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Anxiolytic-like action of neurokinin substance P administered systemically or into the nucleus basalis magnocellularis region

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Abstract

There is evidence that the neurokinin substance P plays a role in neural mechanisms governing learning and reinforcement. Reinforcing and memory-promoting effects of substance P were found after it was injected into several parts of the brain and intraperitoneally. With regard to the close link between anxiety and memory processes for negative reinforcement learning, the aim of the present study was to gauge the effect of substance P on anxiety-related behaviors in the rat elevated plus-maze and social interaction test. Substance P was tested at injection sites where the neurokinin has been shown to promote learning and to serve as a reinforcer, namely in the periphery (after i.p. administration) and after injection into the nucleus basalis magnocellularis region. When administered i.p., substance P had a biphasic dose–response effect on behavior in the plus-maze with an anxiolytic-like action at 50 μg/kg and an anxiogenic-like one at 500 μg/kg. After unilateral microinjection into the nucleus basalis magnocellularis region, substance P (1 ng) was found to exert anxiolytic-like effects, because substance P-treated rats spent more time on the open arms of the plus-maze and showed an increase in time spent in social interaction. Furthermore, the anxiolytic effects of intrabasalis substance P were sequence-specific since injection of a compound with the inverse amino acid sequence of substance P (0.1 to 100 ng) did not influence anxiety parameters. These results show that substance P has anxiolytic-like properties in addition to its known promnestic and reinforcing effects, supporting the hypothesis of a close relationship between anxiety, memory and reinforcement processes.

Keywords: Neurokinin; Basal forebrain; Anxiolytic; Anxiogenic; Elevated plus-maze; Social interaction

1. Introduction

The peptide neurotransmitter substance P belongs to the neurokinins, the mammalian members of the tachykinin family of peptides, which are widely distributed in peripheral mammalian tissue and in the central nervous system (Pernow, 1983). Substance P is present in various brain areas, where the neurons that express substance P are colocalized with neurons containing classic neurotransmitters, for example, acetylcholine in the basal forebrain or dopamine in the striatum (Bannon et al., 1991). Mammalian tachykinin receptors have been classified as tachykinin NK_1, NK_2 and NK_3 subtypes, based on whether their putative ligand is substance P, neurokinin A or neurokinin B, respectively (Quirion and Dam, 1988). It is likely that all neurokinin receptor subtypes exist in the central nervous system (CNS), although only the NK_1 and NK_3 sites have so far been mapped in any detail (Guard and Watson, 1991).

The characteristic distribution of substance P and the presence of specific neurokinin receptors in the brain prompted investigations to unravel the role of substance P in brain function. The neurokinin has been implicated in the control of pain perception, in motivated behaviors and behavioral disorders (Otsuka and Yoshioka, 1993) as well as in neural plasticity processes associated with learning and memory formation. Facilitation of learning by post-trial injection of substance P has been observed at injection

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sites where the peptide has also been shown to be reinforcing, namely, in the lateral hypothalamus/medial forebrain bundle, medial septum, nucleus basalis magnocellularis and after systemic administration (Huston et al., 1993; Tomaz and Huston, 1986). Substance P can also promote processes responsible for functional recovery following damage to the CNS (Sprick et al., 1996) and can counteract age-related performance deficits (Huston and Hasenöhrl, 1995).

There is substantial evidence that anxiety and memory for negative reinforcement learning are closely linked (Beuzen and Belzung, 1995; Tomaz et al., 1992, 1993), raising the possibility that substance P, in addition to its promnestic and reinforcing effects, might also play a role in the neural control of fear and anxiety. Few studies have investigated the neurokinin in the context of 'emotional' processes, with contradictory results. Substance P, particularly its N-terminus, appears to exert a modulatory influence on physiological responses to stress (Oehme and Krivoy, 1983) and was even considered to act as a 'physiological tranquilizer' (Starr et al., 1978). Congruent with this hypothesis, an inverse relationship was reported between substance P levels in cerebrospinal fluid and intensity of anxiety in humans (Almay et al., 1988), and injection of certain tachykinin NK_1 receptor antagonists produced anxiogenic-like effects in rodents (Saria et al., 1993; Zernig et al., 1992, 1993). However, tachykinin NK_1 and NK_2 receptor antagonists have also been shown to reduce (File, 1997; Walsh et al., 1995), and substance P receptor agonists to increase, anxiety-related behaviors (Aguiar and Brandao, 1996; Teixeira et al., 1996; De Lima et al., 1997). The reasons for the discrepant findings require clarification. The use of different experimental models of anxiety, species and modes of injection may explain the conflicting results. Moreover, it remains to be determined where in the brain and in interaction with which neurotransmitter(s) substance P is active in modulating anxiety.

The aim of the present experiments was to investigate in more detail the influence of substance P on fear and anxiety. Thus, two experiments were carried out to assess the effects of substance P in the elevated plus-maze (Pellow et al., 1985) and the social interaction test (File, 1993). Both paradigms have been validated using behavioral and/or physiological criteria, and have shown good sensitivity to both anxiolytic as well as anxiogenic drugs. Substance P was tested at injection sites where the neurokinin has repeatedly been shown to promote learning and to serve as a reinforcer, namely, given systemically and injected into the nucleus basalis magnocellularis region (Huston and Hasenöhrl, 1995; Tomaz and Huston, 1986). Furthermore, in order to ensure pharmacodynamic specificity of the effects of substance P on parameters of fear, groups of rats were included which received intrabasalis injections of Met-11-Arg amide, which has the inverse sequence of the substance P molecule.

