Theoretical Medical Sciences Ph.D. Program

BEHAVIOURAL EFFECTS OF SUBSTANCE P INJECTED INTO THE AMYGDALA AND THE GLOBUS PALLIDUS

Ph.D. Thesis

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1. INTRODUCTION

In this dissertation we investigated the behavioral effects of substance P in two different brain structures, namely, in the globus pallidus and in the amygdala. The animal (and human) behavior is defined as whole changes appearing in the internal conditions and/or in the motor pattern of an organism. In this essay, however, we will focus principally on learning and memory processes occurring in rewarding and punishing situations. Brain lesions caused by accidents or circulatory disturbances and neurodegenerative disorders may result in several types of learning and memory deficits. 10-15 % of people older than 65 years is suffer from some degree of decay in learning, memory or cognitive capabilities, thus, this is a common and serious problem.

Our working group has been concerned with the examination of the physiological functions of the globus pallidus (GP) since a long time. In addition to being an important structure of the extrapyramidal motor system, the GP may also be involved in the central control of perceptual, motivational, learning and memory processes [28,30,45]. Electrolytic or excitotoxic pallidal lesions cause learning deficits in several learning tests, including active and passive avoidance situations, Morris water maze, radial arm maze, or visual and non-visual discrimination learning paradigms [13,14,28,34]. Several studies in animal experimental research support the role of the GP in reward-related processes [29,36]. It was shown in humans that not only motor symptoms can be observed after selective bilateral pallidal lesion but anhedonia, depression and the ceasing of the drug addiction also occur [33]. There are several data supporting the structural and functional differences between the ventral-medial and dorsal-lateral part of the GP. The ventromedial part is considered to be a transitional area between the dorsal, motor pallidum and the ventral, limbic pallidum [16,35,37].

It is generally accepted that the amygdala (AMY) is involved in the control of reinforcement and learning processes. The AMY complex, as part of the limbic system, is considered to be also one of the key elements in the regulation of motivational and emotional processes as well as of behaviors related to fear and *anxiety* [7,17,18]. Dysfunctions of this structure may contribute to the etiology of generalized anxiety disorder [17]. Substantial role of AMY has been suggested in the regulation of learning and memory processes and it is an important structure of working or episodic *memory* [40]. Extensive evidence indicates that the AMY is a critical site for affecting several

neuromodulatory systems that can influence memory and, thus, AMY may modulate the storage of memory traces in other brain sites [15,31]. The AMY is also a key component of neural systems regulating *positive reinforcement* and reward-related processes [53]. Several studies suggest the role of this structure in mediation of the effects of drugs of abuse and natural rewards [1,26,38]. The AMY is heterogeneous considering its functions and also its structure. Several data support the differential roles that the central (ACE) and the basolateral (ABL) nuclei play in the aforementioned processes [2,15,18,38,44,52].

Experimental findings show that the acetylcholinergic (ACh) and dopaminergic (DA) systems play essential roles in the regulation of learning and memory. Nowadays became increasingly interesting, however, the investigation of the modulatory roles of neuropeptides, such as substance P (SP), on these processes. The undecapeptide SP, identified in mammalian and non-mammalian species, belongs to the *tachykinin* peptide family [9]. Its receptors have been found in the central and peripheral nervous system and have been classified into three types, namely, neurokinin (NK)1, NK2 and NK3 [10]. SP preferentially acts at NK1 receptors, however, it can bind to and act as full agonist on all three types [10,41]. SP has been implicated in a wide range of behaviors, its *memory facilitating* effects have been found in passive and active avoidance paradigms after its peripheral or intracranial applications [22,27]. *Positive reinforcing* effects have been revealed after peripheral administration of SP or after direct injections into the lateral hypothalamus, the medial septum or the ventral pallidum [20,23,25,47]. In addition, SP can influence animals' behavior related to fear and *anxiety*. Its anxiogenic and also anxiolytic effects were shown, depending on the dose range used and the specific brain region into which it was applied [12,24,48]. SP, like other peptides, often has an inverted U-shaped dose-response relationship [20,48]. SP coexists and closely interacts with several neurotransmitter systems and can modulate the release and/or the effects of other transmitters [5,6,11,21,49]. Changes in the SP content of certain brain regions were revealed in numerous diseases, thus, this peptide probably plays a role in the etiology of some neurodegenerative disorders [3,4,8,54]. The wide distribution of SP and its receptors has been shown in the central nervous system, among others, in the GP, in the ACE and also in the ABL [19,42,43]. Effects of SP administered into these brain areas on behavioral processes, however, haven't been investigated yet.

