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INFLUENCE OF PASSIVE AVOIDANCE LEARNING BY SUBSTANCE P IN THE BASOLATERAL AMYGDALA*

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Neuropeptide substance P (SP) has reinforcing and memory facilitating effects after its peripheral or central application. Rats self-inject SP into the ventromedial caudate-putamen and SP microinjections into the basal forebrain induce place preference with a simultaneous increase of dopamine level. In the amygdaloid body SP positive neurones and terminals have been identified. The aim of the present study was to examine the possible reinforcing effects of SP in the basolateral amygdala (ABL). CFY male rats were conditioned in two-compartment passive avoidance paradigm and place preference was examined in two-compartment-box and in circular open field. Animals were microinjected bilaterally with 10 ng SP, 100 ng SP or vehicle solution (0.4 µl/side) into the ABL. Results showed that post-shock infusion of 10 ng SP significantly enhanced passive avoidance learning while 100 ng SP was ineffective. In two-compartment-box and in circular open field place preference did not develop after SP treatments, however. Our data are the first to demonstrate that SP in the ABL is involved in learning and memory processes related to aversive situations. Results that SP microinjections were not followed by rewarding-reinforcing consequences in place preference paradigms indicate that the local SP network in the ABL is not involved in neuronal circuitry responsible for addictive behaviour.

Keywords: Substance P – learning – passive avoidance – place preference – basolateral amygdala

INTRODUCTION

The undecapeptide substance P (SP) belongs to the tachykinin peptide family and acts primarily at tachykinin NK-1 receptors. Its calcium-dependent stimulation-induced release, neuronal biosynthesis, anatomical localisation, metabolism and receptor-binding characteristics have been analysed in details [12, 24, 29]. Administration of SP affects a wide range of behaviours including locomotor activity, nociception and learning [6, 32, 34]. SP has heterogeneous distribution in the mammalian central nervous system. SP like immunoreactivity located in cell bodies and nerve terminals has been demonstrated in different brain regions including the amygdaloid body (AMY) [20, 27]. Memory facilitating as well as positively rein-

*Dedicated to Professor József Hámori on the occasion of his 70th birthday.

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forcing effects of SP microinjection have been found in the lateral hypothalamus, nucleus basalis magnocellularis, medial septal area and the ventromedial caudate-putamen in rats [4, 8, 18, 22, 33]. In an early study it was shown, however, that post-trial infusion of SP into the medial nucleus of the AMY impaired avoidance behaviour in rats [11]. Since the basolateral AMY (ABL) is involved in the mechanisms of reinforcement and memory processes [30], in the present experiments behavioural consequences of bilateral SP microinjections into the ABL were examined in passive avoidance and place-preference paradigms.

MATERIALS AND METHODS

Subjects and surgery

Subjects were 62 adult male CFY rats (LATI, Gödöllő, Hungary) weighing 280–300 g at the beginning of the experiments. All animals were maintained in a temperature (22 ± 2 °C) and humidity (65–70%) controlled vivarium with a 12 h light-dark cycle (lights on 6:00 a.m.). All animals were cared for in accordance with institutional (Pécs University Medical School) and international standards (European Community Council Declarative 86/609/EEC). Rats were housed individually with standard laboratory food pellets (CRLT/N standard rodent food pellets, Charles River Laboratories, Budapest) and tap water were available ad lib. For surgery, subjects were anaesthetised with ketamine (80 mg/kg i.p., Calypsol, Richter Gedeon, Hungary) supplemented with diazepam (2 mg/kg i.p., Seduxen, Richter Gedeon, Hungary). Stainless steel bilateral guide tubes (22G) were stereotaxically implanted into the dorsal border of the basolateral amygdaloid nucleus (ABL, coordinates referring to the bregma AP: 1.0, ML: 5.2, DV: 6.5 mm ventral from the surface of the dura) according to a stereotaxic atlas [25]. The tips of cannulae were positioned 0.5 mm above the intended injection site. Cannulae were fixed to the skull with acrylic cement and stainless steel screws. When not being used for injections, the guide tubes were occluded with obturators made of 27 G stainless steel wire. Animals were allowed to have 7 days for postoperative recovery before experiments. During this time they were frequently handled daily.

