Anxiolytic effect of spermine microinjected into the dorsal periaqueductal grey in rats

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ABSTRACT. To investigate a possible effect of the spermine and its polyamins receptors in the dorsal periaqueductal grey (DPAG) in anxiety. Groups of rats received microinjections of spermine (2.5 nmol) into DPAG and were tested in the elevated plus-maze apparatus. Results indicated an overall significant increase in the percentage of entries and in the time spent in open arms. In another experiment the anxiolytic effect obtained after the microinjection of spermine (2.5 nmol) into DPAG was block by previous microinjection of polyamines antagonists, arcaine (4 nmol) or ifenprodil (5 nmol) into the same site. These results indicate that the spermine receptor interaction in the modulation of anxiety in rats exposed to the elevated plus-maze. Considering that polyamines are natural component of the brain, these results suggest that polyamine system within DPAG may play a role in aversion and anxiety.

Key words: anxiety, elevated plus-maze, periaqueductal grey, polyamines, rats, spermine.

RESUMO. Efeito ansiolítico da espermina microinjetada na substância cinzenta periaquedutal dorsal de ratos. Investigamos o possível efeito das espermina e receptores das poliaminas na substância cinzenta periaquedutal dorsal, sobre a ansiedade. Grupos de ratos receberam microinjeções de espermina (2,5 nmol) no interior da matéria cinzenta periaquedutal dorsal e foram testados no labirinto em cruz elevado. Os resultados indicam um aumento significativo na porcentagem de entradas e no tempo de permanência nos braços abertos. Outro experimento mostra que o efeito ansiolítico obtido após microinjeção de espermina (2,5 nmol) no interior da matéria cinzenta periaquedutal dorsal, foi bloqueado pela microinjeção prévia dos antagonistas das poliaminas, arcaina (4 nmol) ou ifenprodil (5 nmol), neste sítio. Estes resultados indicam que o receptor da espermina interage na modulação da ansiedade em ratos expostos ao labirinto em cruz elevado. Considerando que as poliaminas são encontradas normalmente no cérebro, estes resultados sugerem que o sistema de poliaminas na matéria cinzenta periaquedutal dorsal possuem um importante papel na ansiedade.

Palavras-chave: ansiedade, labirinto em cruz elevado, matéria periaquedutal, poliaminas, ratos, espermina.

Introduction

The N-methyl-D-aspartate receptor (NMDAR) subtype of the excitatory aminoacid receptor is involved in many physiological roles such as neuronal development, plasticity and excitability. In brief, the NMDAR is comprised of subunits associated with a nonselective (Na^+, K^+ and Ca^{++}) channel. The NMDAR/channel complex has independent recognition sites for NMDA, glycine (GLY), polyamines and cation channel blockers (Johnson, 1996).

The endogenous polyamine spermine is the product of ornithine metabolism found in high concentrations (50-1400 nmoles g^{-1} tissue) into the brain (Shaw and Pateman, 1973). Mainly because polyamines are highly concentrated in intracellular space, neurotransmitter or neuromodulator roles have been suggested adding to their physiological participation in cell growth and proliferation, brain development and metabolism (Seiler and Deckardt, 1978; Williams, 1997). Although electrophysiological studies have shown that polyamines influence NMDAR channel by acting in distinct binding sites from the agonists, glutamate or GLY (Ranson and Stec, 1988; Rock and MacDonald, 1995), the net effect of the polyamines on NMDAR remain quite ambiguous, because, potentiation, inhibition or no effect of polyamines have all been reported (Williams, 1997).

The dorsal part of the periaqueductal grey (DPAG) matter is a brain region involved in defensive/aversive responses and has been considered an important target for the mode of

action of putative anxiolytic drugs (Graeff, 1994). We have shown that microinjection of NMDA antagonists (Schmitt *et al*., 1990; Guimarães *et al*., 1991) or antagonists of the GLY-site of the NMDAR/channel applied into DPAG (Matheus *et al*., 1994), each elicited an anxiolytic-like effect. In addition, microinjection of GLY-site agonists, such as, GLY or D-serine, evoked anxiogenic-like effects (Schmitt *et al*., 1995; Carobrez *et al*., 2001). Complementary, γ-aminobutyric acid/A (GABA/A)-mimetic drugs and benzodiazepines anxiolytics, as well as drugs that facilitate serotonergic neurotransmission, revealed anxiolytic-like effects when injected into DPAG (Graeff, 1994).

