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Research report

Effects of substance P microinjections into the globus pallidus and central nucleus of amygdala on passive avoidance learning in rats

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ABSTRACT

Substance P (SP) has been implicated in learning and memory processes. This peptide facilitated learning when injected peripherally or directly into the ventral pallidum. SP has high affinity for neurokinin-1 (NK-1) receptors. WIN51,708 is a potent NK-1 receptor antagonist that can inhibit the physiological effects of SP. Immunohistochemical experiments showed that the globus pallidus (GP) and the amygdaloid (AMY) body are rich in SP immunoreactive elements. Pallidal lesions cause learning deficits in active and passive avoidance paradigms. Serious memory deficits develop after lesions of AMY and its role in conditioned fear has been suggested. The aim of our study was to examine whether the SP microinjected into the GP or central nucleus of AMY (ACE) can modify negative reinforcement. Male Wistar rats were conditioned in a passive avoidance situation. Animals were microinjected with 0.4 μl of 10 ng SP, 100 ng SP or vehicle solution into the GP or the ACE. Results showed that 10 ng SP significantly enhanced passive avoidance learning in both structures, while 100 ng SP was ineffective. Retention examined 1 week later was diminished in the GP and still significant in the ACE. The possible involvement o NK-1 receptors in the effects of SP microinjected into the ACE was also studied. Prior treatment with WIN51,708 could block the SP effects on passive avoidance paradigm. Our results are the first to demonstrate that SP plays important roles, though in different ways, in learning and memory processes related to the GP and AMY.

1. Introduction

Substance P (SP), belonging to the tachykinin family of neuropeptides, was the first identified peptide shown to act as a neurotransmitter. Several tachykinins have been discovered in non-mammalian species and related peptides, known as neurokinins, have been identified in mammals [14,24]. Neurokinin receptors have been classified into three types in mammals, namely neurokinin-1, neurokinin-2 and neurokinin-3 (NK-1, NK-2 and NK-3, respectively). Substance P preferentially binds to NK-1 receptor, however, it can also bind to and act as full agonist on NK-2 and NK-3 receptors [7,24,56].

A number of experiments have implicated SP in a wide range of behaviors including locomotor activity, learning, memory and anxiety [13,20,22,26,28,41,52]. Reinforcing effects have been found after peripheral [64] or central administration of SP, namely in the lateral hypothalamus [30], in the nucleus basalis magnocellularis [29], medial septal area [62] and in the ventromedial caudate-putamen complex closed to the border of the globus pallidus (GP) [41]. In various tasks the effects of SP on memory processes were dose-dependent.

Results based on immunohistochemistry and bioassays have shown the presence of SP in high concentrations in the central nervous system and in the variety of peripheral organs [53]. SP-immunoreactive cells are distributed in various regions considered to be involved in the control of learning and memory such as the striatum or amygdala (AMY). A number of SP cell bodies are present in the central nucleus of AMY (ACE) and also a substantial amount of fibers and terminals can be found there. ACE has high densities of NK-1 receptors as well. Locally high concentration of SP is also present in the ventral GP-substantia innominata complex. Within the pallidal complex SP appears to be confined to fibers and terminal processes. Rat GP also contains a moderate to high level of NK-1 receptors [61].

The GP plays an important role in normal movement regulation and in the pathophysiology of basal ganglia disorders [11,23]. In addition to being an important structure of the extrapyramidal motor system, the GP is also involved in the central control of feeding [36,43,45,58], food-rewarded learning and memory processes as well [25,37,44,45]. GP lesions were reported to impair conditioned visual discrimination learning [15,16].

The amygdaloid complex is considered to be one of the key elements in the neural control of emotion [9], memory [49], and especially negative reinforcement [39,42]. It is well known that the

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AMY is a key structure for innate and conditioned fear behavior and results of lesion studies implicate the AMY in the acquisition and retention of aversively motivated tasks [9,39,42]. Extensive evidence indicates that memory storage is influenced by post-training intra-amygdaloid injections of drugs affecting several different neuromodulatory and neurotransmitter systems [5,32,33,54]. In our previous experiment bilateral SP microinjections into the basolateral nucleus of the AMY significantly enhanced passive avoidance learning [46].

