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# The Octadecaneuropeptide ODN Induces Anxiety in Rodents: Possible Involvement of a Shorter Biologically Active Fragment

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DE MATEOS-VERCHERE, J.G., J. LEPRINCE, M.-C. TONON, H. VAUDRY AND J. COSTENTIN.*The octadecaneuropeptide ODN induces anxiety in rodents: possible involvement of a shorter biologically active fragment.* PEPTIDES **19**(5) 841–848, 1998.—The octadecaneuropeptide ODN has been originally characterized as an endogenous ligand of central-type benzodiazepine receptors, on its ability to displace the anxiogenic compound $\beta$-[$^3$H]carboline-3-carboxylate methyl ester from its binding sites. The aim of the present study was to investigate the anxiogenic effects of intracerebroventricular administration of ODN in mice and rats. At doses ranging from 10 to 100 ng, ODN increased in mice the latency to explore a white compartment when the animals were placed in a black one. ODN also reduced the first stay duration in the white compartment. These effects were antagonized by diazepam (0.075 mg/kg, SC) as well as flumazenil (1 mg/kg, SC), indicating that ODN acts as an inverse agonist on central-type benzodiazepine receptors. In rats, ODN reduced the latency to enter a black compartment when the animals were placed in the white one. In the plus-maze elevated test, ODN reduced, in both mice and rats, the number of entries and the time spent in the open arm. In mice, ODN (100 ng) increased the thigmotaxis index, i.e. the distance traveled in the peripheral zone of the open field. Time-course studies revealed that a significant effect of ODN (100 ng) in the black/white compartment test was only observed 40 min after the injection and lasted between 3 and 6 h. The effect of a 1000-ng dose of ODN appeared more tardily than that of a 10-ng dose. In addition, a 1000-ng dose of ODN occluded the early effect of a 100-ng dose on the white compartment first stay duration. The COOH-terminal octapeptide of ODN was more rapidly effective than ODN in the black/white compartment test, suggesting that the anxiogenic effect of the peptide requires the formation of biologically active proteolytic fragment. © 1998 Elsevier Science Inc.

Octadecaneuropeptide (ODN) Diazepam-binding inhibitor Endozepines Rat Mouse Anxiety Elevated plus-maze Black/white compartment Thigmotaxis

DIAZEPAM-BINDING INHIBITOR (DBI) is an 86 amino-acid polypeptide which has been initially isolated from the rat brain on the basis of its ability to displace diazepam from its binding sites (10). Proteolytic cleavage of DBI generates several biologically active fragments including the triakontatetraneuropeptide $DBI_{17–50}$ (TTN) (24) and the octadecaneuropeptide $DBI_{33–50}$ (ODN) (7) which are all designated by the generic term endozepines (26). Intracerebroventricular (ICV) injection of endozepines induces proconflict behavior and reverses the anticonflict action of diazepam

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(7,8,10). In particular, ODN has been reported to increase aggressive interaction in mice (12).

The mechanism of action of endozepines is not fully understood. It has been initially proposed that these peptides act as inverse agonists of central-type benzodiazepine receptors (8) thus inhibiting the activity of the $GABA_A$-chloride channel complex (4,14,25). Subsequently, endozepines were found to interact with peripheral-type benzodiazepine receptors (16,24) and to stimulate cholesterol transport into the mitochondria (15). Consistent with this finding, endozepines have been shown to stimulate biosynthesis of neurosteroids from normal brain tissue (6) and glioma cells (16). More recently, it has been shown that ODN, acting through a non-benzodiazepinic membrane receptor coupled to phospholipase C (9,17) causes an increase in cytosolic calcium concentration in rat astrocytes (13).

Although the term ≪ anxiety peptide ≫ has been coined to designate ODN (3), very few studies have been conducted to investigate the anxiogenic effects of endozepines. It has been reported that immunization of rats against a fragment of DBI reduces reaction of fear and anxiety in a stress model of open field and in the conflict Vogel test (2). A positive correlation between the cerebrospinal concentration of DBI and corticotropin-releasing hormone has been found in depressed patients (20), suggesting that endozepines may play a role in the stress response in humans. The aim of the present study was to investigate the anxiogenic effect and the mechanism of action of intracerebroventricularly administered ODN in mice and rats.

## METHOD

### *Animals*

Experiments were carried out on male Swiss albino CD1 mice (22–25 g) and male Sprague–Dawley rats (180–220 g). Animals were purchased from Charles River (Saint-Aubin lès Elbeuf, France). Mice and rats were housed by 30 and 4, respectively, in Makrolon cages (L = 38 cm, W = 24 cm, H = 18 cm), with free access to water and food (U.A.R., France). The animals were kept in a ventilated room at a temperature of 21 ± 1°C, under a 12 h light/12 h dark cycle (light on between 7:00 a.m. and 7:00 p.m.). Experiments were carried out between 9:00 a.m. and 5:00 p.m. Animal manipulations were performed according to the recommendations of the French Ethical Committee and under the supervision of authorized investigators. Each animal (mouse or rat) was used only once, for a single test, at a single time after treatment.

