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Inhibition of neophobia-stimulated c-Fos expression in the dorsomedial part of the prefrontal cortex in rats pretreated with midazolam

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Abstract:
The effect of an anxiolytic drug, midazolam, on the expression of c-Fos protein (the product of the immediate early gene, *c-fos*) in the rat brain was studied in animals that were exposed to the stress of neophobia using the open field test. Midazolam (0.5 mg/kg, *ip*) selectively and significantly attenuated the neophobia-induced increase in the number of Fos-like immunoreactive neurons in the dorsomedial part of the prefrontal cortex, but not in the primary motor cortex, the piriform cortex or the amygdalar nuclei. Overall, the effects of midazolam indicate that the prefrontal cortex is a likely candidate region in which drugs exert their anxiolytic action, and that the dorsomedial part of the prefrontal cortex may participate in the formation and expression of acute innate fear responses.

Key words:
open field test, neophobia, c-Fos, prefrontal cortex, midazolam

Abbreviations: ACd – dorsal anterior cingulated, M2 (Fr2) – secondary motor cortex, OFT – open field test

Introduction

The synthesis of c-Fos, the protein product of the immediate early gene, *c-fos*, is routinely used to map the neural substrates of many types of anxiety in the brain, as well as the sites of action of anxiolytic drugs [1, 2, 3]. Beck and Fibiger [1] found that exposing rats to an environment that was previously paired with a footshock significantly increased the number of Fos-like immunoreactive neurons in nearly 50 brain regions, both cortical and subcortical, thus mirroring the neural circuits involved in the expression of fear responses [1]. Furthermore, pretreatment of rats with diazepam produced a dose-related decrease in the conditioned stress-induced c-Fos expression [1]. Similarly, Izumi et al. [6] showed that another anxiolytic drug, citalopram (10 and 30 mg/kg, *ip*), attenuated the aversive context-induced c-Fos expression in the secondary motor cortex, the primary somatosensory cortex, and the basolateral nucleus of the amygdala [6].

These data indicate the important role of the cortical and subcortical areas in the effects of clinically used classes of anxiolytic drugs during conditioned fear responses. Recently, we have also shown that the serotonergic innervation of the dorsomedial part of the prefrontal cortex (the Fr2 or M2 – secondary motor cortex) is important for processing emotional input by other brain centers [9, 10]. In rats selected arbitrarily according to their behavior in the 'flinch-jump' pain pre-test and characterized by a stronger freezing response in the conditioned fear test, it was found that the selective serotonergic lesion of the M2 area significantly disinhibited rat behavior controlled by fear (a conditioned freezing response), enhanced c-Fos expression in the dorsomedial prefrontal area, and increased the concentration of γ-aminobutyric acid (GABA) in the basolateral amygdala, which was measured *in vivo* after the conditioned fear testing session [10]. This study highlighted the important role of the serotonergic innervation of the dorsomedial part of the prefrontal cortex (M2 and ACd – dorsal anterior cingulated areas according to Uylings et al., [17] in the control of emotional input to the brain).

A review of available literature shows a paucity of data on the influence of unconditioned fear stimuli and anxiolytic drugs on the activity of brain cortical areas (a search of the PUBMED database with the following key words gave negative results: prefrontal cortex, unconditioned fear, neophobia, c-Fos). Previously, we have found that pretreatment of rats with midazolam at the dose of 0.5 mg/kg enhanced rat exploratory behavior in the open field test (OFT) and inhibited neophobia-related stimulation of c-Fos in the CA-1 and CA-3 areas of the hippocampus [19].

Taking into account all these findings, the aim of the present study was to examine the effects of midazolam, administered at the anxiolytic dose established in the OFT [14–16, 19], on the activity of the dorsomedial part of the prefrontal cortex and amygdalar nuclei in rats subjected to the open field stress of neophobia.

Materials and Methods

Animals

The experiment was performed on adult male Wistar rats (200 ± 20 g) that were bought from a licensed breeder. Animals were housed in standard laboratory conditions under a 12 h : 12 h light : dark cycle (lights on at 7 a.m.), in a constant temperature (21 ± 2°C) and 70% humidity. The rats were given free access to food and water. The experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609 EEC). The Local Committee for Animal Care and Use at Medical University of Warsaw, Poland, approved all experimental procedures using animal subjects.

