

# Substance P in the medial amygdala: Emotional stress-sensitive release and modulation of anxiety-related behavior in rats

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Increasing evidence implicates the substance P (SP)/neurokinin-1 receptor system in anxiety and depression. However, it is not known whether emotional stimulation alters endogenous extracellular SP levels in brain areas important for processing of anxiety and mood, a prerequisite for a contribution of this neuropeptide system in modulating these behaviors. Therefore, we examined in rats whether the release of SP is sensitive to emotional stressors in distinct subregions of the amygdala, a key area in processing of emotions. By using *in vivo* micropush-pull superfusion and microdialysis techniques, we found a pronounced and long-lasting increase (150%) in SP release in the medial nucleus of the amygdala (MeA), but not in the central nucleus of the amygdala, in response to immobilization stress. SP release in the MeA was transiently enhanced (40%) in response to elevated platform exposure, which is regarded as a mild emotional stressor. Immobilization enhanced the anxiety-related behavior evaluated in the subsequently performed elevated plus-maze test. Bilateral microinjections of the neurokinin-1 receptor antagonist [2-cyclopropoxy-5-(5-(trifluoromethyl)tetrazol-1-yl)benzyl]-(2-phenylpiperidin-3-yl)amine into the MeA blocked the stress-induced anxiogenic-like effect, supporting a functional significance of enhanced SP release. In unstressed rats, the neurokinin-1 receptor antagonist displayed no significant anxiolytic effect but reversed the anxiogenic effect of SP microinjected into the MeA. Our findings identify the MeA as a critical brain area for the involvement of SP transmission in anxiety responses and as a putative site of action for the recently discovered therapeutic effects of SP antagonists in the treatment of stress-related disorders.

The neuropeptide substance P (SP) and its preferred neurokinin-1 (NK<sub>1</sub>) receptor have been proposed as possible targets for new antidepressant and anxiolytic therapies. Several preclinical studies have demonstrated a range of anxiety-related behaviors and defensive cardiovascular changes in response to central administration of SP agonists (1–6). Conversely, NK<sub>1</sub> receptor antagonists have been shown to produce anxiolytic-like effects after intracerebroventricular (4) or systemic administration (5, 7, 8). In addition, mice with selective deletion of the gene encoding the NK<sub>1</sub> receptor or the peptide itself also showed decreased anxiety-related behaviors (9–11). Thus, these findings suggest that SP acting as neurotransmitter/neuromodulator (for review, see ref. 12) may be of relevance in the regulation of emotional states including anxiety-related behavior. Confirmation of this proposal was obtained by using a highly selective NK<sub>1</sub> receptor antagonist MK-0869, which relieved the symptoms of depression and anxiety in patients with major depressive disorder and a significant degree of anxiety (5). Although it has been shown that emotional and physical stressors modulate SP tissue levels or SP immunoreactivity in brain areas that are implicated in fear and anxiety (13–17), a change in extracellular SP levels, which is a direct and dynamic marker of SP neurotransmission, cannot be reliably predicted from these studies. Hence, at present it is not clear whether emotional stimuli actually do alter the *in vivo* release of SP, which is a prerequisite for a contribution

of this neuropeptide system in modulating emotional states. Since stressful life experiences are thought to play a role in precipitating episodes of neuropsychiatric disorders including anxiety and depression (18, 19), and laboratory stressors can produce behavioral and physiological changes resembling these mental disorders (20), it is of particular interest to examine the effect of emotional stressors on SP neurotransmission.

The amygdala is critical for the processing of emotions including fear and anxiety (21, 22), and SP-containing neurons as well as NK<sub>1</sub> receptors are highly expressed in this brain area (23–26). The present studies aimed to establish whether endogenous SP is released in the amygdala after immobilization, a severe emotional stressor (27). Furthermore, we investigated whether a mild stressful stimulus would also be sufficient to trigger SP release. Although various subregions of the amygdala (e.g., central, basolateral, and medial) seem to play a role in emotional behavior (21, 22, 28), it is the medial nucleus of the amygdala (MeA) particularly that is activated by emotional stress as estimated using Fos expression as a marker (29). Because of this and because the MeA contains the highest amounts of SP within the amygdala (24), we chose this area together with one additional subregion (the central nucleus of the amygdala, CeA) to reveal whether SP release is sensitive to emotional stress. In addition, we investigated whether local activation or blockade of SP neurotransmission in the amygdala would modulate anxiety-related behavior in the elevated plus-maze (EPM) test.