### *Intracerebroventricular Injections*

In mice, ICV injections (10 μl) were made in the left ventricle, within about 3 s, with a microsyringe (Hamilton, 50 μl) connected to a needle (diameter 0.5 mm) of which the median part of the bevel protruded only 3.5 mm from a guard, limiting its penetration into the brain of manually immobilized mice, according to the procedure of Haley and McCormick (11).

Rats were anesthetized with chloral hydrate (400 mg/kg, IP) and a small area of the skin (about 25 $mm^2$) was dissected out. An incomplete drilling of the skull was made with a dentary miller at the following coordinates: L = 2 mm posterior and 1.8 mm lateral to bregma, according to Albe-Fessard et al. (1). An organo-mercurial antiseptic was applied for preventing infection. Forty eight hours later, an ICV injection (20 μl) was made free-hand in the left ventricle, with a microsyringe (Hamilton 50 μl) connected to a needle (diameter 0.5 mM) of which the median part of the bevel protruded 5 mm from a guard limiting its penetration into the brain. ICV injections were performed by an experienced investigator. Pilot experiments which consisted in injecting a dye solution (diluted India ink) had shown after brain sectioning a ventricular localization in more than 95% of the trials.

### *Black/White Compartment Test*

This test was performed by an investigator who ignored the treatment administered to mice or rats.

The apparatus for mice consisted of an enclosure divided in two compartments, each measuring L = 32 cm, W = 22 cm, H = 18 cm. One compartment was dark (painted black and covered with a lid). The other compartment (not covered) was painted white and strongly lit by a 40-W bulb set 50 cm above the floor. The compartments communicated through an opening (W = 5 cm, H = 5 cm) located at the base of the middle of the partition wall. The testing procedure was as follows. After the ICV injection, mice were placed in individual cages; 90 min later they were introduced into the black compartment (the animal looking at a corner of the wall opposed to the aperture), the cover was applied. A first chronometer was started and was stopped when the animal entered for the first time entirely into the white compartment (all four paws inside). At this time, a second chronometer measured the duration of the first stay in the white compartment. The overall trial never lasted more than 5 min. The latency to enter for the first time the white compartment as well as the first stay duration in this white compartment were measured. If the mouse entered immediately the white compartment (latency < 5 s), the measure was not taken into account and the animal was discarded because such an instantaneous entry shunts the integration of environmental cues and thus does not allow to mouse to arbitrate between curiosity and anxiety and thus to elaborate an adapted response.

The apparatus for rats was similar except that it had larger dimensions:

L = 50 cm, W = 45 cm, H = 22 cm. The dimensions of the opening located at the base of the middle of the partition wall were W = 8 cm, H = 10 cm. Since saline ICV-injected

rats introduced into the black compartment do not usually enter the white compartment during the 5 min of the test, rats were introduced into the white compartment. The sash door was closed during the first 30 s of the stay in the white compartment; then, the door was opened and the chronometer was started to measure the delay before the rat entered the black compartment. In rats the measures were performed 90 min after injection of ODN.

In mice, in order to compare the effects of a low (10 ng) and a high dose (100 ng) of ODN, on the black/white compartment test, the measures were performed 20, 90, 180 min after injection of ODN.

*Time Course of the Effects of ODN on the Black/White Compartment Test in Mice*

A time course of the effect of ODN, at the 100-ng dose, as compared to saline injected controls, was established between 20 min and 9 h, in 5 different experiments, using 4 saline- and 4 ODN-treated mice, for each considered time after ICV injection (28 mice per experiment).

Two animals (one saline- and one ODN-treated) were tested simultaneously in two apparatus, by the same investigator. Each apparatus was used successively for a saline- and an ODN-treated mice. This time-course study led us to select a 90-min interval between the injection of ODN and each behavioral testing.

The effect of the octapeptide fragment of ODN was only tested at the 100-ng dose and the behavioral response was examined 20 and 40 min after ICV injection, in two different experiments, using 10 saline- and 10 octapeptide-treated mice, for each considered time after ICV injection (20 mice per experiment).