Gathering all these facts, it is possible that polyamines regulate the expression of behaviors induced by DPAG manipulation by acting on NMDAR/channel complex. In order to test this hypothesis, we analyzed the effects of spermine microinjected into DPAG on the performance of rats in the elevated plus-maze model of anxiety.

Material and Methods

Subjects

Naïve male albino rats of an outbreed Wistar Strain, weighing 250-300 g at the beginning of the experiments, were used. Rats were housed individually in Plexiglas cages (35 x 30 x 15 cm) containing a layer of woodshavings, with free access to food and water, under conditions of constant ambient temperature (23±1°C), constant humidity and normal light/dark rhythm (with lights on from 7:00-19:00 hours).

Surgery

Subjects were anaesthetized with Equitesin® (3 mL kg^{-1}, IP) and fixed in a stereotaxic frame. A stainless steel guide cannula (o.d.: 0.7 mm; length: 13 mm) was implanted, aimed to the DPAG, for direct intracerebral administration of drugs. The guide cannula was introduce 1.9 mm lateral to the lambda at an angle of 22° with the sagittal plane (horizontal skull), until the cannula tip was located 2.0 mm below the skull surface. The cannula was attached to the bone with stainless steel screws and acrylic cement. To prevent obstruction a stiletto was introduced inside the guide cannula.

Drugs

Drugs used: Spermine hydrochloride (RBI), arcaine sulphate (RBI) and ifenprodil tartrate (RBI). All drugs were dissolved in artificial cerebral spinal fluid and a total volume of 0.4 μl, were microinject 5 min prior to testing in each animal.

Apparatus

The behavioral testing procedures were performed using the elevated plus-maze (EPM), according to the method described by Pellow *et al*. (1985) and previously used in our experiments (Mendes-Gomes and Nunes-de-Souza, 2005). The equipment consisted of a "plus" wooden shaped maze with two opposite open arms (50 x 10 x 1 cm) and two enclosed arms (50 x 40 x 10 cm) extended from a central platform (10 x 10 cm). The floor of the maze was painted with impermeable epoxy resin, to avoid urine impregnation. The maze was elevated to a height of 50 cm above the floor. A 1 cm high edge of Plexiglas circumscribed the open arms to avoid incidental falls. All testing was performed in a sound attenuated room with low indirect illumination (44 lux). All experiments were realized between 8:00 and 12:00 hours.

Procedures

Between seven to ten days after surgery, the rats were randomly assignee to one of each experimental group (Experiment 1 or 2). Microinjection of compounds into DPAG was performing through a dental needle (o.d.: 0.3 mm; length: 16.3 mm) inserted into the guide cannula. A polyethylene catheter (PE 10) was interposed between the outside end of the needle and a 2.0 μl Hamilton® microsyringe. The forward movement of a small air bubble inside the PE 10, confirmed drug flow. For experiment 1, each rat received only one 0.4 μl microinjection of artificial cerebral spinal fluid (F) or spermine (S). For experiment 2, each subject received 2, microinjections (0.2 μl each, 5min. apart), according to the following schema: F/F; F/S; arcaine (4 nmol, A)/F); ifenprodil (5 nmol, I)/F; A/S; I/S (Reynolds, 1990; Chida *et al*., 1992). Five min after the microinjections, each rat was placed inside a wooden arena (60 x 60 x 35 cm), for 5min., in order to increase the overall activity in the EPM, as suggested by Pellow *et al*. (1985). The rats were placed onto the central of the EPM, facing an enclosed arm, and they were allowed to explore the apparatus for another 5min. An observer, stayed 1 m away from the EPM, recorded the number of entries into open and enclosed arms, as well as the times spent in each of them. For analysis, the activities recorded on the open arm were transformed into percentage of entries (ratio between open entries and total entries x 100) and into percentage of time spent on open arms. Drug induced increases in either percentage of entries or time spent on open arms, or both, are indicative of an anxiolytic action, whereas the opposite effect implied on anxiogenic action.