2. Materials and methods

The experiments were carried out in accordance with the German Law on the Protection of Animals and were approved by the state authority (Bezirksregierung Düsseldorf). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.1. Experiment 1: Effect of systemic injection of substance P on exploratory activity in the elevated plus-maze

2.1.1. Animals

Male Wistar rats ($n = 64$; TVA, University of Düsseldorf), weighing 250–350 g, were used for this experiment. Rats were housed in groups of 6 to 8 per cage under standard laboratory conditions with food and water continuously available. A light–dark schedule (12 L:12 D lights on 0700 h) was imposed and all behavioral testing was done during the rats' daylight period between 0900 and 1600 h. Rats were tail-marked and handled daily for 5 min during the three last days before the experiment.

2.1.2. Apparatus

The elevated plus-maze consisted of two open (50×10 cm) and two enclosed arms ($50 \times 10 \times 40$ cm) with an open roof, arranged such that the two arms of each type were opposite each other. The maze was elevated to a height of 50 cm (for details see Pellow et al., 1985). A 40-W red bulb was suspended 150 cm above the center of the maze and provided illumination of 0.203 lux in the open and 0.115 lux in the enclosed arms. The temperature during behavioral testing was 19°C in the open and 20.5°C in the enclosed arms. Wide-spectrum masking noise (68 dB) was provided by a noise generator. The behavior of the animals throughout the experiments was recorded by a video system. After each trial the apparatus was cleaned with water containing 0.1% acetic acid. Behavioral recordings were carried out with the observer unaware of the treatment of the rats.

2.1.3. Drugs and injection procedure

Undecapeptide substance P (molecular weight 1347.80, Peninsula Labs, USA) was dissolved and diluted in physiological saline 1 h before use. Materials in contact with substance P were acid washed. The doses of substance P were 5 (3.7), 50 (37), 250 (185) and 500 (370) μg/kg (nmol/kg), injected i.p. in a volume of 0.5 ml/kg; the same volume was used for injecting the vehicle. The dose range of substance P was selected on the basis of previous experiments assessing the role of the neurokinin in learning, memory and reinforcement processes (Huston and Hasenöhrl, 1995).

2.1.4. Behavioral procedure

Rats were assigned to the following groups: saline ($n = 16$), substance P: 5 μg/kg ($n = 12$), 50 μg/kg

($n = 16$), 250 μg/kg ($n = 12$) and 500 μg/kg ($n = 8$). Each rat received an i.p. injection and was then placed in the center of the plus-maze, facing one of the enclosed arms. The animals were observed for 5 min, during which the number of entries into and time spent on the open and enclosed arms of the elevated plus-maze were measured (arm entry defined as all four paws into an arm). Furthermore, in order to examine the 'anxiolytic profile' of the treatment, the frequency and duration of scanning (protruding the head over the edge of an open arm and fanning with the vibrissae in any direction), risk assessment (protruding from an enclosed arm with the forepaws and head only) and end-activity (amount of time spent at the end of an open arm) were determined post-hoc for the 5-min experimental session for rats injected with the different doses of substance P or with vehicle. Typically, scanning and end-activity are decreased by anxiogenic drugs but are increased by anxiolytics; risk assessment is typically decreased by anxiolytic drugs (Cruz et al., 1994; Hasenöhrl et al., 1996).

2.1.5. Statistical analysis

The data were analyzed by the Kruskal–Wallis test. Whenever the Kruskal–Wallis test was significant, further comparisons between vehicle- and drug-treatment groups were performed using the Mann–Whitney *U*-test (two-tailed). The level of significance adopted was $\alpha = 0.05$.

2.2. Experiment 2: Test for anxiolytic-like effects of substance P in the nucleus basalis magnocellularis region

2.2.1. Animals

Male Wistar rats ($n = 104$; TVA, University of Düsseldorf) weighing 250–350 g were used for this experiment. Rats were housed two per cage under standard laboratory conditions as described in Experiment 1.

2.2.2. Surgery and histology

Animals were food-deprived for 24 h prior to surgery. They were anesthetized with a combination of ketamine and xylazine (1–1.5 ml/kg; i.p.) and secured in a stereotaxic frame (David Kopf Instruments). Rats were unilaterally implanted with a chronic guide cannula (22 gauge; Hamilton Products) aimed at the nucleus basalis magnocellularis (bregma coordinates: −1.3 posterior; (±2.8 lateral; 7.0 mm depth; Paxinos and Watson, 1986) and positioned 0.5 mm above the final injection site. The cannula was secured with dental acrylic, anchored with stainless-steel screws, and protected from blockage with a stainless steel stylet, which extended 0.1 mm beyond the tip. Post-operatively the animals were allowed to recover for at least 1 week before initiation of behavioral testing. During this interval the rats were handled and weighed daily. At the beginning of the behavioral tests, all animals had recovered to at least 90% of their preoperative body weight. At the end of testing the animals were deeply anesthetized with Nembutal (3 ml/kg; i.p.) and received an intrabasalis injection of an aqueous thionine solution for histological verification of the injection site. Following injection, rats were decapitated and the brains were stored in a 30% formalin-sucrose solution for at least 1 week. Histological verification of the injection site was subsequently made on 50-μm coronal sections with the aid of the rat brain atlas of Paxinos and Watson (1986). The injection sites, as revealed by injection of thionine, were located mainly in or near the intended region of the nucleus basalis magnocellularis. Nevertheless, with regard to the histological findings and the known spread of intracerebral injections (Myers, 1966), it is not possible to rule out that the injections made into the nucleus basalis diffused into neighboring structures, namely, into globus pallidus, internal capsule or entopeduncular nucleus.