*Elevated Plus-Maze Test*

The elevated plus-maze test was performed according to Pellow et al. (18). The apparatus for mice consisted of a wooden Greek cross (dimension of each arm: L = 18 cm, W = 6 cm) placed 50 cm above the floor. Two opposite arms were surrounded by walls (H = 6 cm; closed arms) while the two other arms were devoid of enclosing walls (open arms). The whole device was painted black and the room was brightly lit. Animals were injected 90 min before the test. The mouse was placed at the center of the maze, its head facing a closed arm. The measurement (which lasted 5 min) was automated using an image analysis system (described further on). The number of entries (all four paws on the floor of an open arm) and the time spent in the open arms were measured. The plus-maze was wiped clean after each animal trial.

The apparatus for rats was similar, except it had larger dimensions (for each arm: L = 110 cm, W = 10 cm. Two opposite arms were surrounded by walls (H = 31 cm). The device was placed 80 cm above the floor.

*Thigmotaxis Assessment*

The thigmotaxis test was only performed in mice, according to Simon et al. (23). The experimental device was a square open-field (40 × 40 cm) surrounded by walls (H = 30 cm) and covered by fine wire netting. All these elements were painted black, except for one of the walls which was in Plexiglas. The room was very dimly lit. The testing session, lasting 10 min, was preceded by a 5-min period of habituation. The animals were injected 90 min before the introduction into the open-field. The thigmotaxis behavior (i.e. moving along the wall) was assessed by calculating the ratio of the distance traveled in the peripheral zone to the total covered distance and was expressed as a percentage. The measurement was automated using an image analysis system (see further on).

*Image Analysis System*

The plus-maze elevated test as well as the thigmotaxis test were carried out using the Videotrack 512 system (Viewpoint, Lyon, France). The apparatus consisted of video cameras positioned above the experiment field, a video interface and a microcomputer. The system converted the video input signals into binary images in such a manner that each animal corresponded to a white spot against a black background. Virtual windows on a computer screen corresponded to different areas of the open-field or to the open or the closed arms of the elevated plus-maze.

*Drugs and Solutions*

Rat ODN (Gln-Ala-Thr-Val-Gly-Asp-Val-Asn-Thr-Asp-Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys) has been utilized at 10–100-1000-ng doses and the octapeptide (Arg-Pro-Gly-Leu-Leu-Asp-Leu-Lys) has been utilized at 100-ng dose and were synthesized by the solid phase methodology as previously described (9). The peptides were dissolved in saline just before ICV injection.

Chloral hydrate at 400 mg/kg-dose (Sigma, Saint-Quentin Fallavier, France) was dissolved in distilled water. Diazepam (Valium®) at 0.075–0.15-mg/kg dose and flumazenil (Anexate®) at 1-mg/kg dose (injectable solutions; Roche, Neuilly-sur-Seine, France) were diluted in saline.

*Statistics*

The data are expressed as mean ± SEM. Differences between groups were assessed by Student's *t*-test, by $\chi^2$ test, by Dunnet's *t*-test and by two-way analysis of variance (ANOVA).

## RESULTS

*Black/White Compartment Test*

In mice, 90 min after ICV injection of 10-, 100- or 1000-ng doses of ODN, a significant decrease in the first stay duration in the white compartment was observed, whatever the

TABLE 1

EFFECTS IN MICE OF INCREASING DOSES OF ODN ON THE ENTERING LATENCY AND THE FIRST STAY DURATION IN A WHITE COMPARTMENT FROM A BLACK ONE

| Treatment | White Compartment Entering Latency (s) | White Dompartment First Stay Duration (s) |
|---|---|---|
| Saline 10$\mu$l | 22 ± 2 | 18 ± 1 |
| ODN 10 ng/ 10$\mu$l | 28 ± 3$^{ns}$ | 10 ± 1* |
| ODN 100 ng/ 10 $\mu$l | 39 ± 3* | 7 ± 0* |
| ODN 1000 ng/ 10$\mu$l | 23 ± 2$^{ns}$ | 13 ± 1* |

Mice were injected ICV with saline or ODN (10, 100 or 1000 ng) and, 90 min later, the animals were introduced into the black compartment. Data are means ± SEM for 20 mice per group.

* $p < 0.01$; $^{ns}$ not significantly different as compared to the saline group.

dose administered. In contrast, a significant increase in the white compartment entering latency was observed only at the 100-ng dose of the peptide (Table 1; means ± SEM of 20 mice per group; Dunnet's *t*-test; $p < 0.01$).

In control rats, 90 min after ICV saline injection, the latency to enter the black compartment after opening the sash door was 72 ± 13 s; 90 min after ICV injection of 100 ng ODN, the latency to enter the black compartment was significantly reduced to 21 ± 13 s (mean ± SEM of 9 rats per group; Student's *t*-test; $p < 0.01$).