Drug application

Substance P (SP; S 6883, Sigma Chemical Co) was dissolved in 0.15 M sterile saline containing 0.01 M sodium acetate and phosphate buffer (pH 7.4) and used in the appropriate doses for bilateral intraamygdaloid microinjections in a volume of 0.4 µl per side. SP or vehicle were microinjected through a 30 G stainless steel injection tube extending 0.5 mm below the tips of the implanted guide cannulae. The injection cannula was attached via polyethylene PE-10 tubing to a hand held Hamilton

microsyringe. SP or vehicle were injected for 1 min, and the injection cannula was left in place for an additional 1 min to reduce the amount of drug drawn up the cannula track. Awake animals were injected in their home cage.

Behavioural experiments

Passive avoidance

In the experiments two-compartment passive avoidance test was used. During the habituation trial rats were placed into a white painted brightly lit (100 W bulb) start box (60×60×60 cm). The latency to enter the black painted, dark shock compartment (15×15×15 cm) of the apparatus through a guillotine trap door was measured. Subjects were allowed a maximum time of 300 s to enter and/or stay the dark compartment. In the following day conditioning trial was performed. After the entrance into the dark shock compartment the trap door was closed and the rat received three times a 0.5 mA inescapable foot-shock for 1 s each. Immediately after conditioning rats received bilateral intraamygdaloid microinjection of 10 ng SP or 100 ng SP or vehicle solution (controls). Twenty-four h later latency to enter the dark compartment was measured (test trial).

Place preference in two-compartment-box

The apparatus (a rectangular box; 50×25×50 cm) consisted of two compartments of equal size [10]. One compartment (25×25×50 cm) was painted white while the other was painted black. Around its central transverse axis the apparatus could be tilted 0.5 cm toward the direction of either the white, or the black compartment. A home-made equipment containing microswitches measured the time the rats spent in the white or the black compartment. Experiments were performed on consecutive days. During habituation trial rats were placed into the centre of the apparatus and they had free access to all parts of both compartments for 10 min. Twenty-four h later conditioning trial was performed. Treatment (bilateral microinjection of 10 ng SP, 100 ng SP or vehicle) was given in that compartment where the animals had spent less than 50% of their time during habituation trial (treatment compartment). After injection the rats were forced to remain in this non-preferred compartment for 10 min. In the following day test trial was performed. Animals had free access to both compartments for 10 min and the time the rats spent in the white or the black compartment was recorded.

Place preference in circular open field

A circular open field (85 cm in diameter, wall height 40 cm) was used for the experiments [10]. Two crossed lines marked 4 quadrants of equal size and identical floor and wall textures. Outside of the open field external visual cues were provided to the

animals. Experiments were performed on consecutive days. Each trial lasted 10 min. During the habituation trial the rat was placed into the centre of the apparatus. The time spent in each of the 4 quadrants and entries into the quadrants were recorded. The treatment quadrant was determined for each rat to be the quadrant in which the rat spent neither the most, nor the least time. During the conditioning trial transparent plexiglas barriers were inserted into the field forcing the animal to remain in the treatment quadrant. Rats received bilateral intraamygdaloid microinjection of 10 ng SP or 100 ng SP, or vehicle solution (controls). After 1 min delay rats were placed into the treatment quadrant for 10 min. In the following day the test trial was made. The Plexiglas barriers were removed and the animal was placed into the centre of the apparatus and it had free access to the 4 quadrants for 10 min. Throughout the experiments the behaviour of animals was recorded by a specific video camera (EthoVision Video Tracking System, Noldus Information Technology, The Netherlands). Data were stored and motion analysis was made by means of a PC computer using EthoVision Basic software.