Based on the aforementioned data we investigated whether SP administration into these brain structures influence learning and memory processes. Furthermore, we studied the participation of NK1 receptors in the mediation of the effects of SP by means of administration of a specific receptor antagonist.

2. OBJECTIVES

1. Positive reinforcing effects of SP were revealed after its injections into several brain sites. The ventral-medial part of the GP plays a role not only in the control of movement-regulation but also in reward-related learning. The ACE and the ABL have important roles in rewarding - positive reinforcing processes and in the mediation of the effects of certain psychostimulant drugs. We investigated, therefore, the possible positive reinforcing effects of SP injected into the GP or these two AMY nuclei in Conditioned Place Preference paradigm.
2. In the Place Preference test animals spend more time in a certain part of the apparatus than in the others. This could be a result of hypoactivity, which may be due to anxiogenic effects of the substance received. SP can have anxiogenic and also anxiolytic effects, depending on the dose used and the site of action in the brain. We examined, therefore, whether SP injections into the above-mentioned structures have any effect on anxiety in the Elevated Plus-maze test.
3. Learning disturbances can be found after electrolytic or excitotoxic lesions of the GP in passive and active avoidance situations. The AMY nuclei have important roles in negative reinforcement learning in aversively motivated tasks. Learning improvement and also impairment can be obtained after SP administrations. We studied, therefore, the effects of SP injected into the GP, the ACE or the ABL on learning in Passive Avoidance paradigm using weak shock. In addition we examined the effects of SP on memory in Passive Avoidance paradigm after strong shock.
4. NK1 receptors can be found in the GP, the ACE or the ABL in medium to high density. We attempted, therefore, to unravel the role of these NK1 receptors in mediating the rewarding - positive reinforcing, anxiolytic or anxiogenic, and learning influencing effects of SP in these brain structures by means of pretreatment with a specific NK1 receptor antagonist.

3. MATERIALS AND METHODS

3.1. Animals

570 adult male Wistar rats weighing 280-320 g were used in the experiments. Rats were housed individually and kept in a light-, temperature- and humidity-controlled room (12:12 h light-dark cycle, 22 ± 2 °C and 55 ± 10 %). Tap water and standard laboratory food pellets (CRLT/N standard rodent food pellet, Charles River Kft, Budapest, Hungary) were available ad libitum. All behavioral testings were done during the rats' daylight period between 08:00 and 17:00 h. Animals were cared for in accordance with institutional (Pécs University, Medical School) and international standards (National Institutes of Health Guidelines for Laboratory Animals).

3.2. Surgery

Animals were stereotaxically implanted bilaterally with 22 gauge (0,644 mm) outer diameter stainless steel guide cannulae, directed toward the ventral part of the GP, or the dorsal border of the ACE and ABL. The cannulae terminated 1.0 mm above the target areas. Coordinates according to the rats' stereotaxic atlas of Paxinos and Watson [39] were the following: *GP*: AP: -1.4 mm, ML: ±3.4 mm, DV: -6.4 mm; *ACE*: AP: -2.3 mm, ML: ±4.1 mm, DV: -6.5 mm; *ABL*: AP: -2,8 mm, ML: ± 5,0 mm, DV: -6,6 mm.

3.3. Materials

Substance P (S 6883, Sigma-Aldrich Co.) was injected bilaterally in 10 or 100 ng (7.42 or 74.2 pmol) doses in a volume of 0.4 µl. The peptide 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 NK1 receptor antagonist WIN51,708 (W-103, Sigma-Aldrich Co.) was applied in 5 ng (11 pmol) dose in a volume of 0.4 µl. The antagonist was diluted in 0.15 M saline solution containing 0.3 % dimethyl-sulfoxide and 0.01 M PBS, and its vehicle solution (*Veh2*) was used for bilateral Control injections in the same volume to those of antagonist injections. Tubes containing solutions were kept in +4 °C before application.

In the experiments with SP animals were assigned to the following groups: SP 10 ng: rats receiving 10 ng SP, SP 100 ng: rats receiving 100 ng SP, Control: rats receiving *Veh1*. In the experiments with the NK1 receptor antagonist the following groups were used: ANT: rats receiving 5 ng WIN51,708 and then vehicle of SP (antagonist + *Veh1*), ANT+SP: rats receiving 5 ng NK1 receptor antagonist and 10 ng SP (antagonist + SP), SP: rats receiving the vehicle of the antagonist and then 10 ng SP (*Veh2* + SP), Control: rats receiving two vehicle injections (*Veh2* + *Veh1*). The antagonist or *Veh2* were applied 15 min prior to SP or *Veh1* injections.

