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Neurokinin 1 Receptor Antagonism Promotes Active Stress Coping Via Enhanced Septal 5-HT Transmission

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Antagonists of the substance P (SP) preferring neurokinin 1 receptor (NK1R) represent a promising novel class of drugs for the treatment of stress-related disorders such as depression and anxiety disorders; however, the involved neuronal pathways releasing SP in response to stressors are ill defined. By using *in vivo* microdialysis in combination with a highly sensitive and selective radioimmunoassay we found that exposure to forced swim stress increased SP release in the rat lateral septum (LS), a key area in processing emotions and stress responses. Acute administration of the selective NK1R antagonist L-822429 injected either systemically or locally into the LS reduced passive and facilitated active stress-coping strategies in the forced swim test. This effect seems to be mediated by enhanced intraseptal serotonergic transmission via serotonin (5-HT)1A receptors since NK1R blockade reversed the swim stress-induced decrease to an increase in extracellular 5-HT efflux, and furthermore the behavioral effects of L-822429 were blocked by intraseptal 5-HT1A receptor antagonism. A direct heterosynaptic regulation by NK1R on 5-HT release from serotonergic fibers was ruled out by immunocytochemistry at the light and electron microscopic level indicating involvement of GABAergic interneuron(s) in this interaction. Taken together, our data identify the LS as a critical brain area for the involvement of SP transmission in the modulation of stress responses and demonstrate that NK1R blockade can elicit a functionally significant facilitatory effect on 5-HT transmission, which does not necessarily involve the previously proposed interaction with neuronal firing at the cell body level of raphe neurons.
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INTRODUCTION

The neuropeptide substance P (SP) and its preferred target the neurokinin 1 receptor (NK1R) have been suggested to be involved in the modulation of emotional processes including stress reactions (Culman and Unger, 1995; Rupniak, 2002; Ebner and Singewald, 2006). This idea was initially based on the finding that SP acts as neurotransmitter and/or neuromodulator within the brain (Otsuka and Yoshioka, 1993; Hökfelt *et al*, 2001) and on the evidence that changes in SP content occur in brain areas known to be implicated in processing emotions and stress reactions in response to aversive situations (Herpfer and Lieb, 2005; Ebner and Singewald, 2006). The activation of NK1Rs by central injection of SP or NK1R agonists was shown to produce a range of aversive behavioral and physiological reactions that were largely blocked by selective NK1R antagonists (Rupniak, 2002; Ebner and Singewald, 2006). These findings could be confirmed by studies on mice with genetic disruption of NK1R function or of the peptide itself as these animals exhibit less anxiety- and depression-related behaviors in various behavioral tasks (Rupniak *et al*, 2001; Santarelli *et al*, 2001; Bilkei-Gorzo *et al*, 2002). On the basis of these findings, SP/NK1R pathways have been proposed to be involved in the etiopathology of a number of stress-related diseases such as depression and anxiety disorders (Herpfer and Lieb, 2005; McLean, 2005). Confirmation of this proposal was obtained by clinical findings that selective NK1R antagonists have antidepressant as well as anxiolytic efficacy in patients with major depressive disorder and with moderately high anxiety levels (Kramer *et al*, 1998, 2004; Furmark *et al*, 2005). However, since some conflicting results have been obtained more recently (Keller *et al*, 2005), additional studies are necessary to better understand the precise mechanisms mediating such effects.

Although there is strong evidence for a role of the central SP/NK1R system in the modulation of stress reactions, there have been few attempts to explore the neuronal substrates underlying such modulation. Since the lateral septum (LS), a key brain structure implicated in stress, anxiety, and depression (Sheehan *et al*, 2004), contains one of the densest SP innervations (Ljungdahl *et al*, 1978; Sakanaka *et al*, 1982; Gall and Moore, 1984; Szeidemann *et al*, 1995;

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Hökfelt *et al*, 2004), the aim of the present study was to investigate whether the exposure to a severe emotional stressor like forced swimming triggers an increase in SP release within this area. Furthermore, we studied the physiological significance of intraseptally released SP by administration of a selective NK1R antagonist into the LS and monitored behavioral stress-coping ability. The LS might be a primary receptor site for SP since this area contains a moderate-to-dense number of NK1Rs (Nakaya *et al*, 1994; Saffroy *et al*, 2003), and intraseptal SP administration has been found to modulate anxiety-related behavior in rats (Gavioli *et al*, 1999). In an attempt to shed light on the mechanisms involved in the behavioral effects elicited by modulation of SP transmission in the LS, we further characterized the septal localization of NK1Rs as well as interaction of SP with other neurotransmitter systems possibly involved in behavioral stress coping. Since septal serotonin (5-HT) appears to play a particularly prominent role in regulating behavioral responses to swim stress (Schreiber and De Vry, 1993; Kirby and Lucki, 1997), we examined whether SP interacts with this neurotransmitter in the LS.

METHODS

Animals

Experiments were carried out on adult male Sprague–Dawley rats (250–350 g). Before use, the animals were housed in groups of 4–6 under controlled laboratory conditions (12:12 h light/dark cycle with lights on at 0700, $21 \pm 1^{\circ}$C, 60% humidity, pelleted food, and water *ad libitum*) for at least 1 week after delivery from the supplier. The experimental studies described here were approved by the local Ethical Committee on Animal Care and Use and are in compliance with international laws and policies.

Surgery

A homemade, U-shaped microdialysis probe consisting of an 18-kDa dialysis membrane (Hemophan, Membrana, Wuppertal, Germany) was stereotaxically implanted under sodium pentobarbital (40 mg/kg, i.p.) and ketamine (50 mg/kg, i.p.) anesthesia. The probe was positioned according to the stereotaxic atlas of Paxinos and Watson (1998) with its tip reaching the right LS (Figure 1; implantation coordinates: 0.8 mm rostral to bregma, 1.6 mm lateral to the midline, 7.2 mm below the surface of the skull with an angle of 10° to avoid sagittal sinus damage) and fixed to the skull with two jeweler's screws and dental cement. The two endings of the probe were attached to 5-cm-long pieces of polyethylene tubing for connection with the infusion pump. Postoperatively, rats received a one-time injection of buprenorphine (0.1 mg/kg i.m.).

