Anxiogenic effects in the rat elevated plus-maze of 5-HT$_{2C}$ agonists into ventral but not dorsal hippocampus

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The effect of the non-selective 5-HT$_{2C}$ receptor agonist trifluoromethyl-phenylpiperazine (TFMPP, 0.75, 1.5 and 3.0 μg) and the preferential 5-HT$_{2C}$ agonist 6-chloro-2 (1-piperazinyl)pyrazine (MK-212, 0.1, 0.3 and 1.0 μg) microinjected into the ventral or dorsal hippocampus was investigated in anxiety measures of rats exposed to the elevated plus-maze test. Ventral hippocampal (VH) microinjections of the 0.75 or 1.5 μg doses of TFMPP reduced open-arm exploration without affecting the number of closed-arm entries, indicating a selective anxiogenic profile. The highest dose (3.0 μg) reduced open- and closed-arm entries, suggesting interference in locomotor activity. The 0.1 μg dose of MK-212 also caused a selective anxiogenic effect when microinjected into the ventral hippocampus, without disturbing locomotor activity. Microinjections of the two higher doses of MK-212 (0.3 or 1.0 μg) into the ventral hippocampus led to a decrease of exploration in both arms of the maze. In contrast to the anxiogenic effect observed in the VH, neither TFMPP nor MK-212 significantly changed anxiety measures when microinjected into the dorsal hippocampus. These results suggest that activation of 5-HT$_{2C}$ postsynaptic receptors located in the ventral, but not in the dorsal, hippocampus play an important role in anxiety triggered by the elevated plus-maze test. *Behavioural Pharmacology* **15:37–43 © 2004 Lippincott Williams & Wilkins.**

Behavioural Pharmacology 2004, **15**:37–43

Keywords: elevated plus-maze, 5-HT$_{2C}$ receptors, TFMPP, MK-212, ventral hippocampus, dorsal hippocampus, animal models of anxiety, rat

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Sponsorship: A.P.M.C. and J.L.-F. are recipients of research grants from Conselho Nacional de Desenvolvimento Citentíficoe Technológica (CNPq) (350214/1998-0 and 522720/95-1). S.H.A. was the recipient of student fellowships from CNPq.

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Received 12 September 2003 **Accepted as revised** 16 November 2003

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is known to play an important role in anxiety (for reviews see Iversen, 1984; Griebel, 1995; Lucki, 1996; Naughton *et al.*, 2000; Graeff, 2002). The actions of 5-HT are mediated by a wide family of receptors (Hoyer *et al.*, 1994; Martin *et al.*, 1998), including the 5-HT$_2$ receptors. Evidence supporting a role for 5-HT$_2$ receptors in anxiety arises mainly from studies showing that drugs acting as agonists at 5-HT$_{2C}$ receptors, previously referred to as 5-HT$_{1C}$ receptors (Abramowski *et al.*, 1995; Martin *et al.*, 1998; Clemett *et al.*, 2000), present anxiogenic effects in clinical and experimental forms of anxiety. For example, systemic injections of 5-HT$_{2C}$ agonists, such as *m*-chlorophenylpiperazine (*m*-CPP) or trifluoromethyl-phenylpiperazine (TFMPP), increase anxiety in humans (Mueller *et al.*, 1985; Charney *et al.*, 1987; Kahn *et al.*, 1988) as well as in several animal models of anxiety, including the social interaction test in rats (Kennett *et al.*, 1989), the light–dark transition (Nic Dhonnchadha *et al.*, 2003), the elevated T-maze (Graeff *et al.*, 1998), the elevated plus-maze (Benjamin *et al.*, 1990; Rodgers *et al.*, 1992; Gibson *et al.*, 1994; Griebel *et al.*, 1994; Fone *et al.*, 1996; Wallis and Lal, 1998; Setem *et al.*, 1999) and a modified version of the latter test (Jones *et al.*, 2002).