Statistical analysis

Data were analyzed by single-factor (treatment) analysis of variance (ANOVA), using the Statistic® and the Instat® software packages. *Post hoc* differences between group means of experiment 1 were analyzed using the Dunnett multiple comparisons test and differences between groups of experiment 2 using the Tukey test. The level of significance was $p<0.05$ in all analyses.

Histology

After behavioral tests, the rats were sacrificed under deep Equitesin® anesthesia (4 mL kg^{-1}, IP), and pefused through the left ventricle of the heart with isotonic saline followed by 10% formalin solution. The dental needle was insert through the guide cannula and 0.3 µl microinjection of Evans blue was performing. The brains were removing and immerse in 10% formalin solution. Frozen sections of 50 µm were obtained in cryostat (Cryocut® 1800). Injection sites were localized and compare to Swanson brain maps (1992) where rats receiving microinjections outside the DPAG were exclude from analysis.

Results

Experiment 1: effects of spermine on maze exploration

The microinjection of spermine into DPAG produced significant effects on the percentage open arm entries ($F_{4,41}=2.76,\ P<0.05$), percentage time spent on open arms ($F_{4,41}=2.67,\ P<0.05$), without affecting the number of enclosed arm entries ($F_{4,41}=1.5,\ NS$). Post-hoc analysis showed an increased ($P<0.05$) in the percentage of entries and time spent on open arms at 2.5 nmol (Figure 1), although no specific effect was detected with the Dunnett´s *t*-test for the frequency of enclosed arm entries ($p>0.05$).

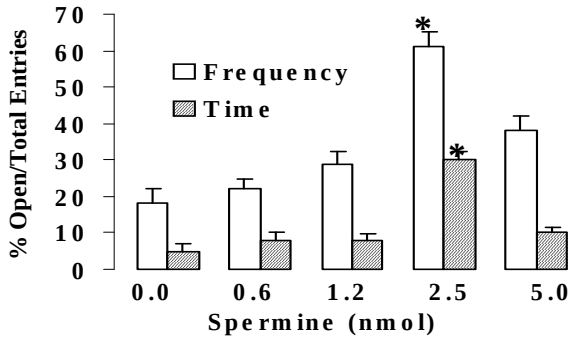

Figure 1. Effects of spermine microinjected into the dorsal periaqueductal grey of rats tested in the elevated plus-maze. Values represent the means ± SEM of the percentage of entries and of time spent on open arms, N = 9 –12 rats per group. ⋆P<0.05 versus control (Dunnett´s test).

Experiment 2: effects of spermine on maze exploration in rats pre-treated with polyamine antagonists

Microinjection of spermine (2.5 nmol) produced significant overall effects on the percentage open arm entries ($F_{6,64}=3.35,\ P<0.05$) and on the percentage time spent on open arms ($F_{6,64}=6.41,\ P<0.0001$), without affecting the enclosed arm entries ($F_{6,64}=1.84,\ NS$). However, spermine (2.5 nmol) failed to increase the percentage open arm entries and the percentage time spent on open arms in rats previously treated with arcaine (4 nmol) or ifenprodil (5 nmol)(Figure 2).