### *Effect in Mice of Diazepam or Flumazenil on the ODN-Evoked Increase in the Entering Latency and First Stay Duration in a White Compartment from a Black One*

Administration of diazepam, at a dose as low as 0.075 mg/kg SC, 30 min before testing, reversed the effect of 100 ng ODN on the white compartment entering latency, as well as on the first stay duration in the white compartment (Table 2). Similarly, flumazenil (1 mg/kg) attenuated the effect of ODN on the white compartment entering latency and abrogated the effect of ODN on the first stay duration in the white compartment. When injected alone, diazepam (0.15 mg/kg) as well as flumazenil (1 mg/kg) significantly increased the white compartment entering latency but did not significantly modify the white compartment first stay duration $F(1, 6) = 4$, $p < 0.05$ and $F(247, 62) = 20$, $p < 0.001$, respectively (20 mice per group; two-way ANOVA analysis). When ODN (100 ng ICV), was opposed to 0.075 mg/kg SC of diazepam, it significantly decreased the white compartment entering latency, $F(5, 2) = 8$, $p < 0.01$, in addition it significantly increased the white compartment first stay duration $F(33, 1) = 6$, $p < 0.05$. Similarly, a significant decrease in the white compartment entering latency was observed when 0.15 mg/kg SC of diazepam was opposed to ODN (100 ng ICV), $F(26, 9) = 12$, $p < 0.001$. When 1 mg/kg SC of flumazenil was opposed to ODN (100 ng ICV), a significant decrease in the white compartment entering latency was observed, $F(19,1) = 10$, $p < 0.01$. Concurrently a statistically significant increase in the white compartment first stay duration was observed, $F(247,62) = 20$, $p < 0.001$ (Table 2).

### *Time-Course of the Effects of ODN on the Black/White Compartment Test*

Administration of 100 ng ODN increased the white compartment entering latency only after a time lag of 60 min: in control mice, 60 min after ICV saline injection, the white compartment entering latency was 29 ± 3 sec while in mice injected with 100 ng ODN the latency was 49 ± 5 s (means ± SEM of 20 mice per group; Student's *t*-test; $p < 0.01$). The effect of ODN vanished between the 3$^{rd}$ and the 6$^{th}$ h. Injection of 100 ng ODN reduced the first stay duration in the white compartment only after a time lag of 60 min: in control mice, 60 min after ICV saline injection, the white compartment first stay duration was 16 ± 1 while in mice injected with 100 ng ODN the duration was 9 ± 1 (means ± SEM of 20 mice per group; Student's *t*-test; $p < 0.01$). This effect disappeared only between the 6$^{th}$ and the 9$^{th}$ hour (Table 3).

The COOH-terminal octapeptide fragment of ODN mimicked both the reduction of the first stay duration and the

TABLE 2

EFFECT IN MICE OF DIAZEPAM OR FLUMAZENIL ON THE ODN-EVOKED INCREASE IN ENTERING LATENCY AND FIRST STAY DURATION IN A WHITE COMPARTMENT FROM A BLACK ONE

| Treatment | White Compartment Entering Latency (s) | White Compartment First Stay Duration (s) |
|---|---|---|
| Saline 10 $\mu$l | 28 ± 3 | 16 ± 1 |
| ODN 100 ng/10 $\mu$l | 41 ± 3** | 6 ± 1*** |
| Diazepam 0.075 mg/kg | 31 ± 3$^{ns}$ | 15 ± 1$^{ns}$ |
| Diazepam 0.075 mg/kg +ODN 100 ng/10 $\mu$l | 30 ± 2$^{a1}$ | 10 ± 1$^{a4}$ |
| Saline 10 $\mu$l | 23 ± 2 | 13 ± 1 |
| ODN 100 ng/10 $\mu$l | 50 ± 3*** | 7 ± 1*** |
| Diazepam 0.15 mg/kg | 37 ± 3** | 13 ± 2$^{ns}$ |
| Diazepam 0.15 mg/kg +ODN 100 ng/10 $\mu$l | 32 ± 4$^{a2}$ | 15 ± 2$^{a5}$ |
| Saline 10 $\mu$l | 22 ± 2 | 23 ± 1 |
| ODN 100 ng/10 $\mu$l | 46 ± 3*** | 7 ± 1*** |
| Flumazenil 1 mg/kg | 31 ± 3* | 26 ± 1$^{ns}$ |
| Flumazenil 1 mg/kg +ODN 100 ng/10 $\mu$l | 35 ± 3$^{a3}$ | 17 ± 1$^{a6}$ |