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 16 h before the experiment, animals were placed in the experimental room and allowed to habituate. Experiments were performed between 0700 and 1600. Exposure to the stressor was always completed between 1100 and 1400 to minimize circadian rhythm-related variations in stress responses.

Effects of Forced Swimming on the Release of SP within the LS

Two days after surgery, the microdialysis probe was connected to a syringe mounted onto a microinfusion pump (TSE-Systems, Bad Homburg, Germany) and superfused with artificial cerebrospinal fluid (aCSF) of the following composition: 140 mM NaCl, 3.0 mM KCl, 1.25 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 0.3 mM NaH_2PO_4, and 3.0 mM glucose (pH 7.2). To detect SP in microdialysates by radioimmunoassay, we need a sample volume of 200 µl. Therefore we used a flow rate of 6.5 µl/min and a sampling interval of 30 min. To minimize the binding of SP to the plastic surfaces and to reduce peptidase activity, bovine serum albumin (0.2%) and bacitracin (0.03%) were included in the perfusion medium. After an equilibration period of 120 min, six consecutive 30-min dialysates were collected directly into Eppendorf vials, which were stored at -80°C until assay. At the beginning of the third dialysis interval, animals ($n = 7$) were placed into a swim tank (square plastic tank, $35 \times 35 \times 42$ cm; filled with water at 20°C up to a height of 30 cm) for 10 min with ongoing microdialysis. Thereafter they were gently dried using a towel and returned to their home cage. After stress exposure, animals were dialyzed for further 110 min. Unstressed control animals ($n = 4$) remained undisturbed in their home cages.

Effects of Intraseptal and Systemic NK1R Antagonist Administration on the Behavioral Stress Coping

In the second series of experiments, microdialysis was used to administer the selective NK1R antagonist (2*S*,3*S*)-*N*-{[2-cyclopropoxy-5-(5-trifluoromethyl)tetrazol-1-yl]benzyl}-(2-phenylpiperidin-3-yl)amine dihydrochloride (L-822429; Ebner *et al*, 2004; Singewald *et al*, 2007) locally into the LS. Therefore, four groups of animals implanted with a microdialysis probe into the right LS were dialyzed with aCSF at a rate of 3.3 µl/min as described above. The animals were then left undisturbed for 90 min, at which time point the perfusate was kept at aCSF, or changed to aCSF containing either L-822429 (1 and 10 µM; $n = 6$ each) or its low-affinity enantiomer (2*R*,3*R*)-*N*-{[2-cyclopropoxy-5-(5-trifluoromethyl)tetrazol-1-yl]benzyl}-(2-phenylpiperidin-3-yl)amine dihydrochloride (1 µM; $n = 6$). Drugs reached the LS after approximately 5–6 min and the stressor was applied 80 min later. During the 10-min forced swimming session under ongoing retrodialysis, the behavior was recorded by a video system and scored by a trained observer blind to the animals' treatment quantifying absolute time measurements. The behavior of the animals was assigned to one of the three following behavioral categories: (1) struggling, defined as movements during which the forelimbs broke the water's surface; (2) swimming, defined as movement of the animal induced by movements of the fore and hind limbs without breaking the water surface; and (3) floating defined

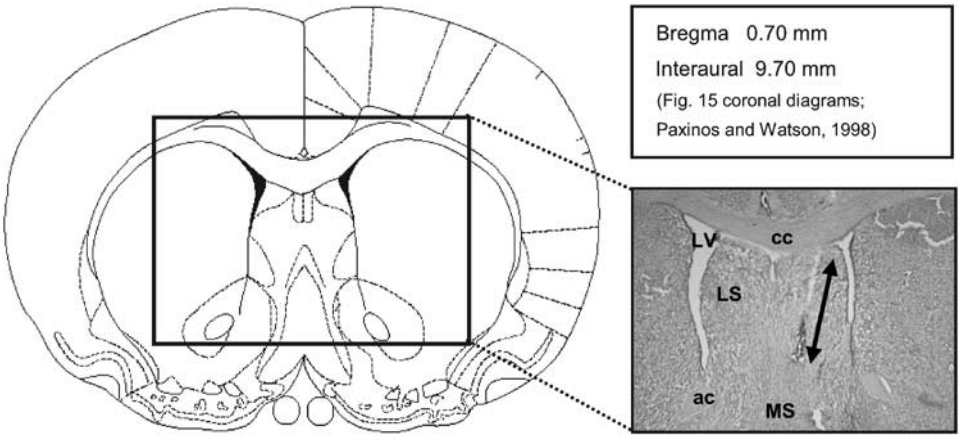

Figure 1 Schematic drawing and representative microphotograph of a cresyl-stained coronal section of the rat brain showing the localization of the active membrane tip (arrow) of a microdialysis probe within the lateral septum (LS). ac, anterior commissure; cc, corpus callosum; LV, lateral ventricle; MS, medial septum.

as the behavior during which the animal used limb movement just to keep its equilibrium without any movement of the trunk. After the stress exposure, the animals were returned to their home cages. In two additional groups of animals, either L-822429 (30 mg/kg i.p.; $n=13$) or saline (controls; $n=10$) was administered systemically 90 min before swim stress exposure. These animals were dialyzed with aCSF as described above. In seven rats of the NK1R antagonist-treated group, the perfusate was changed to aCSF containing the selective 5-HT1A receptor antagonist WAY-100635 (10 μM; Sigma-RBI, Steinheim, Germany). In a further group of animals, the selective $GABA_B$ receptor antagonist CGP 35348 (500 μM; $n=9$; Sigma-RBI) was infused via retrodialysis before, during, and after forced swimming. In all these further groups of animals, behavioral analysis during forced swimming was analyzed as described above. L-822429 binds with high affinity to the rat NK1R and is highly selective for the NK1R over the NK2 and NK3 receptors, respectively. In contrast, the enantiomer demonstrates little to no binding affinity for any of the three NK receptor subtypes (Singewald *et al*, 2007).