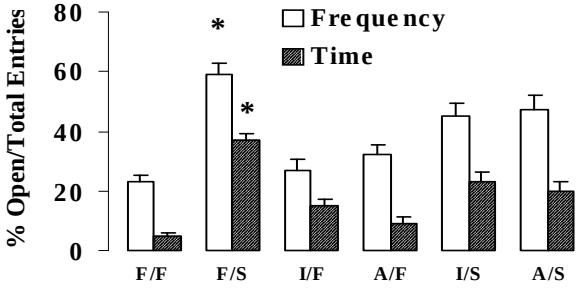

Figure 2. Effects of the spinal fluid (F) and polyamine antagonists, ifenprodil (5 nmol, I) or arcaine (4 nmol, A) on the anxiolytic effect of spermine (2.5 nmol, S) both microinjected into DPAG and further tested in the elevated plus-maze. Values represent the means ± SEM of the percentage of entries and of time spent on open arms, N = 9 –12 rats per group. ⋆P<0.05 versus control (Tukey *HSD* for unequal sample sizes).

Discussion

Using the elevated plus maze test, an anxiolytic profile of action have been detected for compounds that reduce the activity of the NMDAR/channel complex within the DPAG (Schmitt *et al*., 1990; Gimarães *et al*., 1991; Matheus *et al*., 1994), whereas an anxiogenic outline have been observed using compounds that enhance the activity of the NMDAR/channel complex (Schmitt *et al*., 1995). Based on these facts an attractive speculation would suggest this anxiolytic effect of the spermine to be a result of its action on polyamines receptors associated with the NMDAR/channel complex (Johnson, 1996).

The results obtained in this study provided evidence that microinjection of spermine into DPAG increase the percentage of time spent on the open arms. The percentage of open arms entries also increase alongside the same profile described above. Since, altered at the time and entries in the open arms of the elevated plus-maze are indexes of fear/anxiety (File, 1992), microinjection of the

spermine into DPAG caused a selective anxiolytic effect. We propose that intracellular spermine has a direct inhibitory effect on NMDA receptors that is different from calcium-induced NMDA receptor inactivation and spermine induced voltage-dependent inhibition of AMPA/kainate receptors (Ferchmin *et al*., 2000; Turecek *et al*., 2004).

This above suggestion is compatible with the results obtained in experiment 2. Doses of 2.5 nmol of spermina, revealed an anxiolytic effect of the polyamines, this anxiolytic effect was blocked by previous treatment with arcaine (4 nmol) or ifenprodil (5 nmol). Ifenprodil and arcaine are two of the most commonly reported agents of this type and have been termed polyamine antagonists (Reynolds, 1990). Although there is much evidence that these two compounds interact with a polyamine-site on the NMDA receptor (Carter *et al*., 1989; Reynolds, 1990; Rock and MacDonald, 1992; Williams, 1993).The present results are therefore entirely consistent with this earlier work. The doses of ifenprodil and arcaine used here are known to alter activity of the NMDA receptor, and the results therefore suggest that the polyamine site may be involved in NMDA receptor function in anxiety. This conclusion is consistent with the view that different neuronal networks and/or receptor populations are involved in anxiety.

The behavioral effects elicited by various compounds into PAG lies primarily on the relations of this brain area with the animal defensive system and possibly with aversion or anxiety (Graeff, 1994). Concerning, the related cardiovascular pattern (increased blood pressures and heart rate) elicited by the microinjection of NMDA into PAG, seems compatible with the neurovegetative component of the so-called defense reaction. It is important to notice that at the dose range of 0.1-1 μg spermidine reduced the cardiovascular effects of NMDA, an effect blocked by arcaine (Maione *et al*., 1994a and b).

In summary, present findings indicate that microinjection of the polyamine spermine into DPAG elicited an anxiolytic effect detected in the elevated plus-maze model of anxiety. The anxiolytic effect of 2.5 nmol spermine was blocked by previous injection of the antagonists arcaine or ifenprodil. Considering that polyamines are natural component of the brain these results suggest that polyamines system within DPAG may play a modulator role in aversion and anxiety. Current studies are being developed to test whether polyamines are acting through glutamatergic transmission.

Conclusion

These results indicate that the spermine into DPAG play an anxioselective role detected in the elevated plus-maze test. Considering that spermine is natural component of the brain, these results suggest that polyamine system within DPAG may play a role in aversion and anxiety.

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