## Materials and Methods

**Animals.** Experiments were carried out on adult male Sprague-Dawley rats (250–350 g). Before use, the animals were housed in groups of four to six under controlled laboratory conditions (12:12 h light/dark cycle with lights on at 0700 hours 21 ± 1°C, 60% humidity, pelleted food and water ad libitum) for at least 3 weeks after delivery from the supplier. The experimental studies described here were designed to minimize animal suffering and the number of animals used, and were approved by the Bundesministerium für Wissenschaft und Verkehr, Kommission für Tierversuchsangelegenheiten, Austria.

**Surgery.** A push-pull guide cannula (outer diameter, 0.80 mm; inner diameter, 0.50 mm) or a microdialysis probe was stereotaxically implanted in anesthetized rats (40 mg/kg *i.p.* sodium pentobarbital and 50 mg/kg *i.p.* ketamine). According to the stereotaxic atlas of Paxinos and Watson (30), the tip of the guide cannula was positioned 2 mm above: (*i*) the right MeA (*n* = 10; 2.8 mm caudal to bregma, 3.4 mm lateral to the midline, 7.0 mm

Abbreviations: SP, substance P; NK<sub>1</sub>, neurokinin-1; MeA, medial nucleus of the amygdala; CeA, central nucleus of the amygdala; EPM, elevated plus-maze; aCSF, artificial cerebrospinal fluid.

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below the surface of the skull) or (ii) the right CeA ( $n = 7$ ; 2.3 mm caudal to bregma, 4.2 mm lateral to the midline, 6.0 mm below the surface of the skull). The microdialysis probe was positioned with its U-shaped tip reaching the right MeA ( $n = 8$ ; 2.8 mm caudal to bregma, 3.4 mm lateral to the midline, 9.0 mm below the surface of the skull). For injection experiments, guide cannulae (outer diameter, 0.50 mm; inner diameter, 0.30 mm) were bilaterally implanted 2 mm above the MeA. In a separate ("extra-amygdala") group of animals, guide cannulae were bilaterally implanted  $\approx 2$  mm above the MeA into the internal capsule ( $n = 14$ ; 3.3 mm caudal to bregma, 3.4 mm lateral to the midline, 5.0 mm below the surface of the skull). The cannula or microdialysis probes were fixed to the skull with two stainless steel screws and dental cement.

**Experimental Protocol.** After surgery, rats were housed individually in transparent Plexiglas cages until testing. They were handled for 3 min twice daily to familiarize them with the experimental procedure and to minimize nonspecific stress responses during the experiments. At least 24 h before the experiment, animals were kept in the experimental room and allowed to habituate. Experiments were performed between 0900 and 1600 hours.

**Effects of Immobilization Stress on SP Release in the Amygdala.** Two days after surgery, the stylet of the guide cannula was replaced by a micropush-pull system (pull cannula: outer diameter 0.50 mm, inner diameter 0.30 mm; push cannula: outer diameter 0.190 mm, inner diameter 0.075 mm), which was 2 mm longer than the guide cannula, thus reaching the target region, and superfused with artificial cerebrospinal fluid (aCSF, pH 7.2; 140 mM NaCl/3.0 mM KCl/1.25 mM CaCl<sub>2</sub>/1.0 mM MgCl<sub>2</sub>/1.2 mM Na<sub>2</sub>HPO<sub>4</sub>/0.3 mM NaH<sub>2</sub>PO<sub>4</sub>/3.0 mM glucose/0.2% BSA/0.03% bacitracin) at a rate of 15  $\mu$ l/min. After an equilibration period of 80 min, 11 consecutive 20-min superfusates were collected directly into Eppendorf vials, which were stored at  $-80^{\circ}\text{C}$  until assay. The fifth superfusate was collected during the immobilization procedure. Rats were immobilized for 20 min by two felt strings and attached Velcro fasteners on a special device, which allows painless immobilization (31). After stress exposure, animals were superfused for a further 120 min. For the last superfusate aCSF containing 100 mM KCl was used as a positive control to elicit local depolarization to confirm that the assay sensitivity was adequate to detect the release of SP. Animals assigned to the control group remained undisturbed in their home cages.

**Effects of Elevated Platform Exposure on SP Release in the MeA.** In a separate group of animals, U-shaped microdialysis probes (length of exposed dialysis membrane, 1 mm; molecular cutoff of 18 kDa; Hemophan, Gambro Dialysatoren, Hechingen, Germany) were connected 2 days after surgery with a PE-20 polyethylene tubing to syringes mounted onto a microinfusion pump (CMA100, Carnegie, Stockholm) and perfused with aCSF at a rate of 6.5  $\mu$ l/min. After an equilibration period of 120 min, seven consecutive 30-min dialysates were collected directly into Eppendorf vials, which were stored at  $-80^{\circ}\text{C}$  until assay. At the beginning of the fourth dialysate, animals were placed on an elevated platform (circular, 24 cm in diameter, 70 cm elevated above the floor) for 15 min.