Drugs

Midazolam maleate (Hoffmann La Roche, Switzerland) was dissolved in 0.9% NaCl and administered intraperitoneally (*ip*, 2 ml/kg). Control groups received vehicle in the same volume per body weight. The dose of midazolam, 0.5 mg/kg, was chosen based on previous studies [16, 19]. Each experimental group contained seven or eight animals.

Scheme of the experiment

To reduce the non-specific c-Fos expression caused by injection stress, all animals were habituated (handled for 5 min each) and injected daily with saline during the four days prior to the experiment. On the fifth day, the animals were injected with midazolam or saline. The experiments were performed in two parts. In the first part, the animals were exposed to the open field, and in the second, the naive animals were pretreated with midazolam or saline only. The following groups were used in the experiments: the control group not exposed to the open field and given saline only (saline), the control group pretreated with midazolam (midazolam), the control group exposed to the OFT (saline-OFT) and the group of animals exposed to the OFT pretreated with midazolam (midazolam-OFT).

Open field test

The test was performed 30 min after drug administration in a soundproof, air-conditioned chamber under dim light and continuous white noise (65 dB). The open field apparatus consisted of a round arena (80 cm diameter), with 30 cm high walls, illuminated by a 60 W electric bulb hanging 2.5 m above the floor. The central area was defined by the centrally positioned circle, 30 cm in diameter. Open field behavior of the rats was recorded on a video tape, and then the image was

analyzed using the PC-based Videomot System, which tracked the position of each animal (Software-TSE, Bad Homburg, Germany). The box was cleaned after each trial with 95% ethanol. The naive rats were not subjected to the experiment conditions. Animals were exposed to the open field for 10 min. Immediately afterwards, the rats were returned to their home cages.

Immunocytochemical procedure

One and a half hours after the test and 2 h after drug administration, the animals were sacrificed and their brains were removed and stored at –70°C. The immunocytochemical reaction was performed according to a previously described procedure for slide-mounted brain sections [8]. Briefly, coronal 16 μm cryostat sections from each animal were cut and mounted on silan-coated slides and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.4) for 15 min. The specimens were then washed twice (2 × 15 min) in 0.01 M PBS solution (pH = 7.4), incubated in 3% H_2O_2 solution for 30 min, then washed again in 0.01 M PBS solution (pH = 7.4) twice (2 × 15 min), and incubated in a 3% normal goat serum (NGS) blocking solution. Subsequently, slide-mounted brain sections were incubated in rabbit polyclonal c-Fos IgG diluted at 1:20,000 (sc-52x, Santa Cruz, USA) at a temperature of 4–8°C for 72 h, washed in 0.01 M PBS solution (pH = 7.4) three times (3 × 15 min), then incubated with biotinylated anti-rabbit IgGs (Vector Laboratories, CA) for 2 h, rinsed in 0.01 M PBS solution (pH = 7.4) twice (2 × 15 min), and incubated with an avidine-biotine-peroxydase complex (Vector Laboratories, CA) for 1 h. Finally, after being washed in 0.01 M PBS solution (pH = 7.4) twice (2 × 15 min), slide-mounted brain sections were immunoreacted with a solution containing Tris, 0.03% diaminobenzidine hydrochloride (DAB) and 0.003% H_2O_2. The slides were then dehydrated through an alcohol series, cleared in xylene, and coverslipped in the histofluid mountant. Fos-like immunoreactivity was assessed by light microscopy (Olympus BX-51 light microscope, Camedia Master C-3040 digital camera) at a magnification of 40X. The number of c-Fos-positive nuclei was counted bilaterally with the use of a computerized image analysis system (Olympus DP-Soft version 3.2 software) from two sections per rat in the following brain regions: primary motor cortex (M1), secondary motor cortex (M2), cingulated cortex area 1 and 2 (Cg1 and Cg2), piriform cortex (AP, 0.48), medial amygdalar nucleus (MeA), and basolateral amygdalar nucleus (BLA) (AP, –3.14). From each animal, two adjacent coronal 16 μm cryostat slices were cut from each section based on the atlas of Paxinos and Watson [12]. c-Fos positive nuclei were counted for each region from each rat, and the area of the outlined region was calculated using DP-soft. The results were expressed as the number of immunopositive nuclei per 1 mm^2. Figure 1 is a schematic diagram of the brain sections, with the regions outlined where the c-Fos positive nuclei were counted.