Histology

After completion of experiments animals were anaesthetised with the same procedure used for surgery and were perfused transcardially with saline (0.15 M) followed by 10% formalin solution. Brains were removed, frozen and serially sliced with a freezing microtome. Sections were stained with Cresyl violet. Injection sites were reconstructed according to the stereotaxic atlas [25]. Rats were excluded from statistical analysis if their cannulae were not correctly positioned into the ABL.

Statistical analysis

Data were analysed by ANOVA followed by a post hoc t-test (Excel for Windows). As non-parametric method, Mann-Whitney U-test was also used to evaluate data in place preference tests. The Wilcoxon matched signed-rank test was used to evaluate differences within groups. Friedman's analysis of variance was performed to compare the time spent in the four quadrants in circular open field to determine the treatment quadrant.

RESULTS

Histological examinations showed that in 50 rats cannula tips were symmetrically located in the dorsal part of the ABL. The tracks of cannulae and the tips were determined on the basis of existence of debris and moderate glial proliferation. In 5 rats the position of cannulae was located outside the target area and these animals were excluded from statistical analysis. In 7 animals cannula tips were asymmetrical and

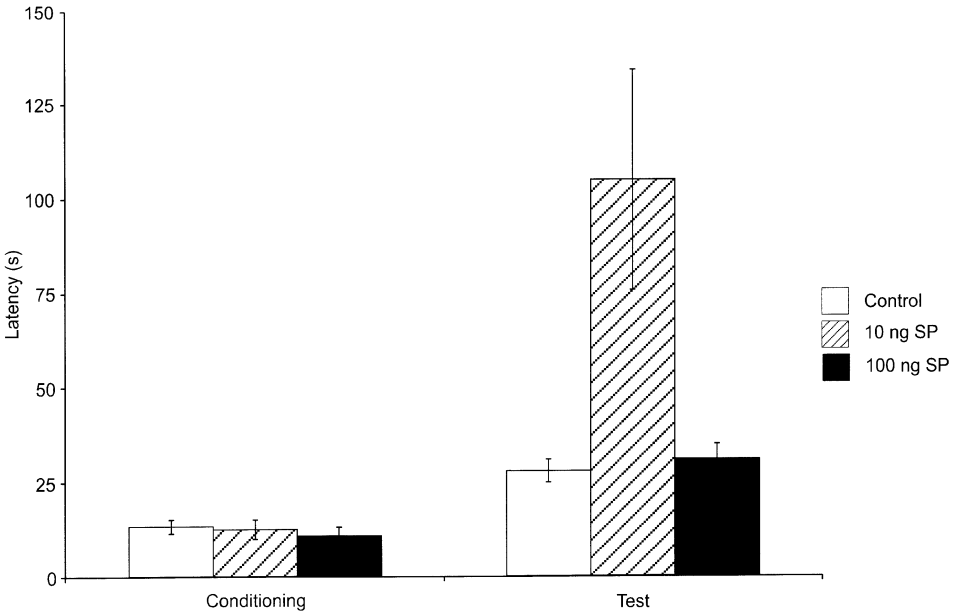

Fig. 1. Passive avoidance learning in two-compartment box after bilateral SP microinjections into the ABL. Columns represent mean latencies (±S.E.M.) during conditioning and test sessions, respectively. Control: vehicle treated rats. 10 ng SP and 100 ng SP: animals microinjected with 10 or 100 ng SP, respectively. For more explanation see the text

somewhat dorsal, so the tips were localised on one side in the central AMY nucleus and in the other side within the lateral AMY nucleus or in the piriform cortex. Data of these rats were also excluded.

In order to achieve a weak learning effect and to prevent freezing-behaviour in the shocked animals a weak electric shock (0.5 mA) was used in the passive avoidance situation. Under such condition the possible reinforcing effect of SP could be examined. For passive avoidance the ANOVA analysis revealed that there was a significant effect for learning [$F(1, 30) = 10.377$, $p < 0.004$, Fig. 1]. While significant difference was not found during the conditioning session, data analysis of test session showed a significant effect among groups [$F(2, 15) = 5.284$, $p < 0.02$]. Analytical comparisons of group means indicated that for shocked rats 10 ng SP ($n = 6$) enhanced learning over controls ($n = 5$, $p < 0.001$) and animals treated with 100 ng SP ($n = 5$, $p < 0.001$; see column Test on Fig. 1). When the non-parametric Wilcoxon test was used it has been revealed that rats increased their latency in every group after the foot shock ($p < 0.03$, respectively).