2.2.3. Peptides and injection procedure

Peptides were purchased from Peninsula Labs (USA). Substance P and Met-11-Arg amide, which has the inverse sequence of substance P (Met–Leu–Gly–Phe–Phe–Gln–Gln–Pro–Lys–Pro–Arg–NH_2; molecular weight 1347.80), were dissolved and diluted to the desired concentration with phosphate-buffered saline (PBS; pH 7.4) shortly before utilization. The doses of substance P and substance P-inverse used were 0.1 (0.074), 1 (0.74) and 100 (74) ng (pmol); rats of the control group received PBS. Intracerebral injections were made into hand-restrained rats in a volume of 0.5 μl administered over 30 s with a 28-gauge Hamilton injection cannula inserted to a depth of 0.5 mm below the tip of the guide cannula. The hemisphere into which an injection was made was balanced within the treatment groups to control for possible lateralization effects. Upon completion of the injection, the injection cannula was left in place for an additional 30 s to ensure sufficient diffusion and to avoid withdrawal of fluid during removal of the cannula. After withdrawal of the injection cannula, another 0.5 μl of the solution was ejected to check for possible clogging.

2.2.4. Apparatus

Two different tests for anxiety were used: the elevated plus-maze (see Experiment 1) and the social interaction test. The social interaction test arena was a gray PVC box (60×60 cm^2) with 35-cm high walls and was lit by bright light (307.2 lux) to maximize the sensitivity of the paradigm to anxiolytic drug effects (for details see File, 1993). The temperature during behavioral testing was 19 to 20.5°C. Wide-spectrum masking noise (68 dB) was provided by a noise generator. The behavior of the animals throughout the experiments was recorded by a video system. The video tapes were analyzed post-hoc by a semi-automated analysis system (Chromotrack, San Diego Instruments). After each trial the apparatus was cleaned with water containing 0.1% acetic acid. All behavioral recordings were carried out with the observer unaware of the treatment of the rats.

2.2.5. Behavioral procedure

The animals were allocated to test pairs on the basis of weight. Both animals of a pair always received the same drug treatment and the rats were assigned to the following treatment groups: PBS ($n = 22$), substance P: 0.1 ng ($n = 16$), 1 ng ($n = 24$), 100 ng ($n = 18$) or substance P-inverse: 0.1 ng ($n = 8$), 1 ng ($n = 8$), 100 ng ($n = 8$). The effects of substance P and substance P-inverse on behavior in the elevated plus-maze and social interaction test were investigated by combining the two paradigms as follows: rats of a pair received an intrabasalis injection with a given drug at 2-min intervals and were then tested, in succession, on the plus-maze for 5 min. Thereafter, both rats were placed in the center of the social interaction box and their behavior was observed for a 4.5-min trial. During the 5-min test session in the elevated plus-maze the following measures were assessed: number of entries into and time spent on the open and enclosed arms as well as duration and frequency of scanning, risk assessment and end-excursion (for description see Experiment 1). Furthermore, from the distance traveled and time spent on the open and enclosed arms, the speed at which the rat moved in these areas of the maze was determined (cm/s). In the social interaction test the frequency and duration of the following behaviors were scored as active social interaction during the 4.5-min trial: sniffing, following and walking around the partner, grooming, climbing over and crawling under partner (for details see Gardner and Guy, 1984). Motor activity was determined by counting the number of walks/runs of approximately one body length for each rat of a pair.

2.2.6. Statistical analysis

The Mann–Whitney *U*-test (two-tailed) was used to test for between-group differences (drug vs. vehicle groups). The level of significance adopted was $\alpha = 0.05$ and adjusted for six tests to $\alpha^* = 0.05/6 = 0.0084$, according to the Bonferroni procedure for multiple comparisons (for details see Krauth, 1988).

3. Results

3.1. Experiment 1: Effect of systemic injection of substance P on exploratory activity in the elevated plus-maze

The Kruskal–Wallis test indicated a significant effect of the substance P treatment upon the time spent on the open arms of the maze ($\chi^2 = 15.72$, $df = 4$, $P < 0.01$). Post-hoc analysis showed that substance P at 50 μg/kg increased the time spent on the open arms ($P = 0.016$), whereas at 500 μg/kg a decrease was observed ($P = 0.032$; Fig. 1).

