

# Anxiolytic effect of methylene blue microinjected into the dorsal periaqueductal gray matter

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## Abstract

The dorsal periaqueductal gray (DPAG) has been implicated in the behavioral and autonomic expression of defensive reactions. Several results suggest that, along with GABA, glutamate and serotonin, nitric oxide (NO) may play a role in defense reactions mediated by this region. To further investigate this possibility we microinjected methylene blue (MB; 10, 30 or 100 nmol/0.5  $\mu$ l) into the DPAG of rats submitted to the elevated plus-maze test, an animal model of anxiety. MB has been used as an inhibitor of soluble guanylate cyclase (sGC) to demonstrate cGMP-mediated processes, and there is evidence that NO may exert its biological effects by binding to the heme part of guanylate cyclase, causing an increase in cGMP levels. The results showed that MB (30 nmol) significantly increased the percent of time spent in the open arms (saline =  $11.57 \pm 1.54$ , MB =  $18.5 \pm 2.45$ ,  $P < 0.05$ ) and tended to do the same with the percentage of open arm entries (saline =  $25.8 \pm 1.97$ , MB =  $33.77 \pm 3.07$ ,  $P < 0.10$ ), but did not change the number of enclosed arm entries. The dose-response curve, however, had an inverted U shape. These results indicate that MB, within a limited dose range, has anxiolytic properties when microinjected into the DPAG.

## Key words

- Anxiety
- Elevated plus-maze
- Nitric oxide
- Animal model

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The dorsal periaqueductal gray matter (DPAG) has been implicated in the behavioral and autonomic expression of defensive reactions (1). Electrical stimulation of this structure leads to flight responses with running, jumping and vocalization accompanied with cardiovascular changes (2). Drugs that enhance or mimic the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), including benzodiazepines, as well as drugs that facilitate serotonergic transmission such as the serotonin (5-HT) uptake inhibitor zimelidine or the 5-HT autoreceptor blocker propranolol, decrease the aversive

consequences of DPAG electrical stimulation (2,3).

Intra-DPAG microinjections of excitatory amino acids such as glutamate also produce a flight reaction in rats (4). In contrast, the NMDA glutamate receptor antagonist AP-7, when injected into this structure, exerts a marked anxiolytic effect in rats submitted to the plus-maze test (5).

A glutamatergic action on NMDA receptors can stimulate the production of nitric oxide (NO) by the activation of the calmodulin-dependent enzyme, nitric oxide synthase (NOS) (6,7). The DPAG displays high levels

of NOS (8) and microinjections into this structure of two NOS inhibitors, L-NAME or L-NOARG, induced anxiolytic-like effects in the elevated plus-maze (9). In addition, injection into the DPAG of NO donors such as SIN-1 and DEA/NO produced a vigorous flight reaction in rats (10). These results suggest that NO may play a role in defense reactions mediated by the DPAG.

To further investigate this possibility we injected methylene blue (MB) into the DPAG of rats submitted to the elevated plus-maze test, an animal model of anxiety. MB has been used as an inhibitor of soluble guanylate cyclase (sGC) to demonstrate cGMP-mediated processes (11), and there is evidence that NO may exert its biological effects by binding to the heme part of guanylate cyclase, causing an increase in cGMP levels (12).

Male Wistar rats (200-250 g) housed in pairs with free access to food and water in a temperature-controlled room ( $23 \pm 1^\circ\text{C}$ ) were used in the experiments. The animals were submitted to stereotaxic surgery under tribromoethanol anesthesia (2.5 mg/kg, *ip*) to implant a stainless steel guide cannula (0.7 mm OD) aimed at the DPAG (coordinates: 1.9 mm lateral to the right side of the lambda at an angle of  $16^\circ$  with the sagittal plane, and 4.0 mm below the surface of the skull). The cannula was attached to the bone with stainless steel screws and acrylic cement. A stylet was introduced into the guide cannula to prevent obstruction. Seven days after surgery the animals received microinjections (0.5  $\mu\text{l}$  in 30 s) of either MB (10, 30 or 100 nmol) or saline and 10 min later were placed in the center of the plus-maze facing an enclosed arm. The maze consisted of two opposite open arms (50 x 10 cm), crossed at right angles by two arms of the same dimensions, enclosed by 40-cm high walls with no roof. It was elevated 50 cm from the floor and had a 1-cm high edge made of plexiglas surrounding the open arms. The number of entries and time spent in open and enclosed

arms of the maze were recorded for 5 min. After the behavioral tests, the rats were sacrificed under deep anesthesia and their heads perfused with isotonic saline followed by 10% formalin. A dental needle was inserted through the guide cannula and a 0.2- $\mu\text{l}$  microinjection of Evans blue was performed. The brains were removed, immersed in 10% formalin for 3 days and 50- $\mu\text{m}$  frozen sections were cut with a cryostat (Cryocut 1800). Injection sites were localized in diagrams of the Paxinos and Watson rat brain atlas. The data for eight rats who received injections outside the DPAG were excluded from the analysis.