Diazepam (0.075 and 0.15 mg/kg) or flumazenil (1 mg/kg) were administered SC 60 min after ICV injection of either saline (10 $\mu$l) or ODN (100 ng). 30 min after the latter injection, the animals were introduced into the black compartment. Data are means ± SEM. for 20 mice per group. $^{a1}$ Significantly different as compared to the ODN-treated group [ANOVA:$F(5,2) + 8$, $p < 0.009$]; $^{a2}$ significantly different as compared to the ODN-treated group [ANOVA: $F(26,9) = 12$, $p < 0.001$]; $^{a3}$ significantly different as compared to the ODN-treated group [ANOVA: $F(19,1) = 10$, $p < 0.004$]; $^{a4}$ significantly different as compared to the ODN-treated group [ANOVA: $F(33,1) = 6$, $p < 0.02$]; $^{a5}$ significantly different as compared to the ODN-treated group [ANOVA: $F(1,6) = 4$, $p < 0.04$] and $^{a6}$ significantly different as compared to the ODN-treated group [ANOVA: $F(247,62) = 20$, $p < 0.001$]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and $^{ns}$ not significantly different as compared to the saline groups.

TABLE 3

TIME COURSE OF THE EFFECTS IN MICE OF ODN AND OF ITS *C*-TERMINAL OCTAPEPTIDE FRAGMENT ON THE ENTERING LATENCY AND THE FIRST STAY DURATION IN A WHITE COMPARTMENT FROM A BLACK ONE

| ICV injection time before testing | White Compartment Entering Latency (s) | | White Compartment First Stay Duration (s) | |
|---|---|---|---|---|
| | Saline 10 $\mu$l | ODN 100 ng/10 $\mu$l | Saline 10 $\mu$l | ODN 100 ng/10 $\mu$l |
| 20 min | $26 \pm 8$ | $25 \pm 3^{ns}$ | $11 \pm 1$ | $10 \pm 1^{ns}$ |
| 40 min | $25 \pm 1$ | $27 \pm 2^{ns}$ | $16 \pm 1$ | $14 \pm 1^{ns}$ |
| 60 min | $29 \pm 3$ | $49 \pm 5^{b}$ | $16 \pm 1$ | $9 \pm 1^{b}$ |
| 90 min | $25 \pm 2$ | $39 \pm 4^{c}$ | $18 \pm 1$ | $7 \pm 1^{c}$ |
| 180 min | $28 \pm 4$ | $50 \pm 7^{a}$ | $14 \pm 1$ | $7 \pm 1^{c}$ |
| 6 h | $34 \pm 4$ | $32 \pm 5^{ns}$ | $16 \pm 2$ | $11 \pm 1^{a}$ |
| 9 h | $37 \pm 5$ | $30 \pm 4^{ns}$ | $17 \pm 5$ | $15 \pm 3^{ns}$ |
| | Saline 10$\mu$l | Octapeptide 100ng/10$\mu$l | Saline 10$\mu$l | Octapeptide 100ng/10$\mu$l |
| 20 min | $28 \pm 2$ | $29 \pm 2^{ns}$ | $14 \pm 2$ | $12 \pm 1^{ns}$ |
| 40 min | $31 \pm 2$ | $41 \pm 2^{b}$ | $17 \pm 1$ | $8 \pm 1^{c}$ |

Mice were injected ICV with saline or ODN (100 ng) and were introduced into the black compartment at the times indicated in the left column. Data are means $\pm$ SEM for 20 mice per group. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$; [ns] not significantly different as compared to the saline groups.

increase of the white compartment entering latency evoked by ODN (Table 3). Administered at the same dose as ODN (100 ng), the octapeptide fragment exhibited a significant effect on both parameters after 40 min: in control mice, 40 min after ICV saline injection, the white compartment entering latency was $31 \pm 2$ while in mice injected with 100 ng octapeptide the latency was $41 \pm 2$ (means $\pm$ SEM of 20 mice per group; Student's *t*-test. $p < 0.01$); in control mice, 40 min after ICV saline injection, the white compartment first stay duration was $17 \pm 1$ while in mice injected with 100 ng ODN the duration was $8 \pm 1$ (means $\pm$ SEM of 20 mice per group; Student's *t*-test; $p < 0.001$; Table 3).

### *Comparison of the Effects in Mice of Low and High Doses of ODN on the Black/White Compartment Test*

The decrease of the first stay duration in the white compartment induced by a low dose (10 ng) of ODN was more precocious (20 min) than that induced by a high dose (100 ng) of ODN (90 min). In contrast, the white compartment entering latency was not affected by the 10-ng dose ODN; it was significantly increased only 180 min after injection of the 100-ng dose ODN (Table 4): in control mice, 20 min after ICV saline injection, the first stay duration in the white compartment was $16 \pm 2$ while in mice injected with 10 ng ODN the duration was $6 \pm 0$ (means $\pm$ SEM of 10 to 20 mice per group; Dunnet's *t*-test; $p < 0.01$); in control mice, 180 min after ICV saline injection, the white compartment entering latency was $27 \pm 2$ while in mice injected with 100 ng ODN, the latency was $37 \pm 4$ (means $\pm$ SEM of 10 to 20 mice per group; Dunnet's *t*-test; $p < 0.05$).

