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Research report

# Brain structures implicated in the four-plate test in naïve and experienced Swiss mice using injection of diazepam and the 5-$HT_{2A}$ agonist DOI

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ABSTRACT

Four-plate test-retest (FPT-R) is a useful tool to study aversive memory and abolishment of benzodiazepine effects in experienced mice to four-plate test (FPT), namely one-trial tolerance. In the present study, we have used local injections paradigm, in order to localize structures implied in anxiolytic-like effects of two drugs in naïve and experienced mice: a benzodiazepine, diazepam that is only active in naïve mice; and a 5-$HT_{2A/2C}$ agonist, DOI that exert its anxiolytic-like effect both in naïve and experienced mice. Periacqueductal grey substance, three sub-regions of hippocampus (CA1, CA2 and CA3) and two nuclei of amygdala (BLA and LA) have been studied. Local injections did not cause any modifications of ambulatory activity. DOI injections elicit anxiolytic-like effects only when injected into CA2, in naïve and experienced mice. Diazepam had an anxiolytic-like effect in naïve mice, only when injected into lateral nucleus of amygdala; and in experienced mice when injected into PAG. These results help us to better understand the way of action of these two compounds and the structures functionally involved in their effects and in one-trial tolerance (OTT).



## 1. Introduction

Benzodiazepines are widely implicated in the treatment of anxiety disorders. This extensive use in clinical practice led to the definition of benzodiazepines as a gold standard in animal models of anxiety. Predictive validity of a new model was subsequently linked to the effectiveness of benzodiazepines to exert an anxiolytic-like effect in animals. Development of new animal models of anxiety allowed the discovery of a phenomenon called "one-trial tolerance" (OTT). Previously described by File and Lister, the abolishment of anxiolytic-like effect of benzodiazepines in experienced mice has been widely studied, especially in elevated plus-maze [7,17], one of the most used models in anxiety studies in mice. Among all animal models of anxiety in mice available, the four-plate test-retest (FPT-R) is a good tool for studying the phenomena of OTT with diazepam [15,25,30]. In FPT-R, spatial knowledge of the apparatus seems to be the major factor triggering abolishment of the anxiolytic-like effect of diazepam. On contrary, removing electric punishments did not modify OTT [25]. OTT could be related to a kind of "aversive learning" linked to environment, even if scopolamine or atropine sulfate, used as amnesic agents did not modify OTT in FPT [3,30]. Moreover, memory-enhancing drugs (amphetamine and pentylenetetrazole) seem to enhance OTT observed in the elevated plus-maze in mice when injected post-trial 1 [32]. The "aversive learning" should be considered cautiously, because this phenomenon seems different from fear conditioning and passive or active avoidance. Indeed, there is no conditioned stimulus in this paradigm [26]. The aversive unconditioned stimuli, namely electric foot-shocks, are paired only with exploration of the apparatus. Moreover, in fear conditioning and avoidance protocols, mice are submitted to uncontrollable aversive stimuli. In FPT, they have the possibility to stay away from aversive stimuli, as soon as it is associated with crossing from one plate to another. This absence of real conditioned stimulus brings a new tool for studying neural pathways and structures involved in this kind of learning/conditioning. The next step in the understanding of OTT is to elucidate which structures are involved in the anxiolytic-like effect of diazepam in naïve mice in FPT. Furthermore, we have to investigate structures that could be involved in the "aversive learning" phenomenon and/or in the abolishment of anxiolytic-like effect of diazepam during trial 2 in FPT. To work on this topic, local injections of DOI or diazepam in cerebral structures were used. Indeed when administered intra-peritoneally (i.p.), this 5-$HT_{2A/2C}$ agonist does not undergo OTT and keeps its anxiolytic-like effect in the test-retest with FPT [22,30]. In a previous study, we have shown that the 5-$HT_{2A}$ agonist DOI injected globally in the hippocampus in FPT naïve mice induced anxiolytic-like effect [20]. Furthermore, using the EPM some authors [6] found that PAG injection of midazolam attenuated anxiety in naïve but not in retest mice. Among all brain structures implicated in anxiety process, periaqueduc-