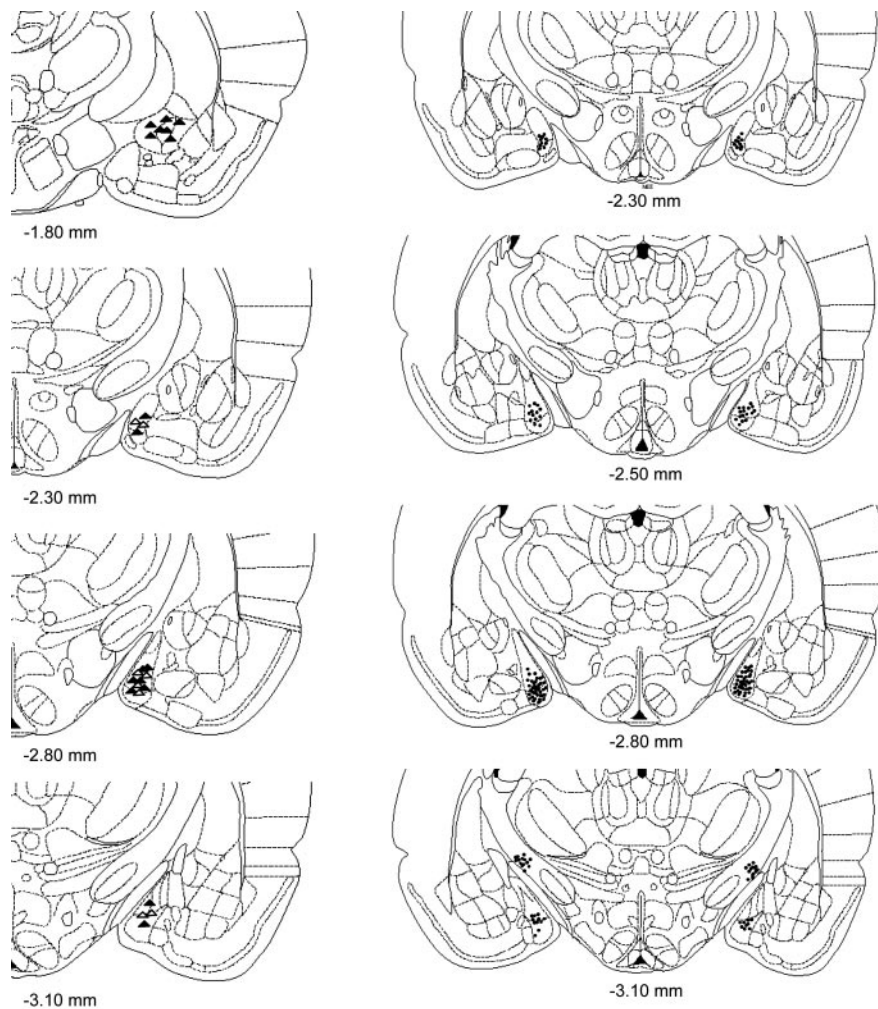
**Effects of Microinjections of SP and/or a NK<sub>1</sub> Receptor Antagonist into the MeA on Anxiety-Related Behavior.** In a separate group of animals, the stylet of the guide cannula was replaced 7 days after surgery by a microinjection cannula (outer diameter, 0.26 mm; inner diameter, 0.13 mm), which was 2 mm longer than the guide cannula, thus reaching the MeA. The injection cannula was connected to a Hamilton microsyringe by PE-20 polyethylene

