) were intraperitoneally (i.p.) injected. Urethane (20%; Sigma, St. Louis, MO, USA) was dissolved in 0.9% saline and injected i.p. Lidocaine was purchased from Astra Zeneca (Osaka), and the formaldehyde neutral buffer solution was purchased from Sigma Aldrich (Tokyo).

Contextual conditioned fear test

A chamber (30 × 30 × 27 cm) constructed from aluminum (side walls) and Plexiglass (rear wall, ceiling, and front door) was used to evaluate fear-related behavior. The floor of the chamber consisted of 27 stainless steel rods wired to a shock generator (Medical Agent, Kyoto) that delivered foot shocks. On day 1, the rats were exposed to the chamber for 5 min and were given five foot shocks (2 s, 0.5 mA, at 30-s intervals). Thirty seconds after the final shock, freezing behavior was counted at 5-s intervals for 5 min. Freezing was defined as the absence of all movement, with the exception of respiration and whisker movements. After an observation of freezing, the rats were returned to their home cages. On day two, vehicle (arabic gum, 2.5 ml/kg, i.p.) or diazepam (0.1 or 0.5 mg/kg, i.p.) was injected. At 20 or 30 min after the injection, the rats were re-exposed to the chamber, and freezing behavior was counted at 5-s intervals for 10 min. The freezing time was calculated as a percentage of the total time.

Measurement of the PSA

Each rat was anesthetized with urethane (1.0 g/kg, i.p.) prior to insertion of a tracheal cannula. Next, each rat was fixed in a stereotaxic frame (Narishige Scientific Instrument Lab., Tokyo). The stereotaxic coordinates (using bregma and lambda in the same horizontal plane) were derived from the Paxinos and Watson's brain atlas. In addition to urethane, the local anesthetic lidocaine was used during the scalp dissection. For each rats, the body temperature was maintained at 37°C using a thermoregulated heating pad (TMO35-1; Takasago Ltd., Kanagawa). Bipolar stimulating electrodes (diameter: 100 μm, resistance: 20 kΩ, material: stainless steel; Eiko Science, Tokyo) with a tip separation of 500 μm were placed in the CA1/subicular region of the ventral hippocampus (Fig. 2: 6.0-mm posterior to the bregma, 5.6-mm lateral, and 4.0 – 7.0-mm ventral from the cortical surface) according to Paxinos and Watson's brain atlas. A recording electrode (diameter: 100 μm, resistance: 20 kΩ, material: stainless steel; Eiko Science, Tokyo) was lowered into the mPFC (Fig. 2: 3.3-mm anterior to the bregma, 0.8-mm lateral, and 3.3-mm ventral from the cortical surface). Electrode positions were optimized to record the maximal field response, which was evoked at a frequency of 0.1 Hz (250-μs duration) with the first negative peak latency of 20.05 ± 0.5 ms (Fig. 1). Field responses were amplified and monitored with an oscilloscope (VC-10; Nihon Kohden, Tokyo). The evoked field potentials were converted from analog to digital data and were averaged with a data analyzing system (Power Lab System; ADI Instruments Pty., Ltd., Castle Hill, Australia). Then, the PSA of the first negative peak was measured (Fig. 1). After electrode insertion, the input/output characterization of each rat was assessed. The test stimuli were delivered every 30 s at an intensity that produced a 60% maximal response that was induced by the maximal stimulation intensity (100 – 640 μA). The vehicle or diazepam was injected after sampling the data for 30 min, and changes in the PSA were observed for 60 min. Seven successive responses were averaged and collected every 5 min throughout the experiment. After the experiments, the brains were removed and fixed in a formaldehyde neutral buffer solution, followed by confirmation of a proper electrode insertion location using a microscope (Fig. 2). The evoked potentials were expressed as a percentage of the baseline level, which was determined immediately prior to drug administration.

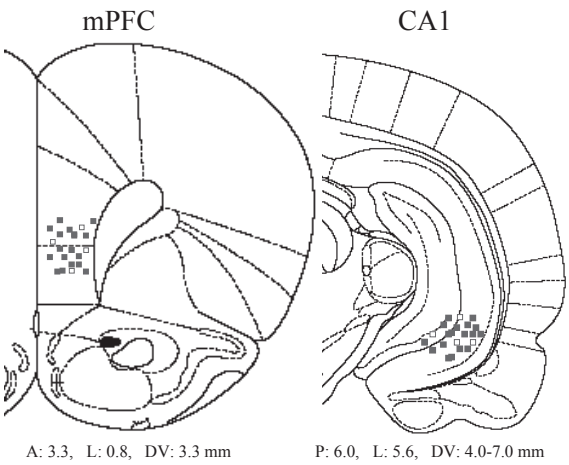


Fig. 2. Schematic representation of the locations of the tips of the recording electrodes in the mPFC and the stimulating electrodes in the CA1/subicular region in the ventral hippocampus (open square: control group and solid square: drug administration group).