Despite showing anxiogenic-like profiles, the underlying neural circuitry related to the effects of systemically administered 5-HT$_{2C}$ receptor agonists remains unclear. This is partially due to the fact that very few studies have examined the effects of centrally administered 5-HT$_{2C}$ receptor agonists in animal models of anxiety (for a review see Menard and Treit, 1999). Nevertheless, in one of these few studies *m*-CPP microinfusions into the dorsal hippocampus (DH) elicited anxiogenic-like effects in the social interaction test in rats. Based on this report, it was important to extend these results to a different animal model, especially because there is strong evidence showing that anxiety defined operationally in a given animal model may differ from that generated by other models, in respect to neural substrate and drug responses for 5-HT ligands (for reviews see File, 1992; Handley and McBlane, 1993; Handley *et al.*, 1993; Belzung and Le Pape, 1994; Griebel, 1995; Rodgers, 1997; Gatch, 2003).

0955-8810 © 2004 Lippincott Williams & Wilkins DOI: 10.1097/01.fbp.0000113327.49506.87

Although both DH and ventral hippocampus (VH) contain 5-HT_{2C} receptors (Pompeiano *et al.*, 1994; Abramowski *et al.*, 1995; Clemett *et al.*, 2000), the two regions are modulated by different 5-HT pathways (Moser and Moser, 1998). DH receives dense 5-HT projections from the median raphe nucleus (MRN), whereas the VH receives 5-HT projection from the dorsal raphe nucleus (DRN; Azmitia and Segal, 1978; Vertes, 1991). In fact, electrical stimulation of the DRN induced 5-HT release in the VH but not in the DH (McQuade and Sharp, 1997). Additionally, it has been shown that aversive stimuli such as footshock (Hajos-Korcsok, 2003) or exposure to the elevated plus-maze (Wright *et al.*, 1992; Voigt *et al.*, 1999) increase the 5-HT levels in the VH. Therefore, it is possible that 5-HT_{2C} receptors located within the DH and VH might have different roles in anxiety behaviors. The present study employed the rat elevated plus-maze test to examine this issue.

Inspired by an earlier elevated Y-maze (Montgomery, 1955) and based on the natural fear of rodents for open spaces (Treit *et al.*, 1993), this test was first introduced in 1984 (Handley and Mithani, 1984) and subsequently validated for use with rats (Pellow *et al.*, 1985) and mice (Lister, 1987). Moreover, factor analysis studies indicated that this test reliably dissociates anxiety (open-arm entries) from locomotor (close-arm entries) effects of several anxiolytic and anxiogenic agents (File, 1992; Cruz *et al.*, 1994). As we previously found anxiogenic-like effects of systemically administered TFMPP on both conventional and ethologically derived measures of rats exposed to the elevated plus-maze (Setem *et al.*, 1999), the first experiment investigated the behavioral effects of TFMPP microinfusions into the DH or VH of rats exposed to this test. To investigate further the role of hippocampal 5-HT_{2C} receptors in the modulation of anxiety-related behaviors in the elevated plus-maze, in a second experiment the effects of infusing the preferential 5-HT_{2C}-receptor agonist, MK-212, into these same hippocampal nuclei were also investigated.

Methods

Subjects

Naïve male Wistar rats weighing 190–260 g were employed as subjects. Animals were housed in pairs and had free access to food and water. Room temperature was controlled and the lighting was maintained on a 12-h on–off cycle. The experiment was conducted during the light phase of the cycle. The experimental protocols employed in the present study were in conformity with *National Institutes of Health Guide for Care and Use of Laboratory Animals* (Publication No. 85–23, revised in 1985).

Surgery and histology

Animals were anesthetized with thionembutal (45 mg/kg i.p.) and fixed in a stereotaxic frame. Each rat was implanted with a single stainless-steel guide cannula aimed at 0.5 mm above the dorsal ($n = 40$) or ventral ($n = 40$) portion of the hippocampus. Taking bregma as the reference for each plane according to the Paxinos and Watson atlas (1986), the coordinates for the DH were: anteroposterior (AP) = −3.3 mm; mediolateral (ML) = 2.2 mm; dorsoventral (DV) = 2.0 mm; for the VH, the coordinates were: AP = −4.8 mm; ML = 5.0 mm; DV = 6.0 mm. At the end of the surgery, the guide cannula was sealed with a stainless-steel wire to prevent eventual congestion. At the end of the experiment, animals were sacrificed with an overdose of thionembutal and perfused intracardially with saline followed by 10% formalin solution. The brains of all animals were removed and stored in formalin (10%) for at least 2 weeks, and then sectioned using the cryostatic method at 50–60 µm. Brain slices were stained with cresyl violet to localize the cannula position. Only rats with cannula sites into the VH or DH were considered for statistical analysis.