tubing. Compound A, a selective antagonist with high affinity for the rat NK<sub>1</sub> receptor [2-cyclopropoxy-5-(5-(trifluoromethyl)tetrazol-1-yl)benzyl]-(2-phenylpiperidin-3-yl)amine, synthesized by the Department of Medicinal Chemistry, Merck Research Laboratories; IC<sub>50</sub> for displacement of [<sup>125</sup>I]substance P to rat cloned NK<sub>1</sub> receptors = 0.9 nM; G. Chicchi and K. L. Tsao, unpublished observations) or SP (Sigma) were dissolved in a small amount of distilled water and diluted to the desired concentration with aCSF. For drug administration, the prehandled animals were gently held, and a volume of 1  $\mu$ l per side was injected over a period of 30 s. The injection cannula remained in the guide cannula for an additional 30-s period after infusion. Different groups of prestressed or unstressed rats were injected with aCSF, Compound A (100 pmol, 1 nmol), or SP (0.1, 1, and 10 pmol) into the MeA. Prestressed animals were exposed to 20 min of immobilization (see above). aCSF or Compound A (100 pmol) were preinjected into the MeA 15 min before the injection of SP (1 pmol). In an "extra-amygdala" control group of animals, aCSF or SP (0.1, 1 pmol) was injected into the internal capsule. Five minutes after drug injection, animals were placed in the center of the EPM facing one of the enclosed arms. The behavior of the rats was analyzed during the 5-min testing period by using an automatic videotracking system (VideoMOT, TSE, Bad Homburg, Germany). The behavioral parameters scored were: (i) entries into open arms (ratio of open-arm entries to total number of entries into all arms); (ii) time spent on the open arms (ratio of time spent on open arms to total time spent on all arms); and (iii) overall activity (total number of entries into enclosed arms and total distance traveled). After plus-maze exposure, rats were returned to their home cages.

**Histology.** At the end of the experiment, the animals were killed by an overdose of pentobarbital, and their brains were removed. For histological verification of the localization of the push-pull, microdialysis-, or injection cannulae, brains were sectioned and 100  $\mu$ m coronal sections were stained with cresyl violet. The locations of MeA and CeA were determined on the basis of previous definitions of a brain atlas (30). Judgment of successful implantation of the distinct subregions of the amygdala was made before analyzing release and microinjection experiments.

**RIA.** The concentration of SP in push-pull superfusates and microdialysates was measured by a highly sensitive and selective RIA. In each assay, standard curves were constructed for SP by use of known amounts of synthetic SP. Standards and samples were preincubated with 100  $\mu$ l of diluted SP-antiserum RD2 (donated by S. Leeman, Boston University School of Medicine, Boston) for 48 h at 4°C as described (32). This antibody has been shown to display no cross-reactivity with other structurally related mammalian tachykinins (32). After addition of [<sup>125</sup>I]Bolton-Hunter-SP [6,000 cpm in 100  $\mu$ l; approximate specific activity 74 TBq/mmol = 2,000 Ci/mmol (1 Ci = 37 GBq); Amersham Pharmacia], all samples were further incubated for 48 h at 4°C. Antibody-bound radioligand was separated from unbound radioligand by addition of charcoal (1 ml) and centrifugation ( $2,056 \times g$  for 10 min at 4°C). The supernatant was decanted, and the bound radioactivity was counted in a gamma counter (CliniGamma, LKB, Turku, Finland). Separate samples either without standards or without antibodies were incubated simultaneously to measure maximal tracer binding and unspecific binding, respectively. The detection limit of the assay was 0.3 fmol per sample (1.5 fmol per ml).

**Statistics.** Experimental subjects were included in the statistical analysis only if the push-pull, microdialysis or microinjection cannulae were confirmed to be localized in the targeted brain area (Fig. 1). Results are presented as means  $\pm$  SEM. Statistical analysis was performed with a statistical software package



**Fig. 1.** Schematic drawings showing the reconstructed localizations of the push–pull, microdialysis, and microinjection cannulae. Filled and open triangles represent the tips of the push–pull cannulae and microdialysis membranes, respectively, in the CeA and MeA. Filled circles indicate the microinjection sites in the MeA and outside the amygdala in the internal capsule. The numbers on each section indicate the distance from bregma. The drawings of coronal sections were derived from the atlas of Paxinos and Watson (30).

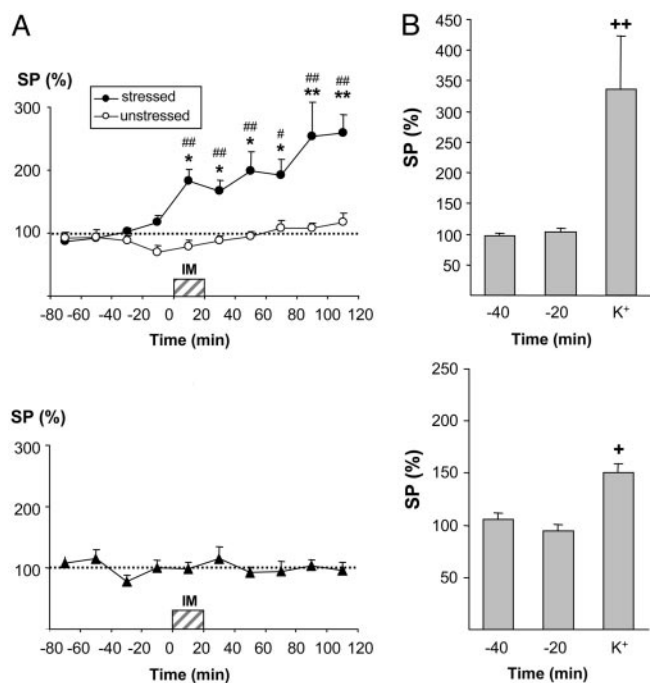
(GB-STAT 6.0, Dynamic Microsystems, Silver Spring, MD). The data obtained from push–pull superfusion and microdialysis experiments are expressed as a percentage of averaged baseline values. Release data were transformed by arc-tangent to fit into a Gaussian distribution. Statistical significance was determined by one-way or two-way (group X time) ANOVA for repeated measures followed by Newman–Keuls test or Fisher’s LSD post hoc analysis. Behavioral measures were analyzed by non-parametric one-way ANOVA (Kruskal–Wallis test) followed by Mann–Whitney *U* test. Statistical significance was accepted if  $P < 0.05$ .