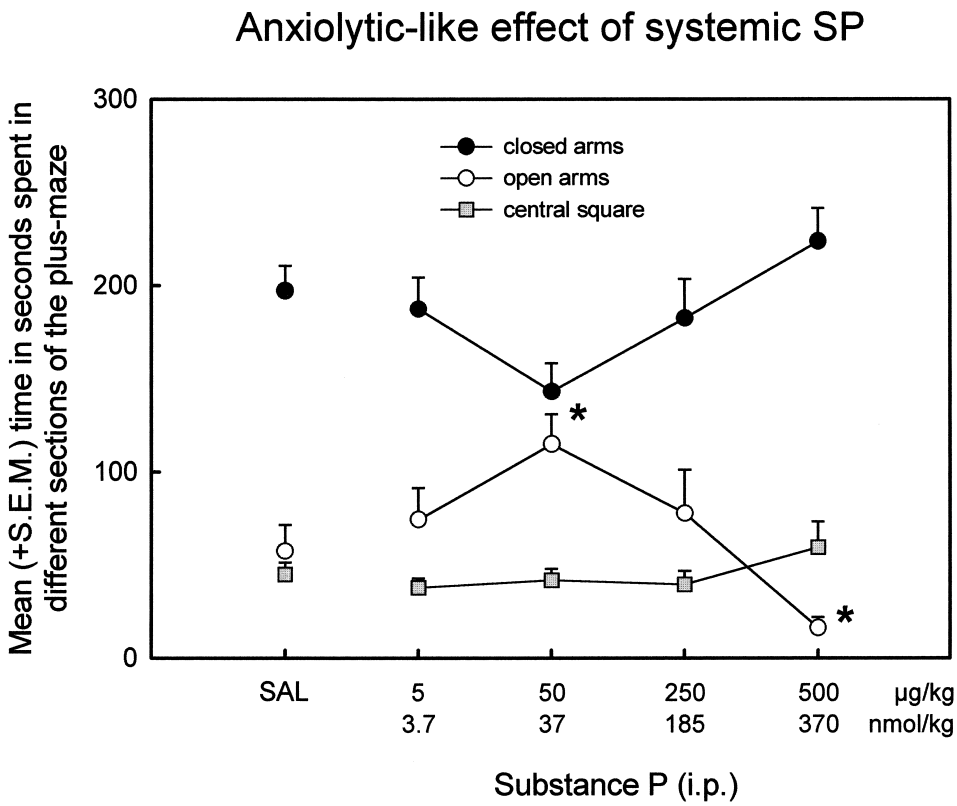

Fig. 1. Mean (+S.E.M.) time in seconds spent in the closed arms, open arms and central square of the elevated plus-maze. After peripheral (i.p.) injection of four different doses of substance P (5 to 500 μg/kg) or vehicle (SAL; 0.5 ml/kg), rats were tested on the elevated plus-maze for 5 min. * $P < 0.05$ vs. corresponding value of SAL-controls.

Table 1
Entries into open and closed arms, frequency and duration of scanning, risk assessment and end-activity for rats injected i.p. with different doses of substance P (SP) or vehicle (SAL) during the 5-min test period in the elevated plus-maze

	SAL	SP			
		5 μg/kg	50 μg/kg	250 μg/kg	500 μg/kg
Open arm entry (*f*)	2.50 ± 0.39	3.42 ± 0.53	3.69 ± 0.29[a]	2.67 ± 0.53	1.38 ± 0.35
Closed arm entry (*f*)	3.88 ± 0.35	4.25 ± 0.36	4.31 ± 0.32	3.58 ± 0.43	2.63 ± 0.35
Scanning (*f*)	3.33 ± 0.96	3.42 ± 0.98	6.13 ± 1.09	3.83 ± 0.96	0.88 ± 0.45[a]
Scanning (*t*)	18.67 ± 5.51	24.75 ± 8.26	52.18 ± 9.75[a]	35.92 ± 15.10	3.13 ± 1.65[a]
Risk-assessment (*f*)	7.80 ± 0.56	7.58 ± 0.48	6.50 ± 0.59	6.08 ± 0.70	5.63 ± 0.90
Risk-assessment (*t*)	72.27 ± 7.45	67.17 ± 11.11	70.75 ± 8.26	89.58 ± 19.52	85.13 ± 21.13
End-activity (*f*)	2.20 ± 0.70	3.17 ± 0.95	4.75 ± 0.89[a]	2.33 ± 0.59	0.63 ± 0.35
End-activity (*t*)	17.07 ± 5.05	28.08 ± 10.91	47.00 ± 8.26[a]	42.17 ± 18.96	3.25 ± 1.97

Values are means ± S.E.M.
(*f*) Frequency.
(*t*) Time in s.
[a] $P < 0.05$, SP vs. SAL.

The treatment with substance P did not significantly influence the amount of time spent in the enclosed arms ($\chi^2 = 9.24$, $df = 4$, $P > 0.05$) and in the central square ($\chi^2 = 1.19$, $df = 4$, $P > 0.05$). There was a significant main effect of the treatment on the number of entries into the open arms ($\chi^2 = 12.77$, $df = 4$, $P < 0.05$) and post-hoc comparisons indicated an increase in the number of open arm entries for rats treated with 50 μg/kg substance P ($P = 0.037$; Table 1). The treatment with substance P did not significantly influence the number of entries into the enclosed arms ($\chi^2 = 8.77$, $df = 4$, $P > 0.05$). A significant main effect of the treatment was observed on the frequency (*f*) and duration (*t*) of open arm scanning ((*f*): $\chi^2 = 11.15$, $df = 4$, $P < 0.05$; (*t*): $\chi^2 = 14.36$, $df = 4$,