In the two-compartment-box paradigm place preference did not develop. Statistical analyses showed that controls ($n = 7$), animals treated with 10 ng SP ($n = 6$) or 100 ng SP ($n = 5$) spent nearly similar time in the treatment compartment during the

test session. When data of habituation session and test session were compared similar non-significant results were obtained.

In circular open field positive reinforcing effect was not found (Fig. 2). No significant changes in time spent in the treatment quadrant on the day of testing or during the habituation session were registered in controls (n = 6), rats treated with 10 ng SP (n = 5) or 100 ng SP (n = 5).

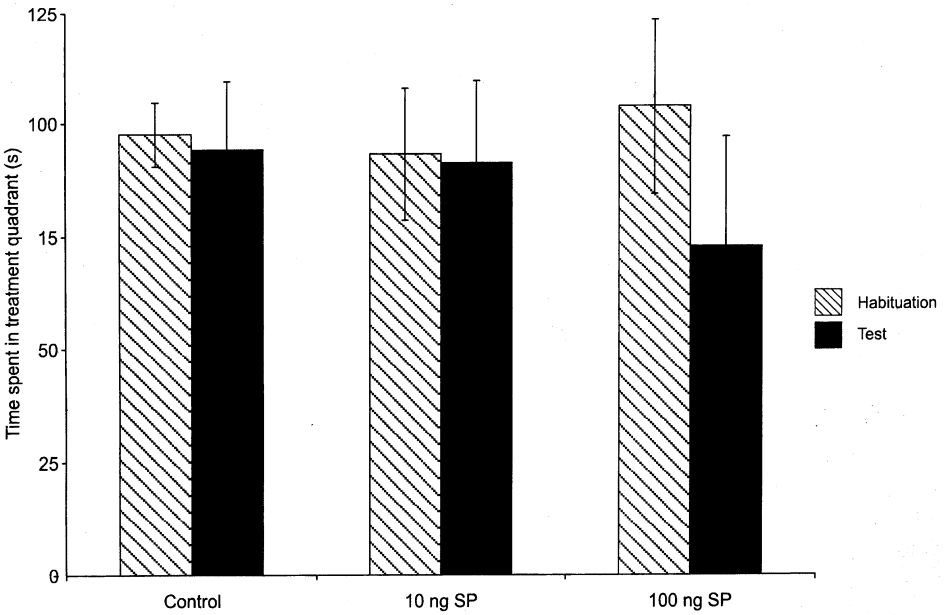

Fig. 2. Experimental results obtained in circular open field. Columns represent mean time (± S.E.M.) spent in the treatment quadrant before (Habituation) or after (Test) bilateral ABL microinjections of vehicle (Control), 10 ng SP or 100 ng SP, respectively. For more explanation see the text

DISCUSSION

Passive avoidance paradigms involve the classically conditioned fear component and an instrumental act. Namely, the passive avoidance response necessitates "not doing something" to avoid the noxious condition. Deficits in learning or retention of avoidance responses are interpreted as being due to emotional changes, cognitive failures, disturbances of reinforcement and memory formation [30]. It is generally accepted that the AMY is involved in the mechanisms of emotion, learning, reinforcement and memory processes. Amygdalectomised rats were found to be impaired in passive avoidance tasks [19, 23] and selective lesions of the ABL or central AMY nuclei (ACE) consistently produced an impaired passive avoidance behaviour [5, 15].