## Results

**Effects of Emotional Stress on SP Release in the Amygdala.** The SP content in push–pull superfusates collected under basal conditions was consistently detected in both the MeA and the CeA ( $9.0 \pm 1.6$  and  $6.5 \pm 1.1$  fmol per 20-min sample,  $n = 6$  and  $7$ , respectively). As shown in Fig. 2A, immobilization stress caused a pronounced and long-lasting increase in the release of SP in the MeA (150%). SP release remained elevated throughout 100 min of poststress testing. This was confirmed by two-way ANOVA, which revealed a statistically significant effect of the factor group ( $F_{1,8} = 21.55, P = 0.0017$ ) and the factor time ( $F_{9,72} = 12.64, P < 0.0001$ ). There was a significant interaction between the factors

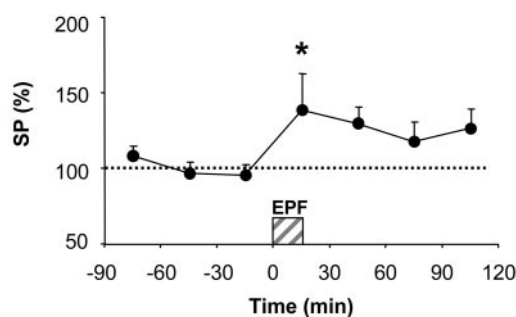
group and time ( $F_{9,72} = 5.78, P < 0.0001$ ). In unstressed control animals ( $n = 4$ ), SP release in the MeA remained unchanged throughout the entire experimental period. Immobilization stress had no effect on the SP release in the CeA ( $F_{9,54} = 0.99, P = 0.45$ ). Superfusion with aCSF containing 100 mM KCl caused an increase in SP release in both the MeA (240%;  $F_{2,10} = 38.43, P < 0.0001$ ) and the CeA (50%;  $F_{2,12} = 7.77, P = 0.006$ ) (Fig. 2B). The sensitivity of the RIA was increased to reliably monitor basal extracellular SP levels in the MeA also by using microdialysis, the most common technique to investigate *in vivo* release. The SP content in microdialysates collected in unstressed (basal) conditions amounted to  $4.6 \pm 0.5$  fmol per 30-min microdialysate ( $n = 8$ ). As shown in Fig. 3, exposure to elevated platform caused a transient increase in SP release within the MeA (40%;  $F_{8,56} = 4.36, P = 0.0004$ ).

**Effects of Microinjections of SP and/or a NK<sub>1</sub> Receptor Antagonist into the MeA on Anxiety-Related Behavior.** The behavioral effects produced by bilateral microinjections of NK<sub>1</sub> receptor ligands into the MeA of rats exposed to the EPM test are shown in Figs. 4 and 5. In unstressed rats, SP injections into the MeA decreased the frequency of open arm entries ( $P = 0.008$ ) and time spent on the open arms of the maze ( $P = 0.022$ ). Post hoc analysis showed that SP at 0.1 and 1 pmol decreased the percentage of time spent