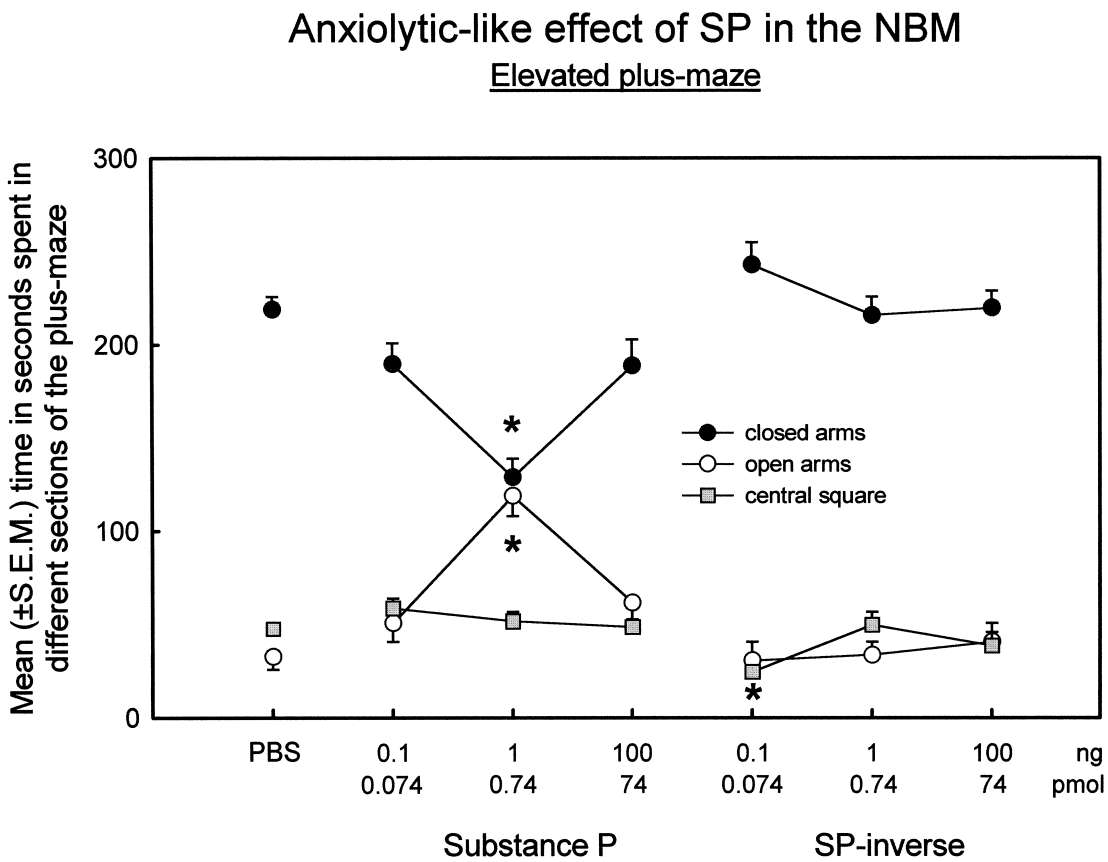

Fig. 2. Mean (±S.E.M.) time in seconds spent in the closed arms, open arms and central square of the elevated plus-maze. After unilateral microinjection of substance P and substance P-inverse (each at 0.1, 1 and 100 ng) or vehicle (PBS; 0.5 μl) into the region of the nucleus basalis magnocellularis, rats were tested on the plus-maze for 5 min. * $P < 0.05$ vs. corresponding value of PBS-controls.

Table 2
Number of entries into and speed on the open and closed arms of the elevated plus-maze

	PBS	SP			SPinv		
		0.1 ng	1 ng	100 ng	0.1 ng	1 ng	100 ng
Open arm entry (f)	3.95 ± 0.44	5.25 ± 0.63	9.50 ± 0.51[a]	5.39 ± 0.92	5.63 ± 0.92	5.50 ± 0.64	4.25 ± 0.80
Open arm speed (cm/s)	2.23 ± 0.19	2.00 ± 0.18	2.38 ± 0.15	2.12 ± 0.21	5.63 ± 1.15[a]	2.50 ± 0.27	3.75 ± 0.73
Closed arm entry (f)	10.55 ± 1.04	11.13 ± 1.52	9.67 ± 0.67	9.50 ± 0.95	15.00 ± 1.60	18.38 ± 0.84[a]	14.75 ± 0.99
Closed arm speed (cm/s)	1.91 ± 0.09	2.13 ± 0.18	2.84 ± 0.33	2.00 ± 0.18	2.63 ± 0.18[a]	2.88 ± 0.25[a]	2.38 ± 0.18

After a single injection of different doses of substance P (SP), the inverse sequence of SP (SPinv) or vehicle (PBS) into the region of the nucleus basalis magnocellularis, the rats were tested on the plus-maze for 5 min.
Values are means ± S.E.M.
(f) Frequency.
[a] $P < 0.05$, SP vs. PBS.

$P < 0.01$) and end-activity ((f): $\chi^2 = 11.49$, $df = 4$, $P < 0.05$; (t): $\chi^2 = 12.65$, $df = 4$, $P < 0.05$). Post-hoc comparisons showed an increase in scanning (t: $P = 0.013$) and end-activity (f: $P = 0.022$; t: $P = 0.013$) for rats treated with 50 μg/kg substance P (Table 1). Rats of the substance P 500 μg/kg group showed less scanning (f: $P = 0.037$; t: $P = 0.014$) and less end-activity even though the respective P-values were not statistically significant (f: $P = 0.14$; t: $P = 0.09$). Frequency and duration of risk-assessment were not influenced by the treatment ((f): $\chi^2 = 5.67$, $df = 4$, $P > 0.05$; (t): $\chi^2 = 0.94$, $df = 4$, $P > 0.05$).

3.2. Experiment 2: Test for anxiolytic-like effects of substance P in the nucleus basalis magnocellularis region

3.2.1. Elevated plus-maze

The effects of the different doses of substance P and substance P-inverse on the time spent in the enclosed arms, open arms and central square of the elevated plus-maze are depicted in Fig. 2. Rats treated with 1 ng substance P showed a significant increase in time spent on the open arms ($P < 0.001$) and a significant decrease in time spent on the enclosed arms of the maze ($P < 0.001$); they did not significantly differ from vehicle controls in the time spent in the central square of the plus-maze ($P = 0.758$; $\alpha^* = 0.0084$). Furthermore, the treatment with 1 ng substance P did not influence the number of entries into the enclosed arms ($P = 0.886$) but significantly increased the number of entries into the open arms ($P < 0.001$; Table 2); the mean speed on the enclosed arms was somewhat increased, but the respective P-value was not statistically significant ($P = 0.026$; $\alpha^* = 0.0084$). Rats treated with 100 ng substance P showed an increase in time spent on the open arms, but the respective P-value was not statistically significant ($P = 0.039$; $\alpha^* = 0.0084$). The treatment with substance P at 0.1 ng did not significantly influence the behavioral pattern of the animals. Rats which were treated with the different doses of the inverse sequence of the substance P molecule did not significantly differ from vehicle-injected controls in the time spent in the open and enclosed arms of the elevated plus-maze (Fig. 2) or in the number of entries into open arms (respective P-values > 0.05; Table 2). Rats treated with 0.1 ng substance P-inverse showed a significant decrease in time spent in the central square of the elevated plus-maze ($P < 0.001$; $\alpha^* = 0.0084$). Furthermore, substance P-inverse significantly increased the number of closed arm entries at 1 ng ($P =$