Apparatus

The elevated plus-maze employed in the present study has been described in detail elsewhere (Cruz *et al.*, 1994; Setem *et al.*, 1999). Briefly, it consisted of two opposite arms (50 × 10 cm) made of wood. The arms crossed at a right angle, with two opposed arms of the same size. The latter were enclosed by walls 40-cm high except for the entrance. The apparatus was elevated 50 cm above the floor. To prevent the rats from falling down, a rim of Plexiglas 1-cm high was made to surround the open arms. The illumination was provided by a 60-W light bulb suspended 175 cm above the maze. The experimental sessions were recorded by a vertically mounted video camera, linked to a monitor and VCR in an adjacent room. A highly trained observer, who remained blind to treatment conditions, analyzed the videotapes. For each animal, the number of open- and close-arm entries was registered.

Experiment 1

One week after surgery the animals received infusions (0.2 µl) of either sterile saline or TFMPP (0.75, 1.5 or 3.0 µg) into the DH or VH, according to a 4 × 2 factorial design. The first factor was related to the doses used and the second was related to the microinjection site into the hippocampus (DH or VH). Drug infusion was made by an internal cannula, which was introduced and lowered 0.5 mm below the guide cannula. The internal cannula was connected to a 10 µl Hamilton syringe via PE tubing, which allowed the experimenter to deliver the drug during a 30 s period. At the end of injection, the internal cannula was held inside the brain for 2 min to prevent drug back-up and allow absorption.

Experiment 2

The second experiment followed exactly the same experimental procedure as Experiment 1, with the

exception that MK-212 (0.1, 0.3 or 1.0 µg), instead of TFMPP, was microinjected into the VH or DH.

Drugs

TFMPP (trifluoromethyl-phenylpiperazine HCl; RBI, USA) and MK-212 [6-chloro-2(1-piperazinyl)pyrazine HCl; Tocris, Ballwin, Missouri, USA] were dissolved in sterile saline (0.9% NaCl) and injected in a volume of 0.2 µl 15 min before the tests.

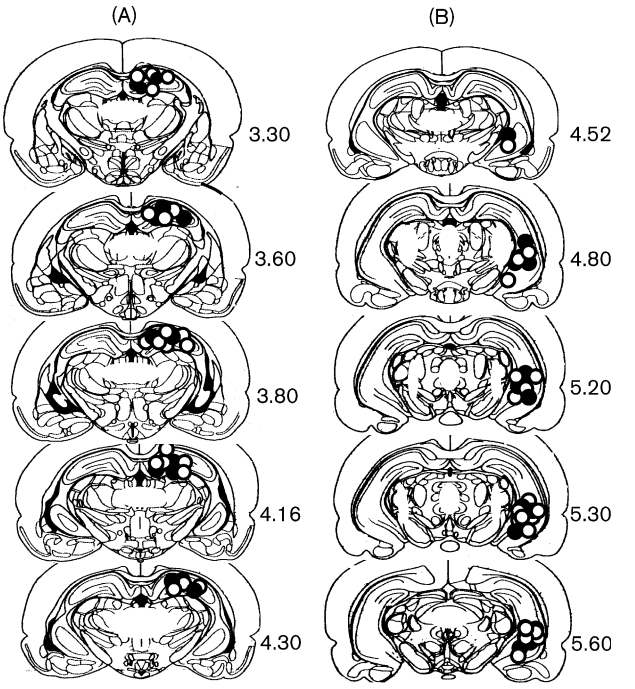
Statistical analysis

The total number of entries (open + enclosed arms), the percentage of open-arm entries (100 × open/total) and number of closed-arm entries were calculated for each animal and analyzed by a 4×2 factorial analysis of variance (ANOVA). Whenever ANOVA was significant, the Bonferroni *post-hoc* test was employed for pairwise comparison. The level of statistical significance was $P < 0.05$.