Effects of Intraseptal and Systemic NK1R Antagonist Administration on the Release of 5-HT in the LS

In a further series of experiments, microdialysis was used to monitor extracellular 5-HT efflux after intraseptal or systemic NK1R antagonist administration. Four groups of animals were dialyzed with aCSF at a rate of 3.3 μl/min as described above. After an equilibration period of 120 min, consecutive 30-min dialysates were collected in Eppendorf tubes, transferred to dry ice, and stored at $-80^{\circ}C$ until measurement. L-822429 (1 and 10 μM; $n=6$–7) and its low-affinity enantiomer (1 μM; $n=7$) were infused via retrodialysis as described above. In two additional groups of animals, either L-822429 (30 mg/kg i.p.; $n=7$) or saline (controls; $n=7$) was administered systemically. To protect 5-HT from decomposition, the tubes used to collect the microdialysates contained 3 μl of the following solution: 1.6 mM $Na_2S_2O_5$ and 0.025 mM EDTA.

Quantification of SP and 5-HT in Dialysates

The concentration of SP was measured in microdialysates by a highly sensitive and selective radioimmunoassay (detection limit: 0.1 fmol/200 μl dialysate; cross-reactivity of the antiserum RD2 with other related peptides, like neurokinin A and B, was <0.01 and 0, respectively) using synthetic SP (Sigma-RBI) as a standard and [^{125}I]Bolton-Hunter-SP (approximate specific activity 74 TBq/mmol = 2000 Ci/mmol; Amersham, UK) as a tracer (for a detailed description see Ebner *et al*, 2004).

Determination of 5-HT and 5-hydroxyindol-3-acid was performed by reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (detection limit: 0.3 pg/50 μl dialysate; Singewald *et al*, 1997). The HPLC system consisted of a Jasco PU-2085PLUS semi-micro pump (Jasco, Tokyo, Japan), operating at a flow rate of 0.08 ml/min and an electrochemical detector (LC-4B, BAS, West Lafayette, USA) set at +600 mV. The analytical column (SepStik microbore column, 150 × 1 mm, 5 μm, C18, BAS) was protected by a guard column (SepStik, 14 × 1 mm, 5 μm, C8, BAS) and was coupled directly to a BAS Unijet 3 mm glassy carbon electrode. Samples of 50 μl were automatically injected by a CMA 200 refrigerated autosampler. The mobile phase consisted of 93% phosphate buffer (PB; 0.1 M NaH_2PO_4, 1 mM sodium octanesulfonic acid, 10 mM NaCl, and 0.5 mM Na_2-EDTA), 7% acetonitrile, and the pH adjusted to 3.5 with *o*-phosphoric acid. The mobile phase was filtered (0.2 μm) before use. Evaluation of 5-HT was carried out by comparing peak heights of samples with a set of external standard solutions by using an integrator (SIC Chromatocoder 12, System Instruments, Tokyo, Japan).

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 microdialysis probes, brains were sectioned (50 μm, coronal sections) and stained with cresyl violet (Figure 1). The locations of probes in the LS were determined on the basis

of previous definitions of a brain atlas (Paxinos and Watson, 1998). Judgment of successful implantation of the LS was made before analyzing release and behavioral experiments.

Immunocytochemistry for Light and Electron Miscroscopy

For immunocytochemical studies, adult male rats were deeply anesthetized with thiopental (thiopentone sodium; 100 mg/kg, i.p.) and transcardially perfused with saline, followed for 15 min by a fixative composed of 4% paraformaldehyde, ~0.2% picric acid made up in 0.1 M PB (pH 7.2–7.4). For immunoelectron microscopy, 0.05% glutaraldehyde was included in the fixative. Brains were removed quickly, rinsed extensively in PB and sectioned on a vibratome at 50 and 70 μm thickness for immunofluorescence and electron microscopy studies, respectively.

Immunofluorescent experiments were carried out according to previously published procedures (Ferraguti *et al*, 2004). Free-floating sections were incubated in blocking solution, composed of 0.9% NaCl buffered with 50 mM Tris (TBS; pH 7.4), 0.1% Triton X-100 (TX), and 20% normal goat or horse serum (NS), for 1 h and then in primary antibodies in combination or alone, made up in TBS, 0.1% TX, and 1% NS for approximately 48 h (4°C). Primary antibodies used in this study were: rabbit anti-NK1R (Chemicon; 1:1500), goat anti-choline acetyltransferase (ChAT) (Chemicon; 1:200), mouse anti-calbindin D-28k (SWant; 1:6.000), and goat anti-ST(C-20):sc-1458 serotonin transporter (SERT) (Santa Cruz; 1:500). Subsequently, after extensive washes in TBS, sections were incubated overnight at 4°C with a mixture of appropriate secondary antibodies: donkey anti-rabbit or anti-mouse Alexa 488 (Molecular Probes; 1:1.000) and donkey anti-rabbit or anti-goat Cy3TM (Jackson Immunoresearch; 1:400). Sections were then mounted onto gelatin-coated slides in Vectashield (Vector). Immunofluorescence was analyzed using a Zeiss Axioplan2 microscope with epifluorescence illumination. Images were displayed using the Openlab software (version 3.1.5; Improvision, Coventry, UK). Brightness and contrast were adjusted for the whole frame and no part of a frame was modified in any way. Procedures for immunoelectron microscopy were as described earlier (Corti *et al*, 2002). Floating sections were incubated with the following primary antibodies: rabbit anti-NK1 (Chemicon; 1:500); goat anti-SERT (Santa Cruz; 1:250), diluted in TBS-1% NS for 72 h and visualized by an immunoperoxidase method (ABC Vector) using 3-3′-diaminobenzidine (0.5 mg/ml). Sections were then treated with 2% OsO_4, contrasted with 1% uranyl acetate and embedded in epoxy resin (Durcupan ACM, Fluca, Sigma). Ultrathin (70 nm thickness) serial sections were collected on pioloform-coated copper grids and analyzed in a Philips CM120 electron microscope.