Table 3
Frequency and duration of scanning, risk-assessment and end-activity

	PBS	SP			SPinv		
		0.1 ng	1 ng	100 ng	0.1 ng	1 ng	100 ng
Scanning (f)	7.04 ± 0.92	7.88 ± 1.16	15.42 ± 1.06[a]	8.95 ± 1.48	9.38 ± 2.10	9.50 ± 1.44	10.00 ± 0.93
Scanning (t)	22.45 ± 3.73	33.31 ± 7.11	64.96 ± 7.92[a]	39.00 ± 8.08	13.75 ± 3.43	18.13 ± 3.33	18.25 ± 4.09
Risk-assessment (f)	16.68 ± 1.73	19.69 ± 3.19	20.00 ± 1.88	15.78 ± 2.44	24.25 ± 3.16	37.00 ± 2.65[a]	22.25 ± 1.60
Risk-assessment (t)	57.54 ± 5.12	52.13 ± 5.36	41.75 ± 3.93	54.50 ± 6.33	32.63 ± 4.56	47.00 ± 5.32	47.13 ± 9.39
End-activity (f)	1.59 ± 0.42	2.81 ± 0.76	6.13 ± 0.80[a]	3.23 ± 0.74	2.50 ± 0.89	1.75 ± 0.59	2.25 ± 0.56
End-activity (t)	15.64 ± 4.66	28.50 ± 8.12	69.38 ± 11.10[a]	32.12 ± 8.72	13.88 ± 6.58	10.00 ± 4.37	18.25 ± 6.43

After a single injection of different doses of substance P (SP), the inverse sequence of SP (SPinv) or vehicle (PBS) into the region of the nucleus basalis magnocellularis, the rats were tested on the plus-maze for 5 min.
Values are means ± S.E.M.
(f) Frequency.
(t) Time in s.
[a] $P < 0.05$ SP vs. PBS.

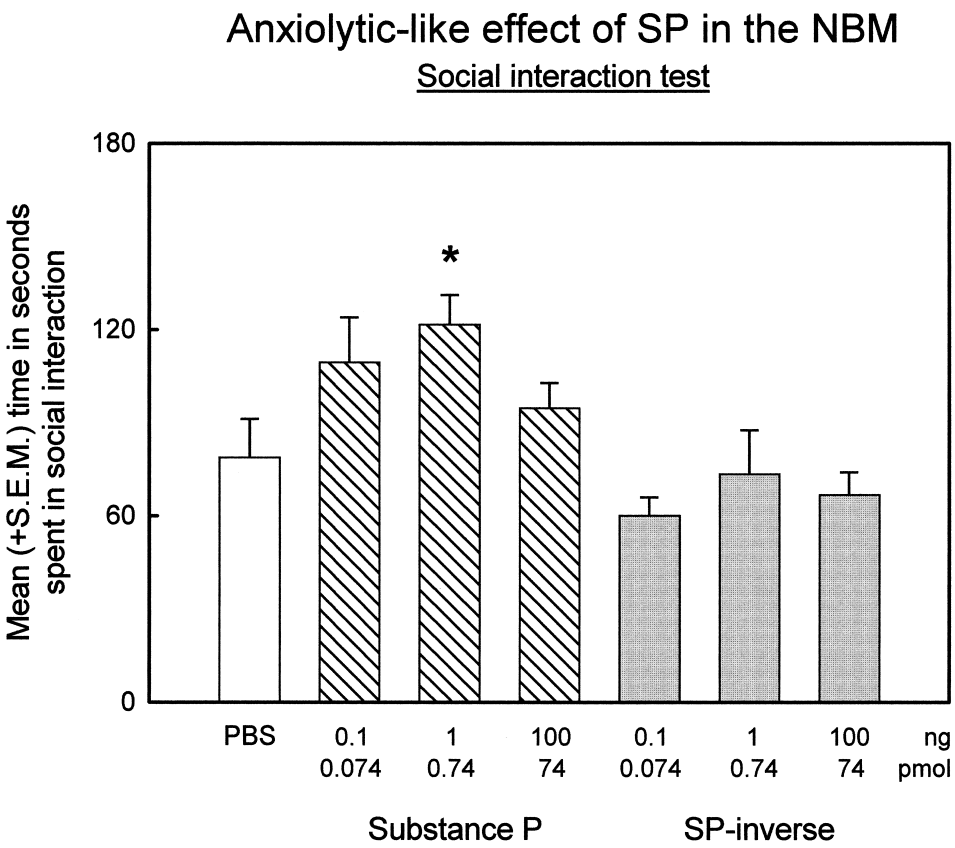

Fig. 3. Mean (+S.E.M.) time in seconds spent in social interaction for pairs of rats after unilateral injection of substance P and substance P-inverse (each at 0.1, 1 and 100 ng) or vehicle (PBS; 0.5 μl) into the region of the nucleus basalis magnocellularis. The time spent in social interaction was scored during the 4.5-min test session. $* P < 0.05$ vs. PBS-controls.