### *Effects in Mice of a High Dose of ODN on the Anxiogenic Response Induced by a Low Dose of the Peptide on the Entering Latency and the First Stay Duration in a White Compartment from a Black One*

The reduction of the first stay duration in the white compartment, observed 90 min after injection of 100 ng ODN, was totally prevented when the animals received a high dose

TABLE 4

COMPARISON OF THE EFFECTS IN MICE OF A LOW AND A HIGH DOSE OF ODN ON THE BLACK/WHITE COMPARTMENT TEST

| ICV injection time before testing | White compartment entering latency (s) | | | White compartment first stay duration (s) | | |
|---|---|---|---|---|---|---|
| | Saline 10 $\mu$l | ODN 10 ng/10 $\mu$l | ODN 100 ng/10 $\mu$l | Saline 10 $\mu$l | ODN 10 ng/10 $\mu$l | ODN 100 ng/10 $\mu$l |
| 20 min | $27 \pm 2$ | $34 \pm 4^{ns}$ | $23 \pm 1^{ns}$ | $16 \pm 2$ | $6 \pm 0^{b}$ | $17 \pm 2^{ns}$ |
| 90 min | $22 \pm 2$ | $28 \pm 3^{ns}$ | $23 \pm 2^{ns}$ | $18 \pm 1$ | $10 \pm 1^{b}$ | $13 \pm 1^{b}$ |
| 180 min | $27 \pm 2$ | $31 \pm 1^{ns}$ | $37 \pm 4^{a}$ | $18 \pm 3$ | $14 \pm 1^{ns}$ | $8 \pm 1^{b}$ |

Mice were injected ICV with saline or ODN (10 or 100 ng) at various times before being introduced into the black compartment. Data are means $\pm$ SEM for 10 to 20 mice per group. [a] $p < 0.05$; [b] $p < 0.01$; [ns] not significantly different as compared to saline group.

TABLE 5

EFFECTS IN MICE OF A HIGH DOSE OF ODN ON THE ANXIOGENIC RESPONSE INDUCED BY A LOW DOSE OF THE PEPTIDE ON THE ENTERING LATENCY AND THE FIRST STAY DURATION IN A WHITE COMPARTMENT FROM A BLACK ONE

| Treatments 90 min and 30 min before testing | White Compartment Entering Latency (s) | White Compartment First Stay Duration (s) |
|---|---|---|
| Saline 10 $\mu$l<br>Saline 10 $\mu$l | 30 ± 3 | 15 ± 2 |
| Saline 10 $\mu$l<br>ODN 1000 ng/10 $\mu$l | 31 ± 3$^{ns}$ | 13 ± 1$^{ns}$ |
| ODN 100 ng/10 $\mu$l<br>Saline 10 $\mu$l | 48 ± 3$^{b}$ | 8 ± 1$^{a}$ |
| ODN 100 ng/10 $\mu$l<br>ODN 1000 ng/10 $\mu$l | 41 ± 5$^{c1}$ | 16 ± 1$^{c2}$ |

Mice were injected ICV twice with saline or ODN (100 or 1000 ng) 90 and 30 min before being introduced into the black compartment. Data are means ± SEM for 20 mice per group. [a] $p < 0.01$; [b] $p < 0.001$; [ns] not significantly different, as compared to the saline group. [c1] Not significantly different as compared to the ODN-treated group [ANOVA:$F(22.6,0.3) = 0.8$, $p < 0.375$]; [c2] significantly different as compared to the ODN-treated group [ANOVA:$F(2.8,6.6) = 18.9$, $p < 0.001$].

of ODN (1000 ng) 30 min before the black/white compartment test, $F(2.8, 6.6) = 18.9$, $p < 0.001$ (20 mice per group; two-way ANOVA analysis; Table 5).

*Thigmotaxis Index*

In mice, ICV injection of 100 ng ODN reduced the total traveled distance by 56%. In control mice, 90 min after ICV saline injection, the index of thigmotaxis was 67 ± 3 while in mice injected with 10 or 100 ng of ODN, the index of thigmotaxis was 66 ± 3 and 80 ± 3, respectively (means ± SEM of 20 mice per group; Dunnet's *t*-test; $p < 0.01$; Table 6).