Results

Figure 1 presents a composite of the internal cannula tip locations aimed at the DH (Fig. 1A) and VH (Fig. 1B) among animals from Experiment 1 (black circles) and Experiment 2 (white circles). Histological examination of the brain slices indicated that most of the cannulae aimed at the DH or VH were located within these nuclei. Animals with cannula outside these areas (DH, $n = 5$; VH, $n = 3$) were removed from the study.

Fig. 1



Composite of internal cannula location aimed at the DH (A) and VH (B). With reference to the Paxinos and Watson (1986) atlas, the numbers on the right-hand side of each plate indicate the distance in millimeters from bregma. Animals from Experiment 1 are represented by black circles whereas animals from Experiment 2 are represented by white circles.

Experiment 1

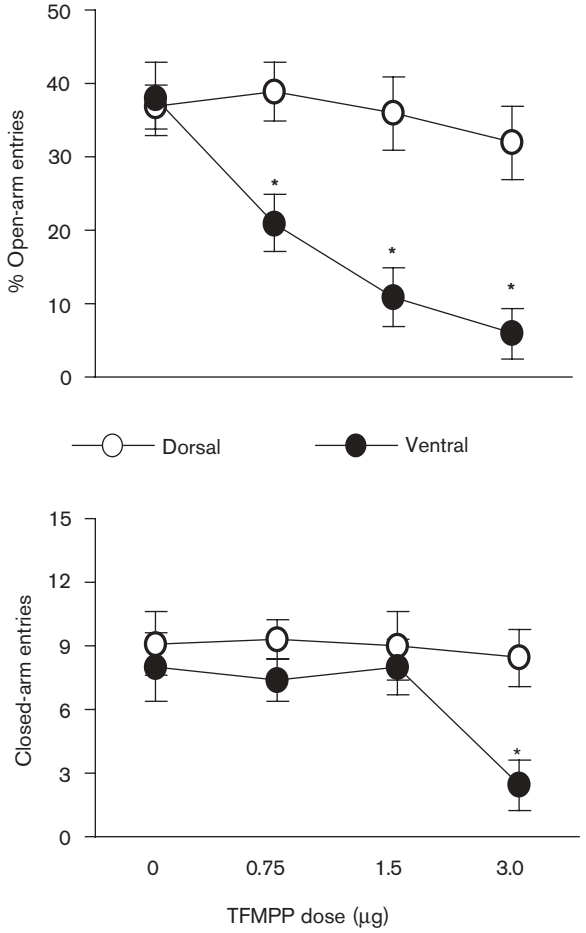
The first experiment investigated the effect of TFMPP microinjected into the VH and DH in the elevated plus-maze. The upper panel of Fig. 2 shows the mean (+ SEM) percentage of open-arm entries among the eight groups derived from the 4×2 factorial design. As can be observed from the figure, all three doses of TFMPP (0.75, 1.5 and 3.0 µg) reduced the mean percentage of open-arm entries when microinjected into the VH but not into the DH. This impression was confirmed through the two-way ANOVA. The analysis revealed an interaction between TFMPP dose and microinjection location [$F(3,65) = 7.72$; $P < 0.01$]. *Post-hoc* comparisons indicated that animals microinjected with 0.75, 1.5 or 3.0 µg of TFMPP into the VH showed a reduction in the percentage of open-arm entries, compared to animals microinjected with control saline (all Ps < 0.01). Moreover, animals microinjected with the three doses of TFMPP into the VH showed a reduction of the percentage of open-arm entries as compared to the animals microinjected with the same TFMPP doses into the DH (all Ps < 0.01). No difference among the groups microinjected with 0.75, 1.5 and 3.0 µg TFMPP microinjected into the VH was detected. Finally, none of the TFMPP doses microinjected into the DH altered the percentage of open-arm entries as compared to the saline control group.