Our results showed that post-shock infusion of 10 ng SP into the ABL significantly enhanced passive avoidance learning in rats, while 100 ng SP was ineffective. Both controls and animals treated with 100 ng SP exhibited definite learning effect measured during the test session, however, their latencies to enter the black compartment were similar. The dose-range of SP applied in our experiments was similar to those used by others [10, 31]. SP often has a dose-related action and most commonly an inverted U-shaped dose-response relationship can be identified when different doses of SP are injected [10]. Although we did not use very low doses (i.e. less than 10 ng), our results in the ABL are consistent with these previous observations. Since SP was administered after the application of foot-shock (see conditioning session) it is unlikely that its effect on learning was due to changes in pain threshold or other non-specific performance variables associated with acquisition.

In our experiments two different place preference paradigms were used for studying possible effects of SP. In two-compartment-box the colour (i.e. white or black) and in addition the kinetic movement (the tilt of the box) were the cues for the rats, while in circular open field external cues were available to guide and locate the animals. In majority of related experiments reinforcing effects of SP were studied in circular open field. Therefore, the present results obtained in circular open field are comparable with those observed after SP injection into the lateral hypothalamus and nucleus basalis magnocellularis [2, 4, 8, 9]. In these limbic structures SP exhibited positive reinforcing effects because rats learned easily to stay in that quadrant of the circular open field in which they had received SP injection before. In the present experiments, however, place preference did not develop, neither in the two-compartment-box, nor in the circular open field. This may suggest that SP in the ABL is involved in learning and memory processes related to aversive situations, while the SP doesn't have rewarding-addictive consequences in the ABL.

Namely, in previous experiments it has been found that rats self-inject amphetamine and dopamine (DA) into the nucleus accumbens [3, 7] showing that the mesolimbic DA system plays essential roles in reward processes and addictive behaviour. Rats self-administer SP into the ventromedial caudate-putamen [18] and SP injections into the nucleus basalis magnocellularis induce place preference in circular open field with a simultaneous increase of DA level [2]. The nucleus accumbens is rich in SP and its receptors and local administration of SP can enhance DA activity there [13]. Thus, the reinforcing effects of SP have been related to DA function [2]. Coexistence of SP with other putative neurotransmitters and related substances including tyrosine hydroxylase [17] has been shown in central and peripheral neurones, and evidence suggests that tachykinins may modulate DA release through presynaptic mechanisms. Electron microscopic immunocytochemistry showed that tachykinin-containing terminals form axo-axonic contacts with thyrosine hydroxylase-labeled DA terminals in the nucleus accumbens [26]. Less is known about SP and related DA mechanisms in the AMY, however. In fact, the mesolimbic DA system innervates the structure, though the DA concentration is significantly lower in basal and lateral nuclei than in the ACE [1]. The SP concentration in the AMY is considerably high in the rat [14]. Numerous SP-positive cells are

found in the area between the central and medial AMY nuclei [20]. These neurones not only make a dense intrinsic plexus within the whole AMY but also project to the bed nucleus of stria terminalis and lateral hypothalamus [28]. While SP containing cell bodies can primarily be found in the ACE, scattered fibres and terminals are present in the ABL [27]. Recently the expression of mRNA for NK-1 receptors was examined in AMY sub-nuclei [21]. A large number of positive cells with moderate to weak intensity signals were found in the ACE and scattered positive neurones with low to moderate intensity of the hybridisation signal were also seen in the ABL [21]. On the basis of these results it is obvious that in both ACE and ABL DA and SP terminals, as well as NK-1 receptors are present, however, the density of them is much higher in the ACE than in the ABL. In our pilot experiments it has been revealed [16] that bilateral SP microinjections into the ACE induce place preference in the same circular open field and evoke anxiolytic effects. As the present results showed in the ABL the SP enhanced only passive avoidance learning and place preference did not develop. One may suggest, therefore, that these different findings can be due to differences in the local neuronal networks and the input-output system of the two AMY nuclei. Further experiments are needed to ascertain the exact and detailed mechanisms of SP and its possible co-action with DA in the AMY, however. Nevertheless, the present results demonstrate that SP in the ABL definitely enhances learning in avoidance situation.

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