3.4. Behavioral experiments

Experiments were conducted in an isolated sound-proof experimental room. Behavioral parameters of animals were measured by means of a PC computer using EthoVision Basic software. (Noldus Information Technology b.v., Wageningen, The Netherlands).

3.4.1. Conditioned Place Preference Test

We used the "corral" method developed by Huston and colleagues for Conditioned Place Preference experiments [20]. During Habituation trial rats had free access to all parts of the apparatus for 10 minutes (600 s). The time that animals had spent in each of the four quadrants was measured. On the next two days the Conditioning trials were executed. One of the four quadrants in which the animal had spent neither the most, nor the least time during Habituation was selected for conditioning (termed as *Treatment Quadrant*). After bilateral injections animals were restricted to the Treatment Quadrant for 15 minutes (900 s) by means of a transparent plexiglass barrier. During Test trial the plexiglass barrier was removed and rats were placed again into the center of the open field in drug-free state and had free access to all parts of the apparatus for 10 minutes (600 s). The time that animals had spent in each of the four quadrants was measured again. Place preference was defined as at least 25 % increase in time spent in the drug-paired quadrant during Test trial compared to the Habituation trial.

3.4.2. Elevated Plus-maze Test

Five minutes after bilateral microinjections the animals were placed on the central platform of the maze and the behavior of the rats was observed for 5 min (300 s). The *time spent* on, the *distance moved* on and the *number of entries* into the Enclosed arms, the Open arms and the end of the open arms (End-arms) were measured. We calculated also the following parameters: the ratio of time spent on the Open arms in proportion to the time spent on the Enclosed arms, the ratio of entries into the Open arms in proportion to entries into the Enclosed arms, and the Total number of entries, which was calculated from the entries into the Open and Enclosed arms. The Total distance moved during 5 minutes was also measured. This latter parameter and the Total number of entries were used for the characterization of the general activity of animals. Each rat was tested only once.

3.4.3. Passive Avoidance Test

The Passive Avoidance procedure consisted of Habituation, Conditioning and Test trials, each lasted maximum of 3 minutes (180 sec). During Habituation trial animals were placed into the large light chamber and had free access to all parts of the apparatus. During Conditioning trial animals were placed again into the light chamber and the latency to enter the dark shock compartment was measured (*Step-through latency*). After entering the dark chamber the door was closed and an inescapable foot shock was delivered to the feet through the floor grid for 3 times, 1 sec each. Conditioning was made by means of weak (0.5 mA) or strong (2.0 mA) electric shock in separate experiments. Immediately after the shock rats had been removed from the apparatus and received bilateral microinjections (SP, antagonist or vehicle). During Test trials rats were placed again in the illuminated chamber and the Step-through latency was recorded again. Test trials were conducted without application of foot shock. In the experiments with weak (0.5 mA) shock Tests were carried out 24 h and one week after Conditioning (Test1 and Test2, respectively). In the experiments with strong (2.0 mA) shock Tests were executed 24 h, one week and two weeks after Conditioning (Test1, Test2 and Test3, respectively).

3.5. Data processing

3.5.1. Histology

At the end of the experiments, rats were anesthetized with urethane and transcardially perfused with isotonic saline followed by 10 % formalin solution. Brains were frozen, cut into 40μm serial sections and stained with Cresyl-violet. Injection sites were reconstructed according to the rats' stereotaxic atlas of Paxinos and Watson. Those animals where the reconstructed cannula placement was outside the target area were excluded from subsequent analysis.

3.5.2. Statistics

Statistical analyses of experimental data were carried out using 'SPSS 15.0 for Windows' computer program. Data were analyzed with one-way or two-way analysis of variance (ANOVA) followed by Tukey's post hoc tests. When the number of experimental groups or number of trials did not allow this comparison, then Student's paired- or independent-samples t-tests were used. Differences were considered significant when p values were less than 0.05.

4. RESULTS

4.1. Globus pallidus

4.1.1. Conditioned Place Preference test

Positive reinforcing effect of SP was found in the Conditioned Place Preference test when injected into the GP. Time that animals had spent in the Treatment Quadrant significantly increased after 10 ng SP injection, while 100 ng SP had no effect. This rewarding - positive reinforcing effect of SP could be blocked by pretreatment with the specific NK1 receptor antagonist WIN51,708. One can suppose, therefore, that this effect is mediated via NK1 receptors.