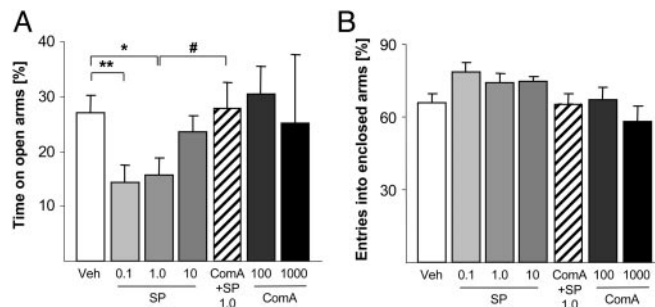


**Fig. 2.** Effects of immobilization (IM) stress (A) and superfusion with 100 mM  $K^+$  (B) on the content of SP in 20-min push-pull superfusates collected consecutively in either the MeA (Upper) or CeA (Lower) of freely moving rats. Animals were immobilized for 20 min during sampling of the fifth superfusate. After 120 min, the superfusion medium was changed to aCSF containing 100 mM KCl. Animals assigned to the control group (unstressed) remained undisturbed and were not exposed to immobilization. Data (means  $\pm$  SEM) are expressed as the percentage of the averaged basal values (dotted line). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. superfusates 1–4; #,  $P < 0.05$ ; ##,  $P < 0.01$  vs. unstressed controls; +,  $P < 0.05$ ; ++,  $P < 0.01$  vs. two samples preceding KCl (Newman-Keuls post hoc test).

on the open arms (Fig. 4A,  $P = 0.009$  and  $P = 0.02$ , respectively), as well as the number of entries into the open arms ( $P = 0.008$  and  $P = 0.004$ , respectively; data not shown). In contrast, animals microinjected with 10 pmol of SP into the MeA (Fig. 3) or with SP (0.1 or 1 pmol) outside the amygdala (data not shown) did not show any behavioral differences compared to the control group that received aCSF. The observed anxiogenic-like effect of SP was blocked by preinjection of Compound A (100 pmol) into the MeA. The frequency of open arm entries and the time spent on



**Fig. 3.** Effects of elevated platform (EPF) exposure on the content of SP in 30-min microdialysates collected consecutively in the MeA of freely moving rats. Animals were exposed to the platform for 15 min during sampling of the fourth superfusate. After stress exposure, animals were superfused for a further 90 min. Data (means  $\pm$  SEM) are expressed as the percentage of the averaged basal values (dotted line). \*,  $P < 0.05$  vs. dialysates 1–3 (Fisher's LSD post hoc test).



**Fig. 4.** Effects of SP and Compound A (ComA) on anxiety-related behavior in unstressed rats. The percentage of the time spent on the open arms (A) and number of entries into the enclosed arms (B) were recorded, starting 5 min after microinjections into the MeA. Data are means  $\pm$  SEM. Vehicle (Veh; aCSF,  $n = 9$ ); SP, 0.1 pmol ( $n = 8$ ), 1 pmol ( $n = 12$ ), and 10 pmol ( $n = 4$ ); ComA, 100 pmol ( $n = 8$ ) and 1 nmol ( $n = 3$ ); ComA + SP 1.0, ComA (100 pmol,  $n = 8$ ) preinjected 15 min before SP injection (1 pmol). \*,  $P < 0.05$  vs. vehicle treated control group; #,  $P < 0.05$  ComA + SP 1.0 vs. SP 1.0 (Mann-Whitney  $U$  test).

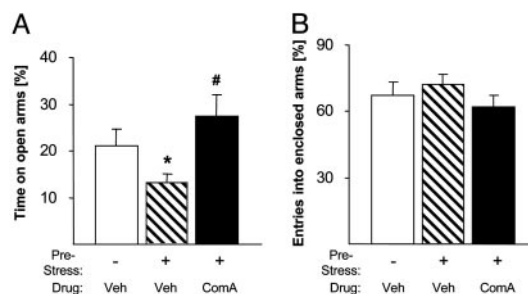
the open arms in animals microinjected with SP (1 pmol) after pretreatment with Compound A was significantly increased compared to SP injected animals ( $P < 0.05$ ) and resembled that seen in vehicle-treated control rats (Fig. 4A). Intra-MeA injection of Compound A (100 and 1000 pmol) did not alter spontaneous behavior on the EPM (Fig. 4A).

Prior immobilization of rats enhanced anxiety-related behavior on the EPM, indicated by a decrease in the percentage of time spent on the open arms (Fig. 5A;  $P < 0.05$ ). This anxiogenic-like effect of immobilization stress was antagonized ( $P = 0.012$ ) by intra-MeA infusion of Compound A (1 nmol; Fig. 5A). Hence, in prestressed rats, Compound A elicited an anxiolytic effect, increasing the percentage of time on the open arms.

The observed behavioral effects of immobilization, SP, or Compound A were not due to changes in general locomotor activity, because entries into enclosed arms (Figs. 4B and 5B) or total path length on the plus-maze (data not shown), reflecting locomotor activity, were not affected by any of the treatments.