Data analyses

All of the results are given as the mean ± S.E.M. To compare the two groups, Student's *t*-test was used. For multiple group comparisons, a one-way ANOVA (followed by a Tukey post-hoc test) was used. The statistical significance was set at $P < 0.05$.

Results

Dose- and time-dependent effects of diazepam on fear-related behavior

Fear-conditioning test was performed to confirm the optimal dose for the anxiolytic-like effect of diazepam. Twenty minutes after administration, 0.1 mg/kg diazepam had no effect on the freezing behavior, whereas 0.5 mg/kg diazepam significantly reduced the freezing behavior compared to that of the control group [one-way ANOVA, $F(3,19) = 4.00$, $P < 0.05$; post-hoc test, control vs. diazepam (0.5 mg/kg), $P = 0.021$) (Fig. 3A1)]. However, 1.0 mg/kg of diazepam did not reduce the freezing behavior. This effect is likely due to the sedative effects of diazepam. In contrast to the 20-min time point, 30 min after administration, 0.5 mg/kg diazepam did not reduce freezing behavior [Student's *t*-test, $F(1,9) = 0.78$, $P > 0.05$) (Fig. 3B)]. In addition, concurrent administration of 0.5 mg/kg diazepam and 30 mg/kg flumazenil, which is a benzodiazepine-receptor antagonist, blocked the effect of 0.5 mg/kg of diazepam on freezing [one-way ANOVA, $F(2,11) = 3.61$, $P < 0.05$; post-hoc test, diazepam (0.5 mg/kg) vs. diazepam (0.5 mg/kg) + flumazenil (30 mg/kg), $P = 0.021$) (Fig. 3A2)], whereas 10 and 30 mg/kg flumazenil alone had no effect on freezing behavior (data not shown).

Effect of diazepam on the PSA in the hippocampal–mPFC pathway

We examined the effects of diazepam on the synaptic efficacy of the hippocampal–mPFC pathway. Systemic administration of 0.1 mg/kg diazepam had no effect on