Statistical analysis

Behavioral and immunocytochemical data were analyzed by one-way ANOVA, followed by the *post-hoc* New-

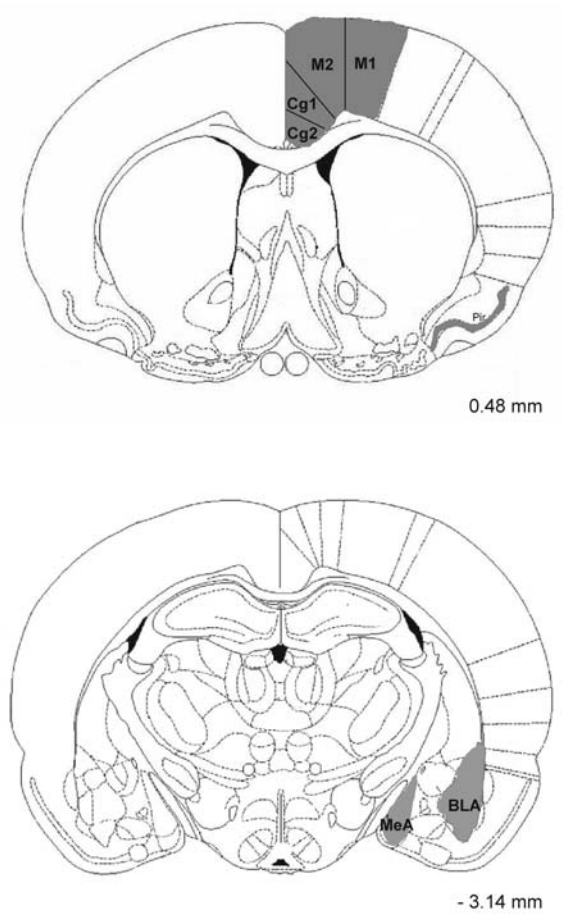

Fig. 1. The scheme of brain sections in which c-Fos expression was assessed. M2 – secondary motor cortex, Pir – piriform cortex, Cg 1 – cingulated area 1, Cg 2 – cingulated area 2, M1 – primary motor cortex, MeA – medial amygdala BLA – basolateral amygdala. (M2, Cg 1, Cg 2 – dorsomedial prefrontal cortex)

man-Keuls test (Statistica for Windows, Release 6, Stat-Soft Inc., USA). The difference between the effects of saline and midazolam on basal c-Fos expression was assessed by Student's *t*-test. The data are shown as the means ± SEM. The probability value of $p < 0.05$ was considered significant.

Results

One-way ANOVA revealed significant differences in the c-Fos expression between groups in the M2 [$F(2, 20) = 15.76$; $p < 0.01$], piriform cortex [$F(2, 19) = 4.59$; $p < 0.05$], Cg1 [$F(2, 20) = 17.95$; $p < 0.01$], and Cg2 [$F(2, 20) = 10{,}46$; $p < 0.01$]. In the M2, Cg1, and Cg2 areas, the Newman-Keuls *post-hoc* test showed a significant increase in c-Fos expression in the control group exposed to the open field (OFT-saline), compared to the naive control group (saline) ($p < 0.01$). This effect was attenuated by the administration of midazolam 30 min before the exposition of the animals to the open field ($p < 0.01$) (Tab. 1A, Fig 2). In the piriform cortex, the Newman-Keuls test showed an increased immunoreactivity in the control group exposed to the OFT (OFT-saline, $p < 0.05$), compared to saline control group, i.e., the influence of neophobia alone. Midazolam slightly, but insignificantly, inhibited this immunocytochemical reaction. In the remaining examined brain areas, BLA, MeA and M1,