*Elevated Plus-Maze Test*

In mice, the 100-ng dose of ODN reduced the total distance traveled in the apparatus by 50%. A concomitant reduction in the number of entries into the open arms of the plus-maze was observed: in control mice, 90 min after ICV saline injection the number of entries into the open arms was 5.6 ± 1.7 while, in mice injected with 10 and 100 ng ODN the numbers of entries were 1.5 ± 0.6 and 1.3 ± 0.4, respectively (means ± SEM of 9 mice per group; Dunnet's *t*-test; $p < 0.05$ and $p < 0.05$, respectively). ODN also induced a reduction in the fraction of time spent in the open arms: in control mice, 90 min after ICV saline injection, the time spent in the open arms was 7.2 ± 1.8 while in mice injected with 10 and 100 ng ODN the times were 1.1 ± 0.7 and 0.3 ± 0.1, respectively (means ± SEM of 9 mice per group; $\chi^2$ analysis; $p < 0.05$ and $p < 0.01$, respectively; Table 7).

In rats, administration of 100 ng ODN did not significantly reduce the total distance traveled but completely suppressed the entry into the open arms (Table 7).

## DISCUSSION

The present study has demonstrated that ICV injection of ODN induces, in both mice and rats, behavioral modifications which may be interpreted as an increase in anxiety. Although a locomotor response was involved in each test, the slight decrease in locomotion elicited by 100 ng ODN in mice does not seem to account for these modifications. It seems more likely that anxiety might influence negatively this exploratory locomotion. For instance in the black/white compartment test a decrease in locomotion would increase the latency for entering the white compartment but it would also increase the first stay duration in this compartment. This was clearly not the case since this duration was shortened. In the thigmotaxis test the total distance traveled by mice injected with 100 ng ODN was indeed significantly reduced but this reduction was more pronounced in the central part (more anxiogenic) of the open field than in the peripheral one (less anxiogenic); as a matter of fact the ≪index of thigmotaxis≫ is the ratio of distances traveled in each zone of the open-field.

The inference that the effects of ODN on these tests depend on an anxiogenic effect is supported by the fact that the three tests which were carried out have been described and validated to detect anxiogenic properties of drugs. In addition, on each test, ODN was effective at the same time and for the same dose (100 ng). Finally, in the black/white compartment test the anxiolytic drug diazepam, at very low doses, inhibited the effect of ODN.

In mice, ODN was found to exert an anxiogenic effect in all behavioral tests applied. In order to determine whether this observation could be extrapolated to rats, we have performed selected series of experiments evidencing that ODN exerts also anxiogenic effects in rats.

The double (black/white) compartment test, applied to mice, was performed in an opposite way as compared to the

TABLE 6

EFFECTS IN MICE OF ODN ON THIGMOTAXIS IN AN OPEN FIELD

| Treatment | Total Distance Traveled (cm) | Index of Thigmotaxis (%) |
|---|---|---|
| Saline 10 $\mu$l | 3430 ± 303 | 67 ± 3 |
| ODN 10 ng/10 $\mu$l | 2698 ± 480$^{ns}$ | 66 ± 3$^{ns}$ |
| ODN 100 ng/10 $\mu$l | 1904 ± 390* | 80 ± 3$^{\dagger}$ |

Mice were injected ICV with saline or ODN (10 or 100 ng) and, 90 min later, the animals were introduced into the open-field. The traveled distances were measured during 10 min, after a 5-min period of habituation. The ''index of thigmotaxis'' is calculated as the distance traveled in the peripheric zone/total distance × 100. Data are means ± SEM for 20 mice per group.

* $p < 0.05$; [†] $p < 0.01$; [ns] not significantly different as compared to the saline group.

TABLE 7

EFFECTS OF ODN IN MICE OR RATS ON THE ELEVATED PLUS-MAZE TEST

| Treatment | Total Distance Traveled (cm) | Number of Entries into Open Arms | Percent of Animals Entering into Open Arms | Percent of Time Spent into Open Arms |
|---|---|---|---|---|
| Mice | | | | |
| Saline 10 $\mu$l | 822 ± 129 | 5.6 ± 1.7 | 100 | 7.2 ± 1.8 |
| ODN 10 ng/10 $\mu$l | 511 ± 137$^{ns}$ | 1.5 ± 0.6* | 60$^{ns}$ | 1.1 ± 0.7$^{\dagger}$ |
| ODN 100 ng/10 $\mu$l | 414 ± 98* | 1.3 ± 0.4* | 66 $^{ns}$ | 0.3 ± 0.1$^{\ddagger}$ |
| Rats | | | | |
| Saline 20 $\mu$l | 1561 ± 312 | 6.0 ± 1.9 | 100 | 6.9 ± 2.9 |
| ODN 100 ng/20 $\mu$l | 1248 ± 302$^{ns}$ | 0.0 ± 0.0** | 0.0$^{\S}$ | 0.0$^{\ddagger}$ |