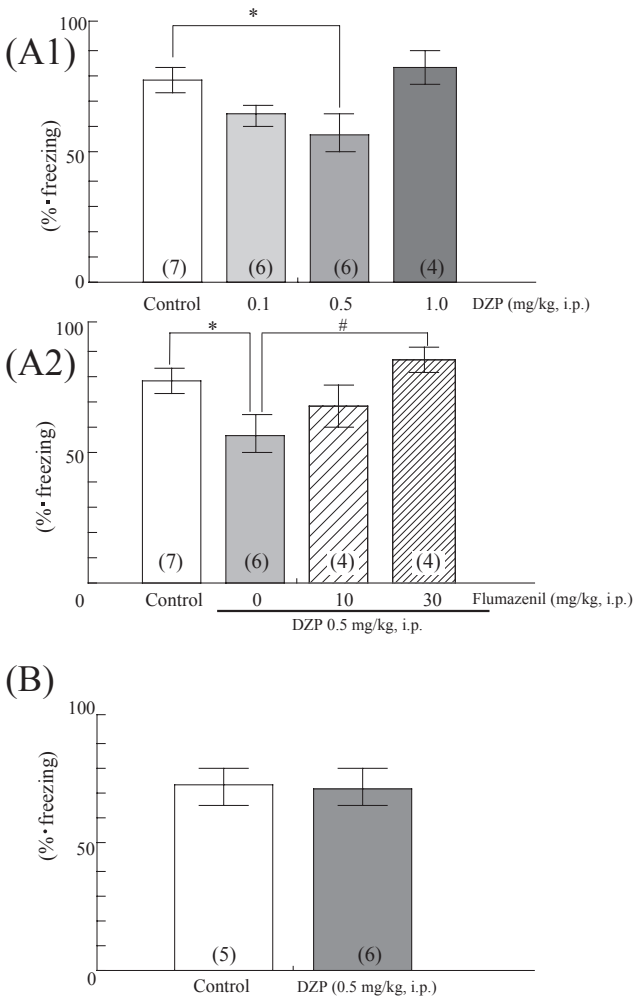


Fig. 3. Effects of diazepam on the experiment evaluating conditioned fear-induced freezing behavior. A1) At 20 min after administration, diazepam (0.5 mg/kg) reduced the freezing behavior of rats in fear conditioning [one-way ANOVA, $F(3,19) = 4.00$, $P < 0.05$; post-hoc test, control vs. DZP (0.5 mg/kg), $P = 0.021$]. A2) Moreover, concurrent administration of diazepam (0.5 mg/kg) and flumazenil (30 mg/kg) did not reduce the freezing behavior [one-way ANOVA, $F(2,11) = 3.61$, $P < 0.05$; post-hoc test, DZP (0.5 mg/kg) vs. DZP (0.5 mg/kg) + flumazenil (30 mg/kg), $P = 0.021$]. B) At 30 min after administration, diazepam (0.5 mg/kg) did not reduce the freezing behavior of rats in fear conditioning [Student's *t*-test, $F(1,9) = 0.78$, $P > 0.05$]. The parenthetic digits are the numbers of rats in these experiments. $^{*}P < 0.05$: control group vs. DZP (0.5 mg/kg) group, $^{\#}P < 0.05$: DZP (0.5 mg/kg) group vs. DZP (0.5 mg/kg) + flumazenil (30 mg/kg) group. DZP, diazepam.

the PSA in this pathway, whereas 0.5 mg/kg diazepam significantly increased the PSA [maximum responses: 167.9 ± 29.9%; one-way ANOVA, $F(2,12) = 4.40$, $P < 0.05$; post-hoc test, control vs. diazepam (0.5 mg/kg), $P = 0.010$]. The PSA value returned to the basal level after 20 min, and the increase of the PSA was significantly inhibited by the concurrent administration of 0.5 mg/kg diazepam and 10 mg/kg flumazenil (Fig. 4).

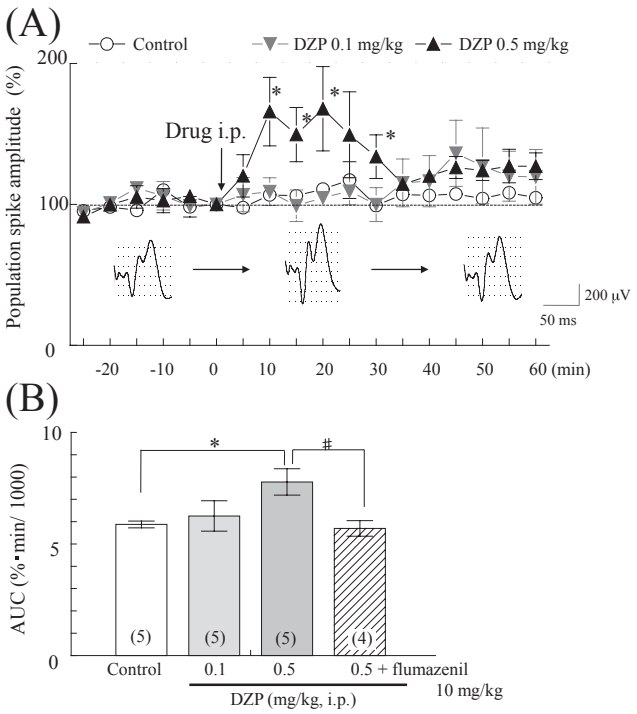


Fig. 4. Effects of diazepam on the time course of the population spike amplitude (PSA) changes (A) and the AUC from 0 to 60 min in the mPFC (B). Diazepam (0.5 mg/kg) increased the PSA in the mPFC. Inset of panel A indicates a representative wave-form of the DZP (0.5 mg/kg) group. The parenthetic digits are sample numbers in these experiments. $^{*}P < 0.05$: control group vs. DZP (0.5 mg/kg) group, one-way ANOVA, $F(2,12) = 4.40$, $P < 0.05$; post-hoc test, control vs. DZP (0.5 mg/kg), $P = 0.010$. $^{\#}P < 0.05$: DZP (0.5 mg/kg) group vs. DZP (0.5 mg/kg) + flumazenil (10 mg/kg) group, Student's *t*-test $F(1,7) = 4.58$, $P < 0.05$. DZP, diazepam.

We did not perform the electrophysiological experiment at 1.0 mg/kg diazepam because sedative effects of diazepam occurred in the conditioned fear tests at this dose.

Discussion

In the present study, we investigated the effect of diazepam, which is widely used clinically as an anxiolytic drug, on conditioned fear, an animal model of anxiety. At a dose of 0.1 mg/kg, diazepam had no effect on conditioned fear-induced freezing behavior. At a dose of 0.5 mg/kg, diazepam significantly reduced freezing 20 min after administration; however, 30 min after administration, 0.5 mg/kg diazepam failed to reduce freezing. In addition, 1.0 mg/kg of diazepam did not reduce freezing. This effect was likely due to the sedative effect at this dose (Fig. 3A1).