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tal grey matter (PAG), baso-lateral (BLA) and lateral (LA) nuclei of amygdale were chosen. Moreover, the three sub-regions of hippocampus (CA1, CA2 and CA3) have been studied due to the major role played by the limbic system in the processing and the storage of information and due to the distribution of $GABA_A$ receptor subunits and 5-$HT_{2A}$ receptors. PAG has been implicated in fear and anxiety process, but also directly in OTT phenomenon, as an increased activity of the PAG could explain the lack of anxiolytic-like effect of drugs elicited by prior apparatus experience [1]. BLA plays a crucial role in the consolidation of information that could lead to insensitivity to benzodiazepines [9]. LA contributes also to contextual or elemental conditioned associations, and interacts with BLA [2]. Hippocampus which has been implicated in the functional state of benzodiazepines receptors in other brain regions [10], shows differential involvements of dorsal CA3 and CA1 hippocampal sub-regions in contextual memory [5], and therefore seems to be a good candidate for the study of OTT and aversive memory. Additionally, synaptic plasticity in BLA has been described after stimulation of the hippocampal formation in vivo [18]. In this study, artificial cerebro-spinal fluid (aCsf), diazepam or DOI were infused in each structure immediately before the test in naïve mice and in experienced mice, in order to evaluate structures implicated in the anxiolytic-like effect of these drugs. It was previously mentioned that localized injections of benzodiazepines in hippocampus may induce changes in anxiolytic-like effect but also in motor activity in mice [16]. To evaluate this phenomenon, infusion protocols were made both for FPT and for actimeter test, with different animals.

## 2. Material and methods

### 2.1. Animals

Male mice (Swiss strain) (Centre d'elevage Janvier, France) weighing 20–24 g were used in this study. They were housed 18 per cage (40 cm × 28 cm × 17 cm) on 12-h light:12-h dark cycle (light on 7 h) and had free access to food and water. The ambient temperature of the room was maintained at 21 ± 1 °C. Experimental groups were composed of 12 mice. All experiments were performed within the guidelines of the French Ministry of Agriculture for experiments with laboratory animals (law no. 87 848). Testing was performed between 8 and 12 h.

### 2.2. Experimental design

#### 2.2.1. Drugs

For i.p. administration, diazepam (RBI, Sigma, France) was mixed in distilled water with a 5% concentration of Tween-80 and injected at a dose of 1 mg/kg. Controls received vehicle treatment only. Diazepam and vehicle were administered 30 min before the test in a volume of 0.5 mL/20 g of body weight. For local infusion, dissolved diazepam (5 mg/mL, Renaudin, France) was employed with injections of 0.3 μL at 0.2 μL/min, at each point of injection.

aCsf was used as vehicle and to dissolve DOI-hydrochloride [(±)-2,5-dimethoxy-4-iodoamphetamine] at 5 mg/mL (RBI, Sigma, France) (for aCsf and DOI, injection of 0.5 μL at 0.5 μL/min, at each point of injection according to [20]). Volume, doses, rates of injection and time interval were chosen after preliminary works [20] and in accordance to literature.

#### 2.2.2. Behavioral procedures

*2.2.2.1. The four-plate test (FPT).* The four-plate test (Bioseb, France) consists of a cage (18 cm × 25 cm × 16 cm) floored by four identical rectangular metal plates (8 cm × 11 cm) separated from one another by a gap of 4 mm. The plates are connected to a device that can generate electric shocks (0.6 mA, 0.5 s). The top of the cage is covered by a transparent Perspex lid that prevents escape behavior. Following a 15 s habituation period, the animal is manually subjected to an electric foot-shock when crossing from one plate to another. The number of punishments is recorded during a 1-min test period. In order to reduce any neophobic response to the situation other than the one linked to the test, the FPT was previously dirtied by mice other than those used during the test. Mice were always tested in a soiled apparatus and there was no cleaning between trials. Every test session was made by the same experimenter in the same room with the same apparatus.

To study the influence of diazepam or DOI infusion in brain structures with naïve or experienced mice, mice were individually tested in the FPT, randomly for drug treatment and test experience. Eight groups were formed according to the treatment (operated mice injected with aCsf, DOI or Diazepam and non-operated mice labeled as "control") and the previous experience of the test (Naïve or Retest mice).