Statistical Analysis

Statistical analyses were performed using a computer software package (GB-Stat 6.0, Dynamic Microsystems, Silver Springs, USA). Microdialysis data were expressed as raw data or as a percentage of averaged baseline values and submitted to a one-way (SP) or a two-way ANOVA (treatment × time, 5-HT) with repeated measures followed by Fisher's LSD or Newman–Keuls *post hoc* analysis. The area under the curve (AUC) describing extracellular 5-HT levels as a function of time was calculated by the trapezoidal method. Mean AUC values and behavioral data were analyzed using a one-way ANOVA for independent measurements followed by the Newman–Keuls test for multiple comparisons or a Student's two-tailed *t*-test for comparison of two groups. Data are presented as means ± SEM. Significance was accepted if $p < 0.05$.

RESULTS

Effects of Forced Swimming on the Release of SP within the LS

The SP content of LS dialysates collected under basal conditions was more than one magnitude above the detection limit of the radioimmunoassay, reaching an average of 1.6 ± 0.4 fmol/dialysate. As shown in Figure 2, swim stress caused a significant increase in the SP content of microdialysates collected from the LS amounting to 192% of the basal release. The evoked SP release remained elevated up to 60 min after onset of the stressor (one-way ANOVA $F_{5,30} = 4.13$; $p = 0.0057$) and returned to pre-stress values within the next 60 min. In contrast, SP content in microdialysates sampled in the LS of unstressed control animals remained unchanged throughout the entire dialysis period (data not shown).

Effects of Intraseptal and Systemic NK1R Antagonist Administration on the Behavioral Stress Coping

As illustrated in Figure 3, intraseptal as well as systemic administration of the NK1R antagonist L-822429 significantly affected the animals' behavior during forced swimming. For the former administration route, statistical analysis by one-way ANOVA indicated a significant difference between groups in active coping ($F_{3,20} = 9.62$, $p = 0.0004$) as well as floating behavior ($F_{3,20} = 9.63$, $p = 0.0004$). *Post hoc* analysis revealed that intraseptal administration of L-822429 via retrodialysis at different concentrations (1 and 10 μM) significantly reduced floating

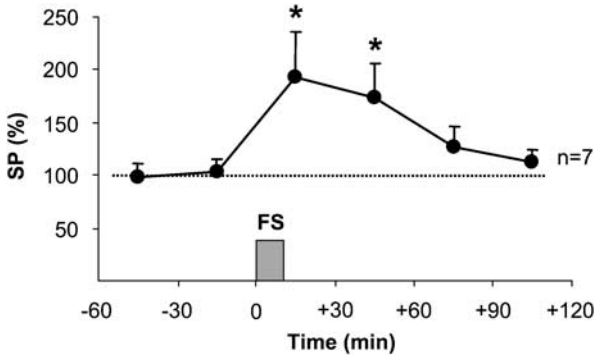

Figure 2 Effect of forced swimming on the content of SP in 30-min microdialysates sampled in the lateral septum (LS) of freely moving rats. Animals were exposed to forced swimming (FS; gay-shaded bar) for 10 min during collection of dialysis sample number 3. Data are expressed as a percentage of baseline (100%, dotted line) + SEM. *$p < 0.05$ *vs* dialysates 1 and 2 (Fisher's LSD *post hoc* test).

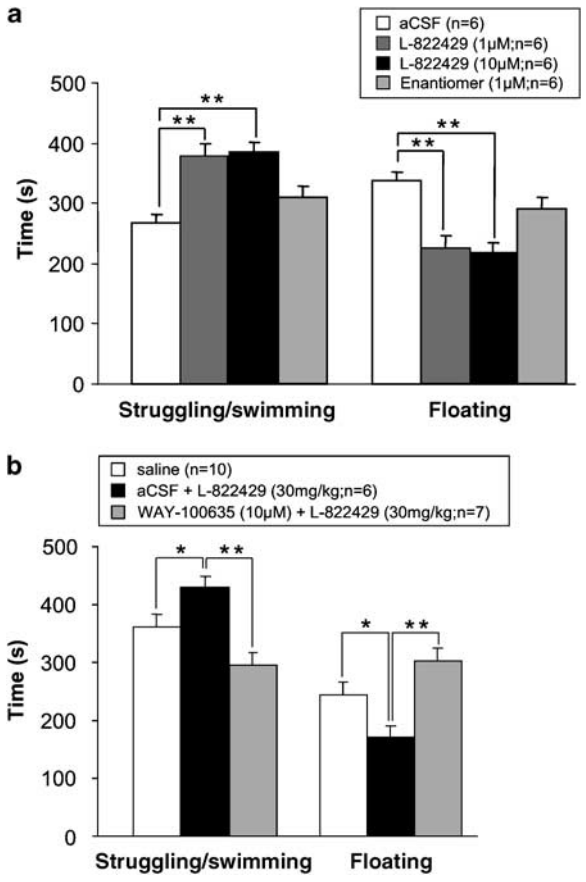

Figure 3 Effect of the NK1R antagonist L-822429 or the low-affinity enantiomer applied either into the LS (a) or intraperitoneally (b) on the time animals spent struggling/swimming (active coping) and floating (passive coping) during 10 min of forced swimming. Data are presented as means + SEM. $^{*}p<0.05$, $^{**}p<0.01$ *vs* vehicle-treated controls (Newman–Keuls *post hoc* test).

time (passive coping; $p<0.01$) during forced swimming by 40% and increased struggling/swimming time (active coping; $p<0.01$) by 60%. In contrast, administration of the low-affinity enantiomer of the NK1R antagonist had no effect on the behavioral stress response, as enantiomer-treated animals did not differ to vehicle-treated controls. Systemic administration of L-822429 (30 mg/kg, i.p.) affected behavioral stress coping in a similar way as intraseptal administration (active coping: $F_{2,20}=8.05$, $p=0.0027$; floating: $F_{2,20}=8.55$, $p=0.0021$; Figure 3b) since antagonist-treated animals showed a reduced floating time ($p<0.05$) and an increased struggling/swimming time ($p<0.05$) compared to control animals. This behavioral effect could be reversed by intraseptal administration of the selective 5-HT1A receptor antagonist WAY-100635 (10 μM). Furthermore, intraseptal administration of a $GABA_B$ receptor antagonist changed the behavioral stress-coping strategy during forced swimming in a similar (antidepressant-like) way as L-822429 (Figure 6).