For statistical analysis the percentage of open arm entries ( $100 \times \text{open}/\text{total entries}$ ) and of time spent in the open arms ( $100 \times \text{open}/\text{open} + \text{enclosed}$ ) was calculated for each rat. These data, together with the number of enclosed arm entries, were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan test for multiple comparisons.

As shown in Figure 1, the injection of 30 nmol MB significantly increased the percentage of time spent in the open arm ( $F_{3,54} = 3.52$ ;  $P < 0.02$ , Duncan test,  $P < 0.05$ , compared to control) and tended to increase the percentage of open arm entries ( $F_{3,54} = 1.98$ ;  $P < 0.10$ , Duncan test,  $P < 0.10$ , compared to control), but did not change the number of enclosed arm entries ( $P > 0.10$ , ANOVA). The results, therefore, suggest that MB has anxiolytic properties (13). The dose-response curve, however, had an inverted U shape, with both higher and lower doses being ineffective. Similar results were recently obtained with the intravenous administration of MB (14).

Several of the biological effects of MB have been related to the antagonism of NO action. For example, MB inhibits sGC, attenuating the NO-mediated increase in cGMP levels (11,12,15). It can also inhibit other iron-containing enzymes such as NOS, acting as a direct NOS inhibitor (16), or directly

antagonize NO through the generation of superoxide anions derived from its oxidative metabolism (17).

Reinforcing a possible involvement of NO in the anxiolytic effect of MB, the exposure of rats to a predator was accompanied by an increase in the production of NO and cGMP in the DPAG (18) and NO donors microinjected into this region induced a vigorous flight reaction (10). Moreover, two NOS inhibitors, L-NAME and L-NOARG, after systemic (19) or intra-DPAG administration (10) showed anxiolytic effects. However, the anxiolytic dose range for these inhibitors was also limited, with the dose-effect curves showing an inverted U shape.

NO has been proposed as a transduction mechanism of NMDA receptor-mediated neurotransmission (6,7) and antagonists of NMDA receptors produce anxiolytic effects in the DPAG (5,20). The present results, showing an anxiolytic effect of an NO antagonist, support this proposition.

In conclusion, the present results show an anxiolytic effect of MB microinjected into the DPAG, supporting the involvement of NO in the modulation of defensive reac-

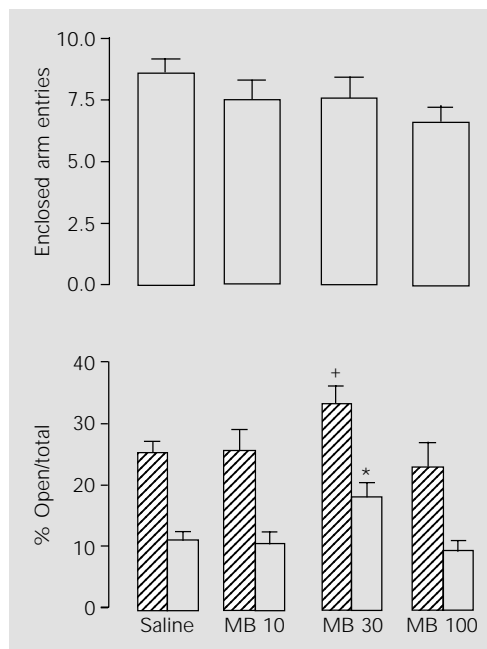


Figure 1 - Anxiolytic effect of intra-DPAG microinjection of methylene blue (MB) in rats tested in the elevated plus-maze. A volume of 0.5  $\mu$ l of saline (N = 22) or MB (10, 30 or 100 nmol, N = 12/group) was injected over a period of 30 s. Ten minutes later the animals were placed in the center of the maze. In the upper panel the columns represent the mean number of enclosed arm entries. In the lower panel the hatched columns represent the mean percentage of entries into open arms and the open columns the mean percentage of time spent in these arms. Bars refer to the SEM of the groups. \* $P < 0.05$  and  $^+P < 0.10$  compared to control (ANOVA followed by the Duncan test).

tion in this region.

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