The lower panel of Fig. 2 shows the mean (± SEM) of closed-arm entries among the groups microinjected with the several TFMPP doses into the VH or DH. Only the highest dose of TFMPP (3.0 µg) microinjected into the VH caused a reduction in the closed-arm entries. Two-way ANOVA revealed an interaction between TFMPP dose and microinjection location [$F(3,65) = 5.2$; $P < 0.01$]. *Post-hoc* comparison indicated that animals microinjected with the 3.0 µg dose of TFMPP into the VH had a decrease in the closed-arm entries as compared to the other groups (all Ps < 0.01).

Experiment 2

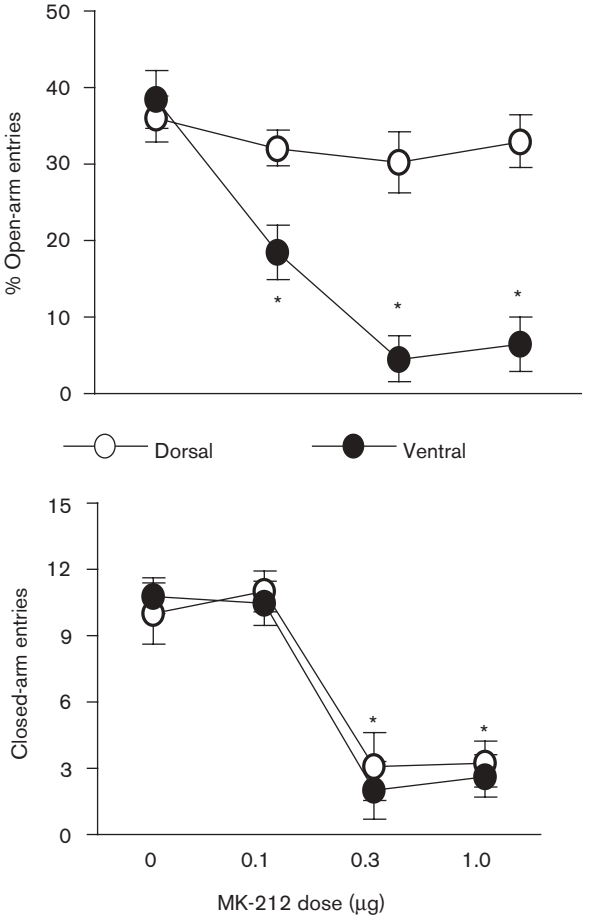
The purpose of the second experiment was to investigate further the role of VH and DH 5-HT$_{2C}$ receptors in the elevated plus-maze through the use of a more selective 5-HT$_{2C}$ agonist. The upper panel of Fig. 3 shows the mean (± SEM) percentage of open-arm entries among the groups microinjected with MK-212 into the DH or VH. ANOVA revealed a significant interaction between the

Fig. 2



Mean (±SEM) percent of open-arm entries (upper panel) and closed-arm entries (lower panel) in the elevated plus-maze, among groups microinjected with TFMPP into the DH (0 μg, $n=9$; 0.75 μg, $n=10$; 1.5 μg, $n=8$; 3.0 μg, $n=10$) or VH (0 μg, $n=8$; 0.75 μg, $n=9$; 1.5 μg, $n=10$; 3.0 μg, $n=9$). * indicates $P<0.01$ (see text for details).

Fig. 3



Mean (±SEM) percent of open-arm entries (upper panel) and closed-arm entries (lower panel) in the elevated plus-maze, among groups microinjected with MK-212 into the DH (0 μg, $n=8$; 0.1 μg, $n=11$; 0.3 μg, $n=9$; 1.0 μg, $n=9$) or VH (0 μg, $n=11$; 0.1 μg, $n=10$; 0.3 μg, $n=11$; 1.0 μg, $n=10$). * indicates $P<0.05$ (see text for details).