Effects of Intraseptal and Systemic NK1R Antagonist Administration on the Release of 5-HT in the LS

Basal 5-HT concentrations in microdialysates collected in the LS were 3.5 ± 0.2 fmol/dialysate. As illustrated in Figure 4, forced swimming significantly reduced 5-HT efflux in the LS by 30% of baseline. Statistical analysis by two-way ANOVA revealed a significant effect of the factor group ($F_{3,23}=22.86$, $p<0.0001$) and time ($F_{8,184}=11.38$, $p<0.0001$) as well as a significant interaction between main factors ($F_{24,184}=21.21$, $p<0.0001$). *Post hoc* analysis revealed significant differences of 5-HT efflux during and after stress exposure between antagonist-treated animals and controls. Intraseptal infusion of L-822429 at a concentration of 1 μM reversed the swim stress-induced decrease of the 5-HT release in the LS and led to a 30% increase of 5-HT release during stress exposure, while the administration of the low-affinity enantiomer had no effect, which was similarly observed in vehicle-treated controls. The facilitatory effect of L-822429 on 5-HT efflux was further enhanced using a higher concentration (10 μM) amounting then to a 100% increase in 5-HT release during forced swimming. At this higher concentration also basal 5-HT release was increased by L-822429. Systemic administration of L-822429 at a dose of 30 mg/kg also reversed the swim stress-induced decrease of the 5-HT release in the LS (Figure 4b). This was confirmed by two-way ANOVA, which revealed a statistically significant effect of the factor group ($F_{1,12}=17.94$, $p=0.0012$) and the factor time ($F_{9,108}=8.99$, $p<0.0001$) as well as a significant interaction between the main factors ($F_{9,108}=16.43$, $p<0.0001$). The cumulative effects of NK1R antagonist treatment on extracellular 5-HT levels in the LS were additionally compared by calculating the AUC values (Figure 4, right panels). Since differences between groups of Figure 4a were transient, we decided to calculate AUC values in this case immediately before and after stress exposure. The AUC values were significantly higher in L-822429-treated rats compared to controls ($F_{3,23}=27.16$, $p<0.0001$).

Distribution of NK1Rs in the Septum and Lack of Association with 5-HT Afferents

In order to establish whether NK1R distribution has a direct anatomical link with release sites for 5-HT in the septum, we investigated—by means of immunofluorescence and immunoelectron microscopy—the localization of NK1Rs and SERT. Moreover, by double immunofluorescence we have examined the neurochemical identity of NK1R-immunopositive neurons in the septum. We observed moderate-to-densely distributed NK1R immunoreactive neurons in both the medial (MS) and LS. The MS and the laterodorsal part of the LS (LSd) were mostly enriched in NK1R-immunopositive neurons, whereas in the intermediate part of the LS (LSi) the density of NK1R-labeled neurons varied considerably at different rostrocaudal levels, with relatively few labeled cells in the more rostral areas (Figure 5a and b). In the MS, but not in the LS, the distribution of NK1R-immunopositive neurons was found to overlap completely with that of ChAT-immunoreactive neurons (Figure 5e), confirming a previous study (Chen *et al*, 2001). We observed that GABAergic neurons expressing the calcium-binding protein calbindin in the LSd (Doutrelant *et al*, 1993) were also immunoreactive for NK1Rs (Figure 5g). Conversely, in the intermediate part, no colocalization was detected between calbindin and NK1R labeled neurons (Figure 5f), suggesting that neurons