Substance P has been implicated in learning and the ACE and GP are involved in the mechanisms of learning and memory processes. Effects of SP administered into these brain areas on learning, however, have not been investigated yet. In the present experiments, therefore, we examined the effects of bilateral SP microinjections into the GP or ACE on negative reinforcement and memory in passive avoidance paradigm. SP was injected in two different doses in order to demonstrate dose-dependent effects. We used specific receptor antagonist to study the involvement of NK-1-receptors in the effects of SP. This was accomplished by examining the effect of prior treatment with the tachykinin NK-1 receptor antagonist WIN51,708. This compound was selected, since it is a competitive antagonist at the NK-1 receptor and several reports have shown that WIN51,708 can inhibit behavioral effects of SP [51,52].

2. Materials and methods

2.1. Subjects

200 adult male Wistar rats weighing 280–320 g at the beginning of the experiments were housed individually and cared for in accordance with institutional (Pécs University, Medical School) and international standards (National Institutes of Health Guidelines for Laboratory Animals). Rats were kept in a temperature- and light-controlled room (22 ± 2 °C; 12:12 h light–dark cycle with lights on at 6:00 a.m.) for 1 week before surgery. Standard laboratory food pellets (CRLT/N standard rodent food pellet, Charles River Kft, Budapest, Hungary) and tap water were available ad libitum. All behavioral testings were done during the rats' daylight period between 08:00 and 17:00 h.

2.2. Surgery

Operations were carried out under combined anesthesia consisting of intraperitoneal injection of a mixture of ketamin and benzodiazepam (Calypsol and Seduxen, Richter Gedeon, Hungary) mixed in a ratio of 4:1 (ketamine: 80 mg/kg body weight, diazepam: 20 mg/kg body weight). Animals were stereotaxically implanted bilaterally with 22 gauge stainless steel guide cannulae, directed toward and 1.0 mm above the dorsal border of the ACE (coordinates relative to bregma: AP: −2.3 mm, ML: ±4.2 mm, DV: −6.2 mm) or the target area in the GP (coordinates relative to bregma: AP: −1.3 mm, ML: ±3.0 mm, DV: −6.4 mm from the surface of dura mater), according to the rats' stereotaxic atlas [55]. Cannulae were fixed to the skull with two stainless steel screws and dental acrylic. When not being used for injection, the guide cannulae were occluded with stainless steel obturators, 27 gauges in diameter. Animals were allowed a minimum of 5 days post-operative recovery before experiments commenced, during which period they were handled daily.

2.3. Drugs and injection procedure

Peptides were obtained from Sigma (Sigma–Aldrich Co.). Substance P (S 6883) was injected in two different doses: 10 ng (7.4 pmol) and 100 ng (74 pmol) in 0.4 μl. Substance P was dissolved in 0.15 M sterile saline solution containing 0.01 M Na-acetate and 0.01 M phosphate buffered saline (PBS, pH 7.4). Control animals received this solution bilaterally as vehicle (Veh1) in equal volume to that used for SP injections. The NK-1 receptor antagonist WIN51,708 [W-103; 5 ng (11 pmol)/0.4 μl] was diluted in 0.15 M saline solution containing 0.3% dimethylsulfoxide and 0.01 M PBS, and its vehicle solution (Veh2) was used for control injections in the experiment with NK-1 receptor antagonist. In this experiment the following groups were used: the antagonist treated group (ANT) received WIN51,708 and then 15 min later vehicle of SP (antagonist + Veh1). The SP injected group pretreated with antagonist (ANT + SP) received WIN51,708 15 min before being injected with 10 ng SP (antagonist + SP). The SP treated group (SP) received vehicle of antagonist and then 10 ng SP (Veh2 + SP). The Control group (Control) received two vehicle injections (Veh2 + Veh1). The antagonist or Veh2 were applied 15 min prior to SP or Veh1 injections. Tubes containing solutions were kept in +4 °C before application.