4.1.2. Elevated Plus-maze test

SP injected into the GP had anxiolytic-like effects. Time spent on and distance moved on the Open arms and End-arms significantly increased in the 10 ng SP treated group, while the general activity of animals was not altered by the treatment. The higher dose (100 ng) of SP did not influence any parameter in the Elevated Plus-maze test. The

anxiolytic effects of the low dose SP were inhibited by the pretreatment with WIN51,708. We concluded, therefore, that NK1 receptors may play a role in the mediation of these anxiolytic effects.

4.1.3. Passive Avoidance test

SP injected into the GP facilitated learning in the Passive Avoidance test. 10 ng SP significantly increased Step-through latency after applying weak shock; this increase indicates that these animals learned better than Controls. The 100 ng dose SP had no effect on learning in this paradigm. The learning improvement by the low dose SP was, however, only short-term since one week after Conditioning there were no significant differences among groups. When strong shock was used Step-through latency significantly decreased after injection of the same dose of SP, indicating that 10 ng SP may interfere somehow with formation or retention of long-term memory.

4.2. Central nucleus of Amygdala

4.2.1. Conditioned Place Preference test

It has been shown in the Conditioned Place Preference test that SP injected into the ACE has positive reinforcing effect. Time that animals had spent in the Treatment Quadrant significantly increased after low dose (10 ng) SP injection. The higher dose (100 ng) of SP did not influence the behavior of animals. The rewarding - positive reinforcing effect of SP may be mediated through NK1 receptors, since this effect could be blocked by pretreatment with the specific NK1 receptor antagonist.

4.2.2. Elevated Plus-maze test

SP injected into the ACE proved to be anxiolytic. Time spent on the Open arms and End-arms significantly increased after the injection of both the lower (10 ng) and higher (100 ng) dose of SP, while the general activity of animals was not altered by the treatments. The anxiolytic effects of SP were not inhibited but only weakened by the pretreatment with the specific NK1 receptor antagonist. Time spent on and distance moved on the Open arms and End-arms decreased after the injection of WIN51,708 but this decrease was not significant compared to the SP injected group. After the antagonist pretreatment, however, the significant differerence in the former parameters disappeared compared to Controls. According to these results we concluded that not only NK1

receptors may play a role in the mediation of the anxiolytic effects of SP in the ACE but the NK3 receptors occurring in low density in this structure may also contribute to these effects.

4.2.3. Passive Avoidance test

It has been shown that SP injected into the ACE facilitated learning in the Passive Avoidance test. The low dose (10 ng SP) significantly increased Step-through latency applying weak shock; significantly better learning was observed in the group treated with low dose SP than in the high-dose SP or Control groups. The learning improvement by the low dose SP was longer-lasting than in the GP since one week after Conditioning there were still significant differences among groups. When strong shock was used Step-through latency was not significantly changed after injection of the same dose of SP, indicating that 10 ng SP in the ACE do not interfere with long-term memory. The memory improving effect of SP in the ACE may be mediated via NK1 receptors since the antagonist could block this effect.

4.3. Basolateral nucleus of Amygdala

4.3.1. Conditioned Place Preference test

We could not found positive reinforcing effect of SP in the Conditioned Place Preference test when it was injected into the ABL. Time that animals had spent in the Treatment Quadrant did not change after low dose (10 ng) SP injection. This time somewhat decreased after the injection of higher (100 ng) dose of SP but this difference was not statistically significant.

4.3.2. Passive Avoidance test

SP injected into the ABL facilitated learning in the Passive Avoidance paradigm. The low dose (10 ng) SP significantly increased Step-through latency after application of weak shock. There were no significant differences among groups, however, one week after the Conditioning. The higher dose (100 ng) of SP had no effect on the learning or memory in the Passive Avoidance test.

5. DISCUSSION

1. Rewarding - positive reinforcing effects of the low dose SP were revealed in Conditioned Place Preference test after microinjections into the GP and ACE but not into the ABL. The present results in the GP and ACE are comparable with those observed by others, i.e. SP can have positive reinforcing properties in other brain structures [20,25,47]. Our results are in accordance with a number of studies supporting the role of the GP and the ACE in the control of positive reinforcing - rewarding processes as well [1,26,29,33,36,38]. In the ABL neither the lower nor the higher dose of SP had positive reinforcing properties. Our finding may suggests, that even if the ABL is involved in the control of positive reinforcing processes according to several authors [26,46], the SP doesn't have rewarding-addictive consequences there. The different findings in the ACE and ABL may be due to different roles of these structures in the positive reinforcing processes. These findings, on the other hand, may be explained by the differences in afferent and efferent connections and/or different SP-ergic innervation or different NK1 and NK3 receptor densities of the two AMY nuclei [42-44,50,51].