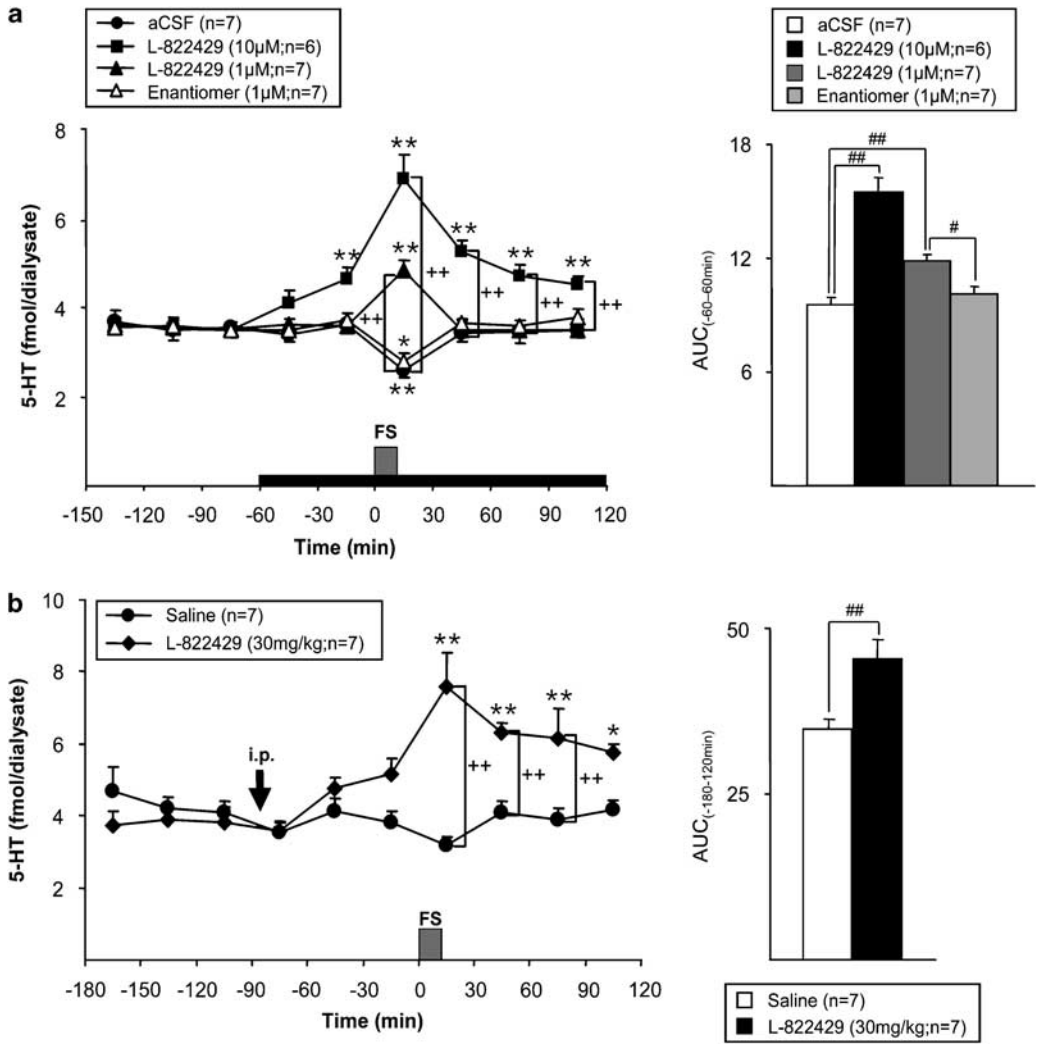

Figure 4 Effects of the NK1R antagonist L-822429 or the low-affinity enantiomer applied either into the LS (a) or intraperitoneally (b) on the concentration of 5-HT in 30-min microdialysates obtained from the LS before, during and after forced swimming (FS; gray-shaded bars). Black bar denotes administration of vehicle (aCSF) or aCSF containing drugs. Data are also presented as AUC representing the integrated time-response curve of the overall effects (right panels). Data are expressed as means + SEM. $^{*}p<0.05$, $^{**}p<0.01$ *vs* dialysate 1–3; $^{++}p<0.01$ *vs* respective value in the vehicle-treated controls. $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* vehicle-treated controls (Newman–Keuls *post hoc* test or Student's *t*-test).

expressing NK1Rs in the LS belong to different neuronal classes. At the ultrastructural level, NK1R immunoreactivity in the LS was primarily associated with postsynaptic elements, including dendritic shafts and spines (Figure 5c and d). However, sporadic presynaptic boutons forming mostly symmetric but also asymmetric synapses were also observed (not shown). 5-HT afferents to the LS, identified by their immunoreactivity for SERT, did not appear to contain detectable levels of NK1Rs nor to target to any significant degree the dendrites or spines of NK1R-immunopositive neurons (Figure 5h and i). These findings rule out a direct heterosynaptic regulation by NK1R on 5HT release in serotonergic fibers.

DISCUSSION

The results of the present study demonstrate that SP is released within the LS in response to swim stress. Furthermore, our data show that intraseptal as well as systemic administration of a selective NK1R antagonist reduces passive and increases active coping strategies in the forced swim test indicative of an antidepressant-like effect. Possible mechanisms of these behavioral actions might include the activation of septal 5-HT1A receptors as the administration of a selective 5HT1A receptor antagonist into the LS blocks the NK1R antagonist effect on stress coping. Further evidence for an involvement of septal 5-HT transmission in the behavioral effects of NK1R antagonism comes from the finding that NK1R blockade reversed the swim stress-induced decrease in septal 5-HT release and from previous observations that 5-HT microinjection or 5-HT receptor activation presumably through postsynaptic 5-HT1A receptors within the LS promotes active coping behavior and produces antidepressant-like effects in different paradigms including the forced swim test (Martin *et al*, 1990; Schreiber and De Vry, 1993).