In electrophysiological experiments, 0.1 mg/kg diazepam had no effect on the PSA in the hippocampal–mPFC pathway. However, 0.5 mg/kg diazepam increased the PSA at 20 min after administration, and the PSA returned

to the basal level at 30 min after administration. From these results, it is conceivable that the effect of diazepam on the synaptic efficacy in the hippocampal–mPFC pathway is similar to its anxiolytic-like effect in a time- and dose-dependent manner. In previous pharmacokinetic studies in rats, after a single intraperitoneal injection, diazepam was rapidly absorbed, and its blood concentration reached the maximum in 10 min (15). In addition, the diazepam was rapidly cleared from the plasma and brain, with an overall half-life of 0.88 and 0.89 h, respectively (16). Specific blood concentrations may be needed for the anxiolytic and electrophysiological effects of diazepam.

A wide consensus exists that the amygdala mediates fear learning (17). In addition, the hippocampus has bidirectional connections with the amygdala. The majority of the projections from the hippocampus to the amygdala originate at the ventral hippocampus (18). Furthermore, several studies have indicated that the hippocampus is involved in the regulation of anxiety and fear. The expression of contextual conditioned fear has been shown to be abolished by a hippocampal-lesion after conditioning (19). The lesions of the ventral hippocampal efferents to the amygdala have been shown to prevent contextual fear conditioning (20), suggesting that ventral hippocampus–amygdala interactions mediate fear learning.

It has been reported that the hippocampus CA1 field unidirectionally projected to the mPFC (3, 21), and this pathway has been speculated to be relevant to spatial short-term memory and working memory (12). Additionally, the mPFC has been implicated in fear memory extinction. For example, electric lesions or chemical inactivation of the mPFC have been shown to impair the extinction process (4, 22), and fear extinction-induced c-Fos expression in the mPFC has been shown to be blocked by an infusion of a protein synthesis inhibitor into the mPFC (5).

Recent studies have also reported the inhibitory control of the mPFC on the amygdala in fear extinction. Tracer studies have revealed the distribution of the mPFC projections to the amygdala (2, 23). Given that most of the mPFC projections to the amygdala are excitatory (24, 25), it has been proposed that the mPFC inhibits the amygdala by activation of inhibitory interneurons within the amygdala (26, 27). It was also reported that mPFC stimulation can suppress the firing of pyramidal cells in the amygdala, which respond to a fear-conditioned stimulus (28, 29).

Synaptic plasticity in the hippocampal–mPFC pathway has been assessed by the formation of long-term potentiation (LTP), which depends on the activity of the specific afferent. Acute stress has been shown to inhibit LTP formation in the hippocampal–mPFC pathway (30). Furthermore, our previous study indicated that the PSA in this pathway was reduced during exposure to the fear-conditioned stimulus and that this reduction was attenuated in parallel with the extinction of conditioned fear (31). Thus, we inferred that the hippocampal–mPFC pathway was important for the regulation of emotion, especially during fear extinction.

The mechanism of the anxiolytic-like action of diazepam commonly involves the enhancement of the inhibition of neurons via gamma aminobutyric acid A ($GABA_A$) receptors (32). The $GABA_A$ receptor has a benzodiazepine binding site, and upon benzodiazepine binding, the frequency of chloride channel opening increases, resulting in the inactivation of neurons. Because of this mechanism, it is plausible that the PSA decreases as a consequence of diazepam administration. In fact, it has been reported that a local injection of the $GABA_A$ agonist muscimol causes a PSA decrease in the hippocampal–mPFC pathway (33).

Unexpectedly, the PSA actually increased in the present experiment. We hypothesized that diazepam did not directly act on the mPFC, but, instead, acted on other brain regions that modulated the hippocampal–mPFC pathway. For example, we previously reported that LTP formation in this pathway was augmented by a 5-HTergic lesion of the whole brain using a 5-HT neurotoxin, 5,7-DHT (34). From these results, it is impossible to determine if diazepam acted on the region that suppressed the mPFC, such as the 5-HTnergic neurons of the raphe, resulting in the increase of the PSA in the hippocampal–mPFC pathway. Therefore, diazepam indirectly activated mPFC neurons; as a result of this effect, neurons in the amygdala were inhibited, and a reduction in freezing behavior was observed.

We believe that the effect of diazepam in the present study was an anxiolytic-like effect, not an amnesic effect, although it has been reported that diazepam could lead to anterograde amnesia. However, by observing animal behavior, we could not infer whether the effect of diazepam on conditioned freezing was derived from its anxiolytic-like effect or from its amnesic effect. We previously reported that another type of anxiolytic agent, fluvoxamine, also increased the PSA in the hippocampal–mPFC pathway. In addition, a clinical study indicated that the anxiolytic effect of fluvoxamine was without amnesia (35). From these studies, there is support for the idea that diazepam decreased freezing through its anxiolytic-like effect, which is the same effect from an SSRI.

In conclusion, the anxiolytic agent diazepam enhances synaptic efficacy in the hippocampal–mPFC pathway in vivo. Based on the results from previous studies, we speculate that the anxiolytic-like effect of diazepam is

derived from the enhancement of the suppressive activity of the mPFC on the amygdala.

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