Test-retest paradigm in the FPT consists of two separate trials (interval between the two tests: 24 h). The two trials are called "trial 1", for test with naïve animals and "trial 2" for experimentation with experienced mice. In trial 1, mice were submitted to the FPT without any injection. During trial 2, the mice were injected, either i.p. 30 min before the test with vehicle or diazepam, or locally in each structure immediately before the test with aCsf, DOI or diazepam, depending on the protocol. Naïve mice and control non-operated mice (which received vehicle or diazepam (1 mg/kg)) were submitted to the test during the same session.

*2.2.2.2. Locomotor activity.* Using an actimeter (Bioseb, France), potential modification of locomotor activity by surgery or infusion was tracked, in order to isolate potential false positive in the FPT study. Locomotor activity was recorded during 75 s immediately after the infusion of aCsf, Diazepam or DOI, to be concordant with the FPT protocol. Controls were made with non-operated mice.

#### 2.2.3. Surgical procedure

*2.2.3.1. Implantation of guide-cannula for micro-infusions.* Mice were anaesthetized with chloral hydrate (400 mg/kg). Stainless-steel guide-cannula (0.60 mm external and 0.35 mm internal diameters) (Unimed, Swiss) were implanted bilaterally for CA1, CA2, CA3, BLA and LA, unilaterally for PAG using standard stereotaxic procedures and apparatus (Bioseb, France).

Following coordinates were used according to the brain atlas of Franklin and Paxinos [12].

CA1: (AP) – 2 mm posterior to bregma, (ML) ± 1 mm, (DV) – 1.5 mm from the skull,
CA2: (AP) – 2.5 mm, (ML) ± 1.75 mm, (DV) – 2 mm,
CA3: (AP) – 2.9 mm, (ML) ± 2.9 mm, (DV) – 2.5 mm,
PAG: (AP) – 4.6 mm, (ML) ± 0 mm, (DV) – 2 mm,
BLA: (AP) – 1.4 mm, (ML) ± 2.9 mm, (DV) – 4.9 mm
LA: (AP) – 2 mm, (ML) ± 3.25 mm, (DV) – 4.25 mm

(Axis: AP: anterior–posterior, ML: medial–lateral axis; DV: dorsal–ventral).

Cannula guides were fixed to the skull with radiopaque posterior glass ionomer restorative cement GC Fuji IX (Henri Schein, France). Stainless-steel dummy-cannula were inserted into guide-cannula to prevent occlusion and left in place until the injections were made. After surgery, mice were allowed at least seven days to recover. After behavioral tests, mice received injections of methylene blue using the cannula guide, they were sacrificed and brains were removed and placed 1 min in 2-methylbutane at −35 °C. Localizations of the implantation of cannula guides were checked by histological procedure on cryostat sections [20].

*2.2.3.2. Intra-hippocampal micro-infusion procedure.* Each infusion cannula (0.30 mm external and 0.15 mm internal diameter) was connected by a polypropylene tube to a 2 μl Hamilton micro-syringe that delivered the solution using an automated pump (Harvard apparatus, France). Behavioral test occurs immediately after injection.

#### 2.2.4. Statistical analysis

Results were expressed as a mean of the number of punished passages (±SEM) for the FPT. Data were tested for homogeneity of the variance and normal distribution. A two-way ANOVA (experience × treatment in trial 2) was employed. If the ANOVA showed a significant interaction between the two factors, a Sidak's test was performed directly. If no significant interaction was shown, ANOVA two-way was performed as an additive model, in order to raise the strength of the analysis of the main factors. Sidak's post-hoc was made on this second ANOVA if needed. All analyses were conducted using the SPSS program for IBM compatible computer.

## 3. Results

### 3.1. Locomotor activity

Results did not reveal any incidence of surgery, injections or injected drug on mice locomotor activity. Statistical analysis did not reveal any significant result in all groups ($p > 0.05$) (results not shown).

Influence of diazepam or DOI infusion in brain structures with naïve or experienced mice.

*Hippocampus CA1* (Fig. 1): Two-way ANOVA revealed no significant interaction between the two factors (experience and treatment). Performed as an additive model, the factor "experience" showed a significant effect on the number of accepted punishments [$F_{(1,91)} = 269.41$; ($p < 0.001$)]. No effect of the factor "treatment during trial 2" was found [$F_{(3,91)} = 1.74$; ($p > 0.05$)]. In naïve mice and in retest mice, surgery and injection of vehicle, DOI or diazepam did not modify the number of punishments accepted by mice,