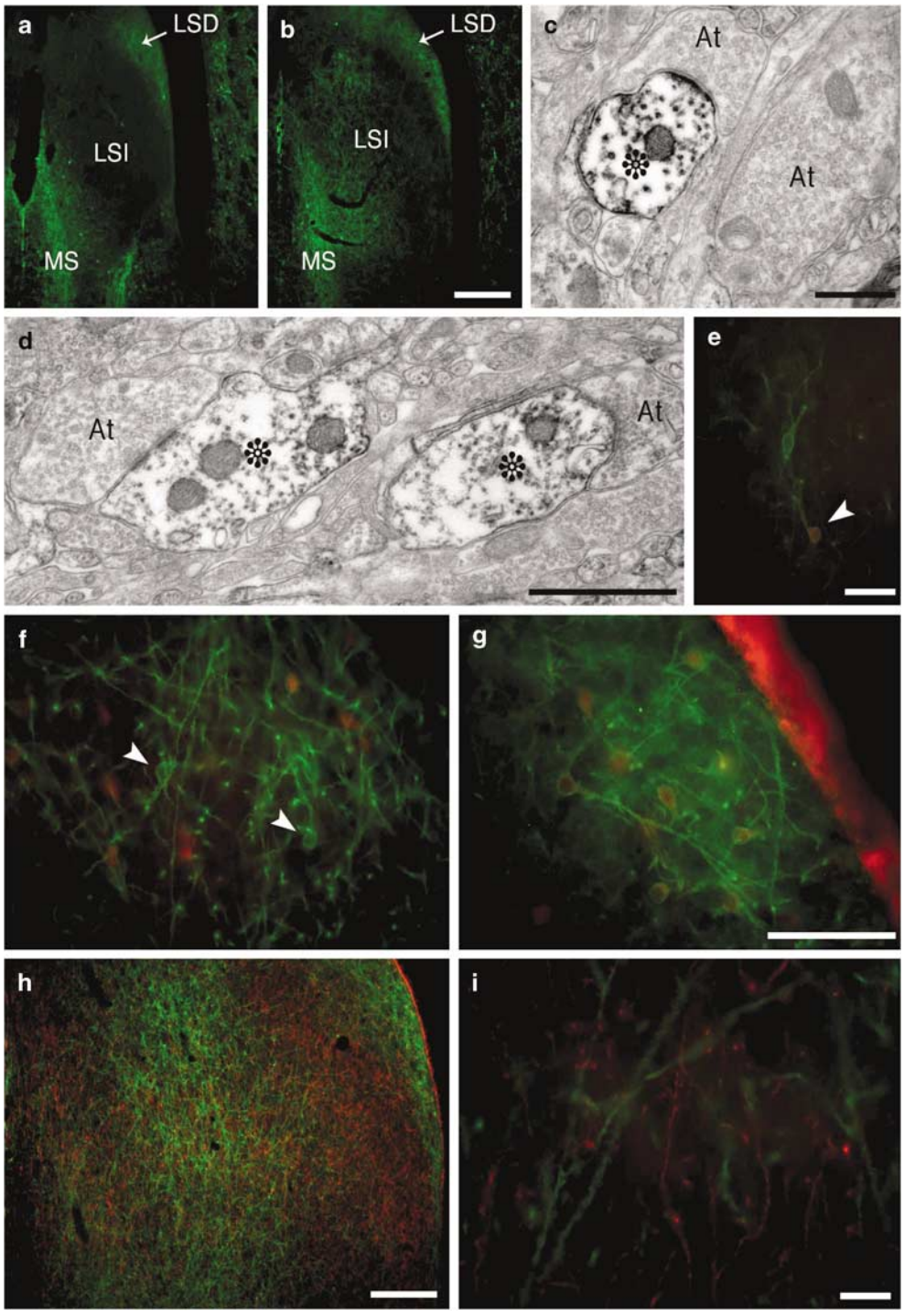

Figure 5 Cellular and subcellular localization of NK1Rs in the septum and codistribution with ChAT, calbindin, and SERT. (a, b) Coronal sections of the LS depicting the immunolocalization of the NK1R at different rostrocaudal levels. The density of NK1-positive neurons in the intermediate part of the lateral septum (LSi) increased moving from rostral to caudal. Intense labeling was also observed in the dorsal part of the lateral septum (LSD) and in the medial septum (MS). (c, d) Electron micrographs showing the immunolocalization of NK1Rs in the LS. Immunoperoxidase labeling was observed primarily in dendritic shafts. NK1 immunopositive dendrites are indicated by asterisks. At, axon terminals. (e) Immunofluorescence image showing labeling for NK1Rs (in green) and ChAT (in red) at the border between LSi and MS. A double-labeled neuron is indicated by the arrowhead. (f, g) Double immunofluorescence labeling for NK1Rs (in green) and calbindin (in red). No colocalization between NK1 (indicated by arrowheads) and calbindin immunostained neurons was observed in the LSi (shown in f). Conversely, in the LSd, nearly all NK1R-expressing neurons appeared to contain calbindin (g). (h, i) Low- and high-magnification immunofluorescence images showing labeling for NK1Rs (in green) and SERT (in red) in the LS. No apparent colocalization was observed between NK1R and SERT in both the LSi and LSd. Scale bars: a, b = 400 μm; c = 500 nm; d = 1 μm; e = 50 μm; f, g = 100 μm; h = 200 μm; i = 10 μm.

Stressor-Induced Release of SP

First indications that septal SP may be involved in stress mechanisms date back to the work of Takayama *et al* (1986) who found decreased SP tissue concentration as well as reduced receptor binding in the septum in response to immobilization stress, which was interpreted as an enhancement of the release of this neuropeptide. This approach, however, was hampered by the fact that no distinction between the extracellular (ie as a signal in interneuronal communication) and the intracellular (ie not related to interneuronal communication) origin of the neuropeptide could be made. In contrast, microdialysis techniques as used in the present study have the advantage of allowing such discrimination as only the extracellular and, thus, signal-acting neuropeptide is monitored (Landgraf *et al*, 1998). However, owing to various technical limitations such as the low recovery of microdialysis probes, very few reports are available demonstrating alteration in SP neurotransmission in brain areas known to be implicated in the regulation of stress and fear/anxiety mechanisms (Ebner and Singewald, 2006). In the case of the LS, however, a sufficient amount of SP was now shown to be accessible even under basal (unstressed) conditions, very likely because the LS contains a high amount of SP-containing fibers and cell bodies (Ljungdahl *et al*, 1978; Sakanaka *et al*, 1982; Gall and Moore, 1984; Szeidemann *et al*, 1995; Hökfelt *et al*, 2004). Notably, previous tracing studies have shown that the SP innervation of the LS derives from both extrinsic as well as intrinsic sources (Sakanaka *et al*, 1982; Szeidemann *et al*, 1995). The present study provides the first evidence that a stressful, emotionally challenging situation such as forced swimming triggers local SP release within the LS, a key area of the limbic stress circuitry important for affective regulation (Sheehan *et al*, 2004; Burgdorf and Panksepp, 2006; Bondi *et al*, 2007).