MK-212 doses and the site of microinjection [$F(3,71) = 10.96$; $P < 0.01$]. As in the first experiment, stimulation of the VH but not DH 5-HT_{2C} receptors, now with the preferential 5-HT_{2C} agonist MK-212 at three different doses (0.1, 0.3 and 1.0 μg), reduced the percentage of open-arm entries in the elevated plus-maze. This impression was confirmed by the fact that *post-hoc* comparisons between each of the three doses of MK-212 microinjected into the VH reliably reduced the percentage of open-arm entries when compared to control saline animals as well as to the matched dose microinjected into the DH ($P < 0.01$). Conversely, none of the three doses of MK-212 microinjected into the DH significantly affected the percentage of open-arm entries when compared to the saline control group.

Finally, the lower panel of Fig. 3 presents the mean (±SEM) of closed-arm entries among the groups microinjected with the three MK-212 doses into the VH or DH. Two-way ANOVA revealed a main effect of MK-212 doses across the DH and VH microinjection sites [$F(3,71) = 5.23$; $P < 0.01$]. *Post-hoc* comparison indicated that animals microinjected with 0.3 and 1.0 μg of MK-212, either into the DH or VH, showed a decrease in the number of closed-arm entries as compared to their respective control saline groups ($P < 0.05$). No other difference was found.

Discussion

Several reports have pointed out that 5-HT activity in the VH is notably associated with anxiety. For example, stressful situations such as footshock (Hajos-Korcsok, 2003) or elevated plus-maze exposure (Wright *et al.*, 1992; Voigt *et al.*, 1999) increase the 5-HT levels in the VH. In

agreement with this view, Experiment 1 found that intra-VH infusions of TFMPP (0.75 or 1.5 μg) increased anxiety in rats exposed to the elevated plus-maze. The fact that the number of closed-arm entries remained unaltered by these two doses of the drug indicates that this anxiogenic effect was not due to locomotor impairment but to increases in the aversion to the open-arms. This finding replicated previous results from our laboratory (Setem *et al.*, 1999) as well as others (Benjamin *et al.*, 1990; Rodgers *et al.*, 1992), showing an anxiogenic effect of systemically administered TFMM in the elevated plus-maze, and suggests that such a behavioral profile might be mediated through the activation of 5-HT$_{2C}$ receptors within the VH. Results from the second experiment supported this suggestion. Thus, microinfusions of MK-212 (0.1 μg) into the VH also led to a decrease in open-arm exploration without affecting the number of closed-arm entries.

The role of VH 5-HT$_{2C}$ receptors in the modulation of anxiety states might involve 5-HT projections from the DRN (Azmitia and Segal, 1978; Vertes, 1991). In this regard, it has been shown that electrical stimulation of the DRN increases the 5-HT levels in the VH (McQuade and Sharp, 1997) and induces anxiety-related behaviors (Sena *et al.*, 2003). Conversely, intra-DRN infusion of the 5-HT$_{1A}$ receptor agonist (±) 8-hydroxy-dipropylamino-tetralin (8-OH-DPAT) decreases the 5-HT levels in the postsynaptic neurons in the VH (Hutson *et al.*, 1989) and attenuates anxiety states in several animal models (for a review, see Menard and Treit, 1999), including the rat elevated plus-maze test (File and Gonzalez, 1996; see also File *et al.*, 1996). Therefore, such bidirectional modulation of anxiety seems to be modulated by the postsynaptic levels of 5-HT within the VH. In agreement with this view, recent reports indicate that rats with neurotoxic VH lesions exhibit a decrease in anxiety-related behaviors in the elevated plus-maze test (Kjelstrup *et al.*, 2002), context fear conditioning and light–dark test (Bannerman *et al.*, 2003).