0.001), the speed of the animals in the enclosed arms at 0.1 and 1 ng (Ps ≤ 0.001), and the speed in open arms at the 0.1 ng dose ($P < 0.001$; Table 2). Table 3 depicts frequency (f) and duration (t) of scanning, risk assessment and end-activity for rats treated with different doses of substance P or the inverse sequence of the substance P molecule. Rats treated with 1 ng substance P showed significantly more open arm scanning ((f) and (t): Ps $<$ 0.001) and end-excursion ((f) and (t): Ps < 0.001), whereas risk assessment was not significantly affected ((f): $P = 0.202$, (t): $P = 0.025$; $\alpha^* = 0.0084$). The behavioral pattern of rats injected with the higher or the lower doses of substance P did not differ from that of the controls (P-values > 0.05). None of the doses of substance P-inverse significantly influenced the frequency or duration of scanning or end-activity (P values > 0.05). However, rats treated with 1 ng substance P-inverse showed a significant increase in the frequency of risk assessment ($P < 0.001$), whereas the duration of this behavior was not influenced ($P = 0.280$). Rats treated with 0.1 ng substance P-inverse showed an increase in frequency and a decrease in duration of risk assessment, but the respective P-values were not statistically significant ((f): $P = 0.054$, (t): $P = 0.018$; $\alpha^* = 0.0084$).

3.2.2. Social interaction test

Fig. 3 depicts the time spent in social interaction by pairs of rats in each treatment group. Rats administered 1 ng substance P showed a significant increase in time spent in social interaction ($P = 0.006$). Rats treated with 0.1 and 100 ng substance P also showed more social interaction, but the respective P-values missed statistical significance (0.1 ng substance P: $P = 0.022$ and 100 ng substance P: $P = 0.073$; $\alpha^* = 0.0084$). The treatment with substance P-inverse did not significantly influence time spent in social interaction; only rats treated with 0.1 ng substance P-inverse showed a tendency to reduce social interactions ($P = 0.076$; $\alpha^* = 0.0084$). Rats of the different treatment groups did not significantly differ from each other in the frequency of social interaction behavior and in the measure of general motor activity (data not shown).

4. Discussion

The results of the first experiment show that substance P, injected peripherally, is active in the elevated plus-maze test of fear and anxiety. Substance P at 50 μg/kg in-

creased the number of entries into and time spent on the open arms of the maze. Furthermore, rats in the substance P 50 μg/kg group showed more excursions into the end of the open arms as well as increased scanning over the edge of the open arms. These effects indicate a reduction in fear, and hence, an anxiolytic-like effect of the neurokinin. Conversely, tachykinin NK_1 receptor antagonists have recently been found to produce anxiogenic-like effects in the mouse light–dark box following peripheral injection (Saria et al., 1993; Zernig et al., 1992, 1993), providing additional evidence for the involvement of the substance P system in fear and anxiety-related behaviors. However, substance P receptor antagonists were also found to interfere with locomotor activity, thereby compromising the supposed anxiogenic effect of this treatment. In contrast, systemic injection of substance P (5 to 500 μg/kg) did not influence closed-arm entry scores, which are often used to control for possible treatment effects on motor activity. Thus, the increase in time spent on the open arms of the maze following administration of 50 μg/kg substance P cannot easily be interpreted in terms of a change in motor activity (for discussion see Dawson et al., 1995). Substance P did not influence the rats' behavior on the elevated plus-maze at doses of 5 and 250 μg/kg, whereas at 500 μg/kg, substance P reduced the time spent on the open arms of the maze and decreased scanning over the edge of the open arms, indicative of anxiogenic effects of the neurokinin in the higher dose range.

The mechanisms by which substance P influenced the exploratory activity on the elevated plus-maze following peripheral injection might have originated in- or outside of the brain. In the periphery, substance P is known to be involved in several functions such as nociception, cardiovascular and gastrointestinal control (Otsuka and Yoshioka, 1993), and it is possible that changes in such functions might be related to the effects of substance P on anxiety-parameters. Although such indirect peripheral effects cannot be ruled out, the results of the second experiment strongly suggest a central site of substance P action. Following injection into the nucleus basalis magnocellularis, the neurokinin was also found to exert anti-anxiety effects. Rats administered 1 ng substance P spent more time on the open arms of the elevated plus-maze and showed an increase in time spent in social interaction, whereas the lower and the higher doses were less active. In both paradigms, the measures of anxiety were not confounded by changes in locomotor activity. Since comparable anxiolytic effects were observed after both systemic as well as intrabasalis injection and substance P can pass the blood–brain barrier (Banks and Kastin, 1985), it is likely that systemically applied substance P directly affected central mechanisms of anxiety. However, in contrast to systemic drug administration, intrabasalis injection of substance P failed to elicit anxiogenic effects at high dosage. Thus, it is feasible that the anxiogenic-like action obtained after systemic substance P was secondary to adverse peripheral side-effects and not primarily related to a central site of action. However, recent studies have shown that substance P, in doses similar to those used for nucleus basalis injection, markedly increased anxiety-related behaviors upon injection into the ventricles (Teixeira et al., 1996) or into the periaqueductal gray (Aguiar and Brandao, 1996). This suggests that the neurokinin can have anxiolytic as well as anxiogenic-like effects, depending on the site of injection in the brain.

Other results support the contention that the region of the nucleus basalis magnocellularis (ventral pallidum/substantia innominata complex) is involved in the neural control of fear and anxiety. The nucleus basalis contains a high density of benzodiazepine binding sites (Yezuita et al., 1988) and there is pharmacological evidence that the amnestic as well as anxiolytic effects of benzodiazepines are mediated through benzodiazepine receptors situated on basal forebrain cholinergic cells (Berntson et al., 1997; Sarter and Schneider, 1988). Furthermore, based on studies performed with lesions of the basal forebrain, the region of the nucleus basalis magnocellularis has been implicated to play a role in the expression of (conditioned) fear (Popovic et al., 1996; Stoehr and Wenk, 1995) and neophobia (Hernadi et al., 1997).