Mice or rats were injected ICV with saline or ODN (10 or 100 ng) and, 90 min later, the animals were placed at the center of the maze. The percentage of time spent in the open arms was calculated by considering only the animals which entered into those open arms. Data are means ± SEM for 9 mice or rats per group. * $p < 0.05$; ** $p < 0.01$; $^{ns}$ not significantly different as compared to the saline groups. $\chi^2$-test $^{\dagger}$ $p < 0.05$, $^{\ddagger}$ $p < 0.01$, $^{\S}$ $p < 0.001$; $^{ns}$ not significantly different as compared to the saline groups.

conventional test, inasmuch as the animals were introduced into the black compartment. We have previously shown that this modality is particularly well adapted for evidencing anxiogenic activity of drugs (22). This test lies on the natural aversion of albino mice for an intensely illuminated environment. Using this procedure, the first stay duration in the white compartment appears to be a more sensitive criterion than the latency to enter the white compartment. In contrast, when rats were placed in the black compartment, they did not enter the white compartment during the maximal 5-min period of the test. Therefore, in rats, the test was performed in an opposite way relatively to mice; in this case, anxiety is associated with a decrease in the latency to enter the black compartment. The elevated plus-maze test as well as the thigmotactic test reflect the need for an anxious animal, introduced into a new environment, to remain in the proximity of peripheral walls in order to remain in contact with them by its vibrissae (21,23). From an anthropomorphical point of view this thigmotactic behavior can be likened to agoraphobia or to escape behavior, i.e. the search for an exit in case of danger.

The anxiogenic effect induced by ICV administration of 100 ng ODN, in the black/white compartment test, culminated at only 90 min. Consistent with this observation, previous studies have shown that the aggressive behavior induced by ICV administration of ODN can be evidenced only after a certain time lag (12). At the 1000-ng dose of ODN, a longer period (3 h) was necessary before an anxiogenic effect was observed. Similarly, 10 ng ODN induced a more precocious anxiogenic effect than a 100-ng dose, i.e., 60 min vs. 90 min. This observation suggests that ODN could act as a prodrug, capable of generating a biologically active peptide fragment. In support of this hypothesis, we found that the octapeptide corresponding to the carboxy-terminal moiety of ODN also exhibited an anxiogenic effect and induced a more precocious response than ODN. Taking into account 1) the long time lag between the ICV injection of 100 ng ODN and its maximal anxiogenic effect (≅90 min) and 2) the efficacy as well as the shorter delay of action of the octapeptide fragment of ODN, it seems that the anxiogenic effect of the peptide would be produced by a fragment of ODN slowly generated by peptidase activity/ies. The octapeptide, or even a shorter fragment, would be candidate for this activity.

Taking now into account 1) the reduction of the time lag between the ICV injection and the appearance of the anxiogenic effect when the dose of ODN was reduced (60 min for 10 ng as compared to 90 min for 100 ng) and 2) the apparent prevention of the effect produced by 100 ng ODN given 90 min before testing by the administration of 1000 ng ODN operated 30 min before testing, one is tempted to propose the following interpretation. The anxiogenic effect linked to a fragment of ODN would be prevented by ODN itself. For instance ODN as well as its active fragment(s) could bind at a same receptor. On this receptor ODN would behave as an antagonist or a very partial agonist whereas the octapeptide, or a shorter fragment, would behave as a full agonist. Thus, when the ratio of ODN and its effective fragment favored ODN the anxiogenic effect was not observed, that corresponds to the time lag for appearance of the anxiety after ODN administration and also to the masking of the effect of 100 ng ODN at the 90$^{th}$ min after its administration by 1000 ng ODN administered 30 min before testing. Previous studies have shown that ODN may interact either with central-type benzodiazepine receptors (8) or with a seven transmembrane G protein-coupled receptor (13,17). The observation that both the benzodiazepine receptor agonist diazepam and the central-type benzodiazepine receptor antagonist flumazenil abolished the response to ODN in the black/white compartment test indicates that the anxiogenic effect of the peptide is mediated through an interaction with central-type benzodiazepine receptors. In fact, ODN (and more likely its cleavage fragment) appears to mimic the anxiogenic activity of ethyl $\beta$-carboline 3-carboxylate (5,7,8,19), indicating that the peptide acts as an inverse agonist on central-type benzodiazepine receptors.

In conclusion, central administration of ODN induces, in

rodents, anxiogenic effects that are mediated by benzodiazepine receptors. The observations that 1) the response of ODN was delayed, especially when high doses of the peptide are administered; 2) the C-terminal octapeptide moiety of ODN mimicked the anxiogenic effect of ODN but induced a more precocious response; and 3) the effect of a moderate dose of ODN was masked by previous administration of a high dose of the peptide, suggest that the mechanism of action of ODN involves the generation of a biologically active proteolytic fragment.

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