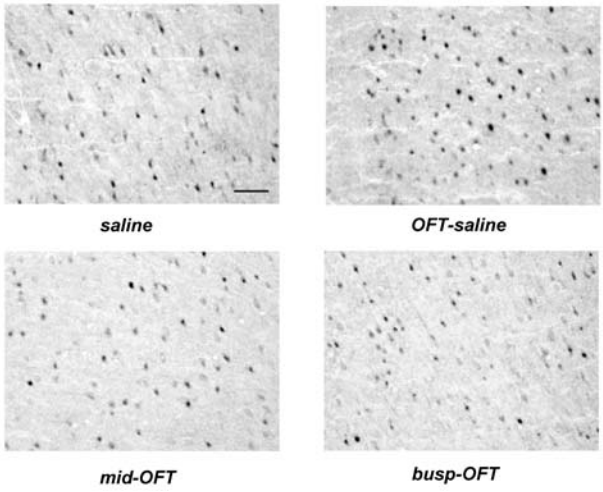

Fig. 2. Microphotographs showing representative expression of c-Fos in the secondary motor cortex – M2 area. Slices were photographed with a 20× lens magnification and then magnified digitally. Bar indicates 175 μm. For more details see "Materials and Methods"

Tab. 1. (A) The effect of the open field test (OFT) of neophobia on c-Fos immunoreactivity in selected brain structures and its changes after pretreating rats with midazolam. Saline – control group not exposed to the open field and given saline only; saline-OFT – control group exposed to the OFT; mid-OFT – group of animals exposed to the OFT and pretreated with midazolam. Data are shown as the means ± SEM. n = 7–8 animals in each group. c-Fos expression is shown as the number of immunopositive nuclei per 1 mm^2. M1 – primary motor cortex, M2 – secondary motor cortex, Cg1 – cingulated area 1, Cg2 – cingulated area 2, Pir – piriform cortex, BLA – basolateral amygdala, MeA – medial amygdala. * $p < 0.05$; ** $p < 0.01$ *vs.* saline; $^{\#\#}$ – *vs.* saline-OFT. **(B)** The effect of midazolam pretreatment on basal c-Fos expression in the secondary motor cortex (M2). Saline – control group given saline only; midazolam – control group pretreated with midazolam. Data are shown as the means ± SEM. n = 7–8 animals in each group. c-Fos expression is shown as the number of immunopositive nuclei per 1 mm^2

A

Brain region	Saline	Saline-OFT	Mid-OFT
M1	115.50 ± 11.64	126.75±10.14	96.14±4.46
M2	110.00 ± 10.89	151.5 ± 7.23**	83.14 ± 6.54$^{\#\#}$
Cg1	134.12 ± 7.08	166.88 ± 11.95**	95.14 ± 4.99$^{\#\#}$
Cg2	100.38 ± 3.42	159.13 ± 15.17**	113.57 ± 4.52$^{\#\#}$
Pir	132.75 ± 14.60	203.75 ± 14.85*	160.16 ± 24.45
BLA	56.25 ± 5.68	65.62 ± 5.48	54 ± 5.14
MeA	61.25 ± 6.24	86.14 ± 14.23	59.4 ± 10.68

B

Brain region	Saline	Midazolam
M2	138 ± 21.48	146.87 ± 27.18

no changes in c-Fos expression were found in response to the stress of novelty. Pretreatment of control rats that were not subjected to the OFT with midazolam did not change the basal expression of c-Fos protein in the M2 area [t = 0.25; df = 15; p = 0.79] (Tab. 1B).