The present data on the anxiolytic-like effects of substance P in the region of the nucleus basalis magnocellularis provide behavioral evidence for a substance P-cholinergic interaction of this area of the brain. The nucleus basalis receives a substantial substance P input from the nucleus accumbens (Lu et al., 1998; Napier et al., 1995) and contains moderate to high levels of substance P receptive sites (Gerfen, 1991; Shults et al., 1984). Electrophysiological studies have demonstrated that substance P can increase the excitability of cultures of nucleus basalis cholinergic cells (Yamaguchi et al., 1990). Previous studies have shown that injection of substance P into the region of the nucleus basalis magnocellularis can facilitate inhibitory avoidance learning and can serve as a positive reinforcer in a place preference task (Huston and Hasenöhrl, 1995). Furthermore, experiments performed with in vivo microdialysis in anesthetized rats revealed that intrabasalis substance P can increase extracellular levels of acetylcholine in frontal cortex (De Souza Silva et al., 1997). This indicates that the anxiolytic, promnestic as well as reinforcing effects of the neurokinin could be mediated, in part, by means of a cholinergic mechanism. Congruent with this hypothesis, activation of the central acetylcholine system by agonists acting at nicotinic receptive sites has been found to result in both anxiolytic as well as memory-facilitating effects (for review see Decker et al., 1995). However, since the nucleus basalis magnocellularis also contains non-cholinergic cells, an involvement of other neurotransmitters/hormones cannot be ruled out. In this context, it is interesting that the reinforcing effects of substance P in the nucleus basalis are related to changes in dopaminergic and possibly serotonergic mechanisms (Boix

et al., 1995), which are also thought to play a significant role in the neural control of learning and anxiety-related processes (Handley et al., 1993).

A further complexity pertains to the binding site(s) that mediate(s) the anxiolytic-like effects of substance P after systemic as well as intrabasalis injection. The inverse sequence of the substance P molecule failed to induce anxiolytic effects in the elevated plus-maze and social interaction test. This suggests, for one, that the anti-anxiety effects of substance P were specific with regard to its amino acid sequence and, secondly, that the behavioral effects of substance P were probably mediated via a specific peptide/receptor interaction and were not the result of, e.g., exposing brain tissue to large amino acid molecules. In line with this suggestion we recently found that WIN51,708, a highly selective tachykinin NK_1 receptor antagonist, can block the anxiolytic as well as reinforcing effects of intrabasalis SP injection (Hasenöhrl et al., in preparation). It is important to note that injection of the inverse substance P sequence was found to increase locomotor activity, which could have masked possible anxiolytic-like effects of the compound. However, this explanation seems unlikely in the light of recent findings (Dawson et al., 1995), showing that psychostimulant compounds at doses that increase locomotor activity constantly produce (false positive) anxiolytic-like effects in the elevated plus-maze (increased open arm entries and time).

A close relationship between anxiety and memory processes has been pointed out. Brain structures thought to be involved in anxiety and in the modulation of memory are extensively overlapped (Tomaz et al., 1992), while compounds that reduce anxiety, like the benzodiazepines, also impair memory (Izquierdo et al., 1990). The present findings provide evidence that substance P can have benzodiazepine-like anxiolytic effects, but, unlike the benzodiazepines, substance P is known to exert hypermnestic, rather than hypomnestic effects, when injected peripherally or into the region of the nucleus basalis magnocellularis (Huston and Hasenöhrl, 1995). Thus, our findings with substance P are in close agreement with those obtained with other recently developed anxiolytics, namely $5\text{-}HT_3$ receptor antagonists, which also suggest a dissociation between anxiolytic and memory-disrupting effects (De Souza Silva et al., 1993).

The mode of action of benzodiazepines is commonly related to their ability to reduce fear. An alternative view is that benzodiazepines, as well as other anxiolytics, have rewarding effects (Spyraki and Fibiger, 1988), raising the possibility that these compounds counteract anxiety by increasing the 'attractiveness' of a situation rather than by decreasing its fearful aspects (for discussion see File, 1986; Widgiz and Beck, 1990). Substance P has been shown to exert reinforcing effects using self-administration (Krappmann et al., 1994) or conditioned place preference (Huston and Hasenöhrl, 1995) as an index for reinforcement. With regard to the reward-enhancement hypothesis of benzodiazepine action, it is feasible that the increase in open-arm activity and social interaction following administration of substance P reflects a peptide-induced amplification of the reward value of exploration and social behaviors rather than a reduction of fear. Previous studies revealed that both the N- and the C-terminus of substance P are involved in mechanisms of learning and reinforcement, however in a different way. Whereas the N-terminal moiety seems to encode for its memory-promoting effects, the C-terminus appears to mediate the reinforcing properties of the substance P molecule (Huston et al., 1993). Thus, with regard to this structure-activity relationship of substance P, testing the effects of N- and C-terminal substance P fragments on anxiety-related behaviors would permit analysis of whether the anti-anxiety effects of the whole substance P molecule are related to its reinforcing or mnemonic action or to both.

In summary, the present results show that substance P can have anxiolytic-like properties it addition to its known hypermnestic and reinforcing effects when applied systemically or into the region of the nucleus basalis magnocellularis. Apart from providing further empirical support for the dissociation of neural subsystems, mediating hypomnestic and anxiolytic effects of drugs, our results could also have implications for the search for novel anxiolytics ('peptoids') devoid of memory disruptive side-effects.

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