The higher dose of TFMPP (3.0 μg) and the two higher doses of MK-212 (0.3 and 1.0 μg) reduced both open- and closed-arm entries when microinjected into the VH. Considering that the absolute number of closed-arm entries has been used as a reliable index of motor activity in the elevated plus-maze (Cruz *et al.*, 1994; Setem *et al.*, 1999), this behavioral pattern suggests an interference of the treatment in behaviors related to locomotor activity. Such findings are not consistent with a previous report, which found that intra-hippocampal infusions of high doses of *m*-CPP through a microdialysis probe increased behaviors related to general motor activity (Takahashi *et al.*, 2001). It must be noted, however, that Takahashi and colleagues microinjected the drug into the whole hippocampus, whereas in our study both 5-HT$_{2C}$ agonists were microinjected into either the VH or DH. Therefore, this issue needs more investigation, because the hypoactivity response produced by systemically administered 5-HT$_{2C}$ receptor agonists in animal model of anxiety is generally accepted to be mediated through activation of central 5-HT$_{2C}$ receptors (Kennett and Curzon, 1988; Heisler and Tecott, 2000; Martin *et al.*, 2002).

The present study also found that microinjections of either TFMPP (Experiment 1) or MK-212 (Experiment 2) into the DH failed to change any anxiety measures in the elevated plus-maze. Such negative results cannot be attributed to the drug potency because the respective TFMPP and MK-212 doses were able to produce anxiogenic effects when microinjected into the VH. In this regard, it is interesting to note that DH neurotoxic lesions also failed to produce changes in behaviors related to anxiety in the rat elevated-plus maze test, while VH lesions produced anxiolytic effects (Kjelstrup *et al.*, 2002).

The fact that intra-DH injections of both TFMPP and MK-212 failed to produce significant effects in the elevated plus-maze contrasts with a previous report showing anxiogenic effects following intra-DH infusion of the 5-HT$_{2C}$ agonist *m*-CPP in the social interaction test in rats (Whitton and Curzon, 1990). Indeed, it has been suggested that the 5-HT system within the DH might play an important role in the social interaction test, but has only discrete effects on the elevated plus-maze (File *et al.*, 2000). It is unclear why activation of DH 5-HT$_{2C}$ receptors modulates some anxiety-like behaviors (social interaction; Whitton and Curzon, 1990) but not others (elevated plus-maze; present study). One possibility is that different animal models might have different sensitivities to different forms of anxiety. In this respect, similar inconsistencies with different 5-HT-acting drugs have been observed in several animal models (for reviews see Griebel, 1995; Rodgers, 1997; Menard and Treit, 1999). For example, intra-DH infusion of 8-hydroxy-2-(di-*n*-propylamine)tetralin (8-OH-DPAT) caused an anxiogenic effect in the social interaction test but no effect in the elevated plus-maze (File *et al.*, 1996; Cheeta *et al.*, 2000).

As a whole, our findings are in accordance with the view that DH and VH might have different functions in defensive behavior (Moser and Moser, 1998). Activation of 5-HT$_{2C}$ receptors located in the DH did not cause any defensive behavior in the elevated plus-maze, whereas activation of the same receptor within the VH resulted in an anxiogenic effect in this test. The role of 5-HT$_{2C}$ receptors in the VH and DH appears to be similar to that of the 5-HT$_{1A}$ receptors within these same areas. For example, naïve rats exposed for the first time to the elevated plus-maze did not show any response to 8-OH-DPAT or the 5-HT$_{1A}$ receptor antagonist tertatolol microinjected into the DH (File *et al.*, 1996), whereas tertatolol caused an anxiolytic effect when microinjected

into the VH (File and Gonzalez, 1996). Similarly, another recent study reported that intra-VH infusions of the highly selective 5-HT_{1A} receptor antagonist WAY-100635 increased open-arm exploration and reduced risk-assessment behaviors in naïve mice exposed to the elevated plus-maze (Nunes-de-Souza *et al.*, 2002). Interestingly, these authors did not find any change in anxiety with intra-DH infusions of WAY-100635, suggesting that 5-HT also does not affect anxiety through actions at 5-HT_{1A} receptors in the DH. Therefore, it seems that 5-HT action at both 5-HT_{1A} and 5-HT_{2C} postsynaptic receptors located within the VH modulates anxiety states of mice and rats in the elevated plus-maze.

Acknowledgements

Thanks are due to Ricardo Nunes-de-Souza for critical reading of, and thoughtful comments on, this work.

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