Drugs or vehicles were microinjected through a 30 gauge stainless steel injection tube extending 1.0 mm below the tips of the implanted guide cannulae. The injection cannula was attached via polyethylene tubing (PE-10) to a 10 μl Hamilton microsyringe (Hamilton Co., Bonaduz, Switzerland). All injections were in volume of 0.4 μl delivered by a syringe pump (Cole Parmer, IITC, Life Sci. Instruments, California) over a 40 s interval. After the injection the cannulae were left in place for an additional 60 s to allow diffusion into the surrounding tissue. During the injections rats were hand-restrained.

2.4. Passive avoidance learning

Assessment of passive avoidance was performed using the one trial step-through paradigm. The apparatus consisted of an illuminated chamber (50 cm × 50 cm × 50 cm) with light-grey walls and a dark chamber (15 cm × 15 cm × 15 cm) with black walls, a movable roof and a metal grid floor for the administration of foot shock. A guillotine door separated the two compartments. A 100 W lamp was positioned above the apparatus and provided the illumination during the experimental period.

The passive avoidance procedure consisted of Habituation, Conditioning and Test trials, each lasted maximum of 180 s. The apparatus was cleaned and dried after each session. All trainings and testings were conducted in an isolated experimental room. In Habituation trial (first day) animals were placed into the light chamber and had free access to all parts of the apparatus for 180 s. During Conditioning trial (second day) animals were placed again into the large chamber and the latency to enter the dark shock compartment was measured (step-through latency). If animal did not enter the dark chamber within 60 s it was excluded from the experiment. After entering the dark chamber the door was closed and an inescapable foot shock was delivered to the feet through the floor grid for three times, 1 s each. In separate experiments Conditioning was made by means of weak (0.5 mA) or strong (2.0 mA) electric shock. Immediately after Conditioning rats had been removed from the apparatus and received bilateral microinjection of solutions described in previous section (see Section 2.3). Test trials were conducted without application of foot shock. Rats were placed again in the illuminated chamber and the latency of entering the dark box was recorded. If the animal did not enter the dark chamber within a 3-min test period, the test was terminated and latency was recorded as 180 s. In experiments with weak shock (0.5 mA) tests were carried out 24 h and 1 week after Conditioning (Test 1 and Test 2, respectively) while in experiments with strong shock tests were executed 24 h, 1 and 2 weeks after Conditioning (Test 1, Test 2 and Test 3, respectively).

Behavior of animals was recorded by a video camera. Data were stored and motion analysis was made by means of a PC computer using EthoVision Basic software (Noldus Information Technology b.v., Wageningen, The Netherlands).

2.5. Histology

At the end of experiments, rats received an overdose of Calypsol and Seduxen mixed in the ratio of 4:1 and were transcardially perfused with isotonic saline followed by 10% formalin solution. After 1 week of post-fixation brains were frozen, cut into 40 μm serial sections and stained with Cresyl-violet. Injection sites were reconstructed according to the stereotaxic atlas of the rat [55]. Only data from rats with correctly placed cannulae were analyzed.

2.6. Statistical analysis

Step-through latencies at both training and testings are represented as means ± standard error of the mean (S.E.M.). In experiments with weak shock two-way ANOVA analyzed between group differences for treatment (10 or 100 ng SP or Control) X trials (Conditioning or Tests), with treatment as between-subject variable and trial as within-subject variable. Post hoc group mean comparisons were conducted using Tukey's post hoc test. In experiments with strong shock one-way ANOVA was used and post hoc group mean comparisons were made by Student's paired- or independent-samples *t*-tests. *P* values less than 0.05 were considered significant. Statistical analyses were conducted using SPSS 11.0 for Windows.

3. Results

3.1. Histology

Histological examination showed that 184 cases of 200 animals the target place of the cannulae were precisely and symmetrically tipped to the target area (GP or ACE). The tracks of cannulae and tip positions were determined on the basis of the existence of debris and moderate glial proliferation. Schematic illustration of cannula placements is shown in Fig. 1.

Considering 16 rats the reconstructed cannula placement was not correctly positioned in the target area, so these subjects were excluded from subsequent analysis. Among these rats, in two cases, the cannulae tips were symmetrically entered into the liquor space