## Discussion

The results of the present study demonstrate that SP is released in a distinct subregion of the amygdala in response to different emotional stressors. Specifically, immobilization stress caused a pronounced increase of SP release in the MeA but not in the CeA. Moreover, a transiently enhanced SP release was also found in response to the mild anxiogenic stimulus of elevated



**Fig. 5.** Effect of Compound A (ComA) on anxiety-related behavior in prestressed rats. Rats were prestressed by exposure to 20 min of immobilization 60 min before microinjection of vehicle (Veh; aCSF,  $n = 9$ ) or ComA (1 nmol,  $n = 7$ ) into the MeA. Unstressed animals injected with vehicle ( $n = 6$ ) were used as further controls. The percentage of the time spent on the open arms (A) and number of entries into the enclosed arms (B) were recorded, starting 5 min after microinjections. Data are means  $\pm$  SEM. \*,  $P < 0.05$  vs. unstressed animals; #,  $P = 0.01$  vs. vehicle-treated prestressed animals (Mann-Whitney  $U$  test).

platform exposure. Immobilization enhanced anxiety-related behavior in the subsequently performed EPM test. Bilateral microinjections of a NK<sub>1</sub> receptor antagonist into the MeA blocked the stress-induced anxiogenic-like effect. In unstressed rats, the NK<sub>1</sub> receptor antagonist displayed no significant anxiolytic effect but reversed the anxiogenic effect of SP microinjected into the MeA.

**Effects of Emotional Stress on SP Release in the Amygdala.** Previously, stress-induced effects on intracerebral SP levels were mainly obtained from brain tissue measurements. Exposure to a variety of emotional, noxious, and painful stressors alters tissue levels or SP immunoreactivity in various brain regions, including the amygdala, septum, periaqueductal gray, and hypothalamus (13–17, 33, 34). However, these stress-induced changes should be interpreted with caution. Tissue measurements of neuropeptides are determined by a number of variables (e.g., synthesis, transport, storage, release, and degradation) and thus do not necessarily reflect the dynamics of local release (for review, see ref. 35). Because neuropeptides such as SP become biologically active only after their release into the extracellular space, attempts to measure intracerebral release focus on approaches that are able to reflect concentrations and their fluctuations in the extracellular fluid. Our finding of high extracellular SP levels in the MeA is consistent with immunohistochemical studies demonstrating a dense plexus of SP containing cell bodies and terminals in this brain area (24, 26). Most previous studies demonstrating *in vivo* release of SP after pharmacological or noxious stimulation by using push–pull or microdialysis techniques were restricted to spinal cord or brain areas known to be involved in nociception and pain transmission (36–39), whereas SP release in forebrain areas involved in stress responses has not been studied. In the present study, an increase in SP release was observed in response to emotional stress in the MeA but not in the CeA, although K<sup>+</sup>-induced depolarization stimulated SP release in the CeA, indicating that a releasable and detectable pool of SP is also present in this brain area. Alternatively, it might be speculated that stimulation with K<sup>+</sup> in the CeA is likely to release SP from more distant sites. The differential sensitivity of MeA and CeA cell populations is also supported by other studies demonstrating that emotional stressors, including immobilization, induce Fos expression (a marker of neuronal activation) to higher extent in the MeA than in the CeA, whereas predominantly physical stressors enhanced Fos expression primarily in the CeA (29, 40, 41).

Immobilization is known to be a severe stressor accompanied by a pronounced ACTH (corticotropin) response (42). Our finding that comparatively mild emotional stress such as elevated platform exposure was sufficient to trigger the release of SP in the MeA contributes to a growing literature that SP functions as a stress neurotransmitter/neuromodulator in the CNS. Exposure to elevated platform elicits a moderate ACTH response (43) and induces anxiety comparable to that following exposure to the EPM, which is known to induce neuronal activation in the MeA, among other brain areas (44–46). Similarly, elevated platform exposure elicited a transient, moderate increase in SP release, suggesting that the degree of SP release is related to the intensity of the stressful stimulus. This is consistent with a previous study demonstrating a graded NK<sub>1</sub> receptor internalization response in the spinal cord depending on stress intensity using noxious thermal stimulation (47). Because increased receptor endocytosis is thought to be associated with increased SP release, it seems likely that the exposure to both stressors used in the present study is accompanied by increased NK<sub>1</sub> receptor internalization in the MeA. Because NK<sub>1</sub> receptor immunoreactivity in the MeA is extremely dense (25, 48), it has previously only been possible to visualize stress-induced receptor internalization in the less densely labeled basolateral nucleus (5, 49, 50).