Discussion

It has been found that pretreatment of rats with midazolam significantly attenuates a neophobia-induced increase in the number of Fos-like immunoreactive neurons in the dorsomedial prefrontal cortex (M2, Cg1, Cg2 fields; Fig. 1, 2). The neophobia-like response was defined as a decrease of rat activity in the central section of the open field, without changing rat activity in the peripheral portion of the apparatus (these data have already been published; midazolam 0.5 mg/kg *ip*, means ± SEM, the number of central visits, saline – 3.0 ± 1.0, midazolam – 6.0 ± 2.0, $p < 0.01$; peripheral distance crossed, saline – 2000 cm ± 150, midazolam – 2100 cm ± 220.0, ns; [19]). It is also important to note that in the present study, the effect of midazolam was confined to the dorsomedial prefrontal cortex (M2, Cg1 and Cg2 areas), with only a slight attenuation of the neuronal response to novelty stress in the piriform cortex. These data confirm previously published findings and indicate that the anxiolytics exert a potent influence on the activity of the prefrontal cortex [1, 11]. They also indicate that the BLA and MeA amygdalar nuclei did not respond to the stress of neophobia. Accordingly, the amygdalar complex has been suggested to be primarily involved in the control of conditioned fear reactions and aversive memories [7].

The issue in locating the cortical area of the rat brain that is homologous to the prefrontal cortex is still not resolved. In this paper, we have followed the criterion proposed by Uylings et al. [17], in which the dorsomedial part of the prefrontal cortex is defined as the M2 (Fr2) – secondary motor cortex, and Cg1 and Cg2 areas, according to the atlas of the rat brain by Paxinos and Watson [12]. In a recent review, Uylings et al. performed a thorough analysis of over 150 papers on the topic of anatomy and morphology of the medial prefrontal cortex [17]. For example, Van Eden et al. [18] have shown with anterograde labeling that Fr2 (M2 area) and ACd (dorsal anterior cingulated – Cg1, Cg2 areas) receive more projections from somatosensory and associational visual cortices than from the primary motor cortex [18]. Uylings et al. have concluded that the majority of anatomical and functional evidence indicates that rats have prefrontal cortex, in which Fr2 and ACd are incorporated [13, 17]. Recently, their opinion has received more support. For example, Hoover and Vertes [5] examined the four divisions of the prefrontal cortex: the medial (frontal) agranular (AGm-Fr2-M2), anterior cingulated (AC), prelimbic (PL) and infralimbic (IL), using retrograde tracing techniques [5]. They found that there is a dorsoventral shift along the prefrontal cortex from the predominantly sensorimotor input to the dorsal prefrontal cortex (AGm and AC), to the primarily "limbic" input to the ventral prefrontal cortex (PL and IL) [5]. These and other authors suggest that the information from widespread areas of the cortex is presumably integrated by the dorsal prefrontal cortex in goal-directed actions. The present study confirms this corollary, extends our knowledge about the role of the prefrontal cortex in fear processing, and underlines the contribution of the dorsomedial part of the prefrontal cortex (M2 and ACd areas) in the expression of rat behavior controlled by unconditioned fear stimuli. In the clinic, a neuroimaging study has recently demonstrated that the balance between left and right dorsolateral prefrontal cortex in major depression is directly linked to negative emotional judgment [4]. All these facts highlight the contribution of the dorsal prefrontal cortex in the control of emotions in different species.

It is also of interest that midazolam (0.5 mg/kg) appeared to be effective at a dose much smaller than that found previously by other authors to decrease the stress-induced expression of c-Fos [1]. This phenomenon can be attributed, at least to some extent, to the difference in the baseline expression of c-Fos, which is dependent on the level of stress (conditioned *vs.* unconditioned fear), since the enhancement of c-Fos by neophobia (by about 130%) was weaker than observed after the conditioned fear (by about 200%, cf. Izumi et al., [6]). Importantly, in the present study, the effect of an anxiolytic was structure-dependent and concerned the dorsomedial prefrontal cortex. Moreover, the control experiment showed that administration of midazolam to naive rats did not change the expression of c-Fos neuronal staining in the M2 cortical area. Accordingly, we have shown in our previous study that midazolam, given to naive rats at the same

dose of 0.5 mg/kg, did not affect the basal expression of c-Fos in other brain structures [19]. The effect of midazolam indicates that the dorsomedial part of the prefrontal cortex is a brain region where this drug may exert its anxiolytic-like action.

Overall, the presented data suggest that the neophobia-related changes in immediate early genes (IEG) expression in the dorsomedial part of the prefrontal cortex may underlie the formation and expression of acute innate fear responses (i.e., neophobia-like fear reaction) that are inhibited by the anxiolytic drug, midazolam.

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