2. Anxiolytic effects of SP have been revealed in the Elevated Plus-maze test after injections into the GP and the ACE. Time spent on and distance moved on the Open arms and the End-arms increased after injections of the low dose SP into these two structures. There were differences, however, in the anxiolytic effects of SP regarding these two structures. Namely, in the ACE the higher dose of SP also proved to be anxiolytic, while in the GP the 100 ng dose of SP had neither anxiogenic nor anxiolytic effect. Based on our results we may conclude, therefore, that the low dose SP does not exert any anxiogenic effects either in the GP or in the ACE. Thus, animals did not spend more time in the previously non-preferred site during the Place Preference test because of the anxiogenic effects of SP. There are only few studies investigating the role of the GP in the control of animals' behavior related to fear and anxiety [32]. Our results are the first to demonstrate, that SP in the GP may play a role in processes related to anxiety.

3. Results of the Passive Avoidance experiments showed learning improvement in this punishing situation after bilateral application of SP into the GP, the ACE and the ABL. Infusion of the low dose SP after electric shock facilitated learning in all the three structures, while the higher dose was ineffective in all cases. Our findings are in good agreement with several data supporting the role of SP, the GP and the different nuclei of the AMY in learning and memory processes [13,14,22,27,31,40]. Nevertheless, the GP and the ACE may participate in these processes in different ways. SP injected into the GP facilitated learning after weak shock, but inhibited somehow the formation of long-term memory or the retention of memory traces. This memory-attenuating effect was also strengthened in the experiment with strong shock. In the ACE the administered SP improved learning and did not attenuate the retention of memory, either in the experiments with weak or strong shock. Thus, mechanisms through which SP may influence learning and memory processes are different regarding the GP and the ACE. It would be necessary to repeat the Passive Avoidance test in the ABL with strong shock to find out whether SP interfere with the formation of long-term memory in this structure as well.

The positive reinforcing, anxiolytic and learning facilitatory effects of SP proved to be dose dependent in our experiments, an inverted U-shaped dose-response relationship could be identified in the GP, the ACE and also in the ABL. This kind of dose-related action of peptides is well known in the literature [20,48].

4. In our next experiments we found that NK1 receptors may play a role in the mediation of the positive reinforcing effects of SP in both the GP and the ACE. The rewarding - positive reinforcing effects of SP could be inhibited by prior treatment with the specific NK1 receptor antagonist in both structures. The anxiolytic effects of SP are also mediated via NK1 receptors in the GP, while in the ACE not only NK1 but also NK3 receptors may participate in the mediation of these effects. Namely, contrary to our results obtained in the GP, in the ACE the NK1 receptor antagonist could not block but only weaken the anxiolytic effects of the low dose SP. We could show that the learning facilitatory effect of SP in the Passive Avoidance paradigm is also mediated through NK1 receptors in the ACE, because this effect could be blocked with the specific NK1 receptor antagonist.

SP coexists and closely interacts with several neurotransmitter systems. The positive reinforcing, learning facilitatory effects of SP may be related to the mesolimbic DA system [6]. One can not exclude the possibility, however, that the interaction with ACh-ergic neuronal elements may participate in the mediation of these effects of SP [5]. According to experimental data interactions of SP with serotoninergic, opiatergic and/or GABA-benzodiazepine transmitter systems could be supposed in the influences of SP on anxiolytic processes [11,21,49]. The learning improving effects of this peptide observed in punishing situation may also be linked to DA-ergic and/or ACh-ergic neurotransmission. Nevertheless, further experiments are necessary to cast light on these mechanisms in detail.

There is growing evidence that SP may play a role in the pathophysiology of some neurodegenerative disorders. SP-immunoreactivity changes in the GP were demonstrated in patients with Parkinson's and Huntington's diseases and also in schizophrenia [4,54]. The changes in SP-erg innervation of the GP, therefore, may contribute to the development of these diseases. Brain level changes of SP content in the AMY have been seen in Alzheimer's disease, schizophrenia or major depression [3,4,8]. Thus, SP-ergic neuronal elements in the AMY may play a role in the etiology of the above-mentioned disorders. The knowledge of the exact role of SP in these processes may contribute to the better understanding of the patomechanism of these disorders and to developing new tools for their treatment.

6. REFERENCES

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7. LIST OF PUBLICATIONS

I. Articles related to the thesis

Lénárd, L. and E. Kertes: Influence of passive avoidance learning by Subtance P in the basolateral amygdala. *Acta Biol Hung* 53(1-2): 95-104, 2002. [IF: 0.416]

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II. Other publications and citeable abstracts

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