**Effects of Microinjections of SP and/or a NK<sub>1</sub> Receptor Antagonist into the MeA on Anxiety-Related Behavior.** In the second part of the present study, we investigated the behavioral significance of the pronounced immobilization-induced SP release in the MeA. We decided to study anxiety-related behavior on the EPM, an established ethological animal model of anxiety that is sensitive to both anxiolytic and anxiogenic drugs (51), because it has been reported previously that immobilization stress induces an anxiogenic-like behavior in this test (27, 52). In accordance with these findings, immobilized rats visited open arms in the EPM less frequently and spent less time on the open arms than unstressed controls, indicating higher anxiety-related behavior in prestressed rats. Microinjections of Compound A, a selective NK<sub>1</sub> receptor antagonist attenuated the enhanced anxiety-related behavior of stressed rats to levels observed in unstressed rats. These findings suggest that the immobilization-induced increase in SP within the MeA, which is pronounced and long-lasting (see above), mediates the enhanced anxiety displayed by these rats. Supporting the anxiogenic properties of SP in this brain area, SP microinjected into the MeA of unstressed rats caused an anxiogenic-like effect, indicated by a decreased frequency of open arm entries and time spent on the open arms. In contrast, no anxiogenic effect was seen when SP was microinjected outside the amygdala into the internal capsule. Notably, the anxiogenic-like effect of SP was obtained after injections of 0.1 and 1 pmol, whereas a higher dose (10 pmol) was ineffective, suggesting a biphasic dose–response effect, which has also been reported in other studies (53, 54). It is likely that at this high dose, SP also activated other receptors in addition to the NK<sub>1</sub> receptor, changing the overall behavioral effect. Indeed, anxiolytic effects have been reported for agonists at NK<sub>3</sub> receptors, which are also present in the MeA (for review, see ref. 55). However, the anxiogenic effects of lower doses of SP are due to stimulation of NK<sub>1</sub> receptors, as they were abolished by pretreatment with Compound A, which had no significant effect under baseline conditions in unstressed rats. The observed behavioral effects of immobilization, SP, or Compound A are not due to nonspecific locomotor effects, because total path length on the plus-maze or enclosed arm entries, a parameter related to locomotor activity, was not influenced.

Our findings are consistent with previous studies demonstrating that NK<sub>1</sub> receptor agonists given *i.c.v.* (4, 56), or locally injected into brain areas including the dorsal periaqueductal gray and septum (3, 6), promote an anxiogenic-like effect in the EPM. Conversely, central or systemic injection of NK<sub>1</sub> receptor antagonists produces anxiolytic-like effects in the EPM in mice (4) and gerbils (8). The present finding that bilateral injections of Compound A into the MeA elicits an anxiolytic-like effect in prestressed rats, whereas no significant effect was observed in unstressed control rats suggests that the NK<sub>1</sub> receptors located in the MeA may be part of a pathophysiologically relevant system that is activated by increased peptide release. Interestingly, clinical effects of NK<sub>1</sub> antagonists were shown in patients with major depressive disorder (5, 57), a patient population in which there is preliminary evidence that SP levels are elevated in CSF (ref. 58; see also ref. 59) and plasma (60). Thus it seems that in normal (unstressed) animals the endogenous SP tone on NK<sub>1</sub> receptors is too low to elicit significant anxiolytic effects by blocking the NK<sub>1</sub> receptor in the MeA alone. Deactivation of NK<sub>1</sub> receptors in larger parts of the amygdala has been shown to reduce the duration of neonatal vocalizations in response to maternal separation in guinea pig pups (50), and to induce anxiolytic-like behavioral effects in the EPM test also in normal rats (61).

The present studies provide the first demonstration that emotional stress triggers the release of SP in the MeA, a distinct subregion of the amygdaloid complex. SP released in response to severe emotional stress causes anxiogenic effects that can be

attenuated by local blockade of NK<sub>1</sub> receptors in the MeA. These findings together with the behavioral effects of SP ligands microinjected into the MeA on the EPM implicate the MeA as an important subregion of the amygdaloid complex where SP mediates stress and anxiety-related responses. This area has been identified as a critical component of the neural circuitry involved in the control of stress responses (62), with afferent connections to other parts of this circuitry including bed nucleus of stria terminalis, periaqueductal gray, and hypothalamic areas (63). Accordingly, previous studies have demonstrated anxiogenic-

like effects after electrical stimulation (64–67) and anxiolytic-like effects after ablation of the MeA (68, 69). The present findings provide further evidence for the amygdala as a possible site of action for the therapeutic effects of NK<sub>1</sub> antagonists in the treatment of stress-associated psychiatric disorders including anxiety disorders and depression.

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