Effect of NK1R Blockade on Stress-Coping Behavior

Dense expression of NK1Rs has been identified in the LS (Figure 5; see Introduction), supporting a possible functional role of SP transmission within this brain area. Indeed evidence has been provided that septal SP is involved in the modulation of anxiety-related behavior (Gavioli *et al*, 1999, 2002). We, therefore, aimed to reveal the possible behavioral significance of the observed swim stress-induced SP release within the LS. The selective NK1R antagonist L-822429 was used to clarify the relevance of intraseptally released SP for stress-coping behavior. This potent NK1R antagonist has previously been shown to be a useful tool to investigate the behavioral impact of endogenously released SP within the rat amygdala (Ebner *et al*, 2004). The present results show that attenuation of SP/NK1R neurotransmission/neuromodulation within the LS can induce acute changes in the behavioral stress response during forced swimming. Drug-treated animals showed prolonged swimming and reduced floating behavior. This suggests that endogenously released SP within the LS promotes passive coping strategies, which can be shifted to more active coping by the NK1R antagonist. Immobility or floating behavior in the forced swim test is thought to represent a measure of depression-like behavior (Porsolt *et al*, 1977; Cryan *et al*, 2005), indicating that NK1R antagonist treatment elicited an antidepressant-like effect by reducing immobility. This action seems to be mediated selectively via NK1Rs since the inactive (low-affinity) enantiomer had no effect. Moreover, we could show similar behavioral effects after systemic administration of L-822429, indicating that the modulatory action within the LS is a critical component for stress-coping modulation by systemic treatment with NK1R antagonists. Our data confirm previous findings demonstrating similar antidepressant-like effects after acute systemic application of different NK1R antagonists in rats (Zocchi *et al*, 2003; Dableh *et al*, 2005) or genetic disruption of NK1R function in mice (Rupniak *et al*, 2001). While pharmacological antagonisms of NK1R in mice had no effect, it potentiated the antidepressant-like activity of various classical antidepressant agents in the forced swim test (Chenu *et al*, 2006). The forced swim paradigm used in the present study provides a relatively severe and naturally occurring stressful stimulus that evokes anxiety (Heinrichs *et al*, 1994; Korte and De Boer, 2003). Thus, it is conceivable that intraseptally released SP is also involved in processing the anxiogenic nature of a stressful episode. As mentioned above, previous studies indeed have shown that microinjections of SP into the LS promote an anxiogenic-like effect in the elevated plus-maze test in rats (Gavioli *et al*, 1999). Interestingly, the same authors found that intraseptal administration of a NK1R antagonist blocks the anxiogenic effect of intracerebroventricularly injected SP, whereas the antagonist treatment '*per se*' had no anxiolytic effect (Gavioli *et al*, 2002). Thus, it seems that the endogenous SP tone on NK1Rs in the LS during elevated plus-maze exposure is too low to elicit significant behavioral effects by blocking NK1Rs. However, it is conceivable that intraseptal NK1R blockade is effective to modulate anxiety responses to more severe stressors, such as forced swim stress, which clearly activates the intraseptal SP system, as shown in this study. This possibility is currently under investigation.

Behaviorally Significant Interaction between SP and 5-HT Systems within the LS

Next we wanted to gain further insight into the mechanism(s) involved in the effects on stress coping elicited by modulation of SP transmission in the LS. The LS, especially the dorsolateral part, receives dense 5-HT innervation from the dorsal raphe nucleus (Köhler *et al*, 1982; Vertes, 1991; Waselus *et al*, 2006). Various 5-HT binding sites, including prominently the 5-HT1A receptor subtype, have been localized within the LS (Pazos and Palacios, 1985; Pazos *et al*, 1985; Pompeiano *et al*, 1992; Waeber *et al*, 1994; Gustafson *et al*, 1996; Morales *et al*, 1998; Lanfumey and Hamon, 2000). Importantly, 5-HT1A receptor activation within the LS was shown to produce antidepressant-like effects in different paradigms including the forced swim test (see above). Our data suggest an involvement of septal 5-HT1A receptors in the antidepressant efficacy of NK1R antagonists, as the administration of the selective and silent 5HT1A receptor antagonist WAY-100635 into the LS blocked the antidepressant-like effect elicited by the NK1R antagonist. Notably, administration of WAY-100635 alone had no effects on stress-coping behavior during forced swimming (GM Singewald *et al*, unpublished data)

confirming previous studies after systemic administration (O'Neill and Conway, 2001; De Vry *et al*, 2004; Cryan *et al*, 2005). In an attempt to further characterize the interaction of 5-HT and SP during stress, we were able to demonstrate a pronounced decrease in septal 5-HT efflux in response to a 10-min forced swim stress, confirming results of previous studies (Kirby *et al*, 1995; Kirby and Lucki, 1997, 1998). Since we exposed animals to the swim stress only for 10-min of the 30-min sampling interval, the measured stress-induced transmitter concentration may not reflect the maximum degree of transmitter release. Intraseptal NK1R blockade reversed the stress-induced septal decrease in 5-HT release in an enantiomer selective manner and led to an increase in 5-HT release indicating an inhibitory effect of endogenous SP on 5-HT release in the LS. Thus, our data indicate that antidepressant-like efficacy of NK1R antagonism is associated with an increased septal 5-HT release. The assumption that antidepressant effects coincide with increased 5-HT levels in the LS is supported by previous findings demonstrating that inescapable shock exposures reduce 5-HT release in septal slice preparations that can be reversed by antidepressant treatment (Sherman and Petty, 1982). This interpretation is consistent with other studies demonstrating that animals resistant to shock-induced learned helplessness show higher levels of septal 5HIAA, a 5-HT metabolite, than helpless subjects (Ronan *et al*, 2000) and that intraseptal 5-HT1A receptor activation produces antidepressant-like effects (Martin *et al*, 1990; Schreiber and De Vry, 1993). Together these findings suggest that stress precipitates a depression-like phenotype by blunting 5-HT release into the LS, which can be reversed by antidepressant treatments. However, it should be mentioned that other researchers suggested that increased 5-HT release in the LS promotes passive coping strategies in the forced swim test since on the second swim day, when the tendency toward immobility is increased, the reduction in stress-induced septal 5-HT release was attenuated (Kirby and Lucki, 1997, 1998). Thus, further investigation is necessary to clarify these discrepancies.

The increased extracellular 5-HT efflux by NK1R antagonism is likely caused by interaction with release mechanisms rather than by interfering with 5-HT reuptake or degradation because neither 5-HT transporters nor monoamine oxidase activity is modulated by different NK1R antagonists (Conley *et al*, 2002; David *et al*, 2004; Lieb *et al*, 2005). To determine the functional sites for septal SP/5-HT interactions, we used immunofluorescence and immunoelectron microscopy for precise cellular and subcellular localization of NK1Rs in rat LS. Consistent with previous autoradiographic (Saffroy *et al*, 2003) and immunohistochemical (Nakaya *et al*, 1994) studies we found a moderate-to-dense distribution of NK1R immunoreactive neurons in the LS, mostly in the laterodorsal part. Notably, in this subregion we observed that NK1Rs are highly expressed on calbindin-positive (a marker of GABAergic interneurons) neurons primarily at their soma-to-dendritic domain. This is in line with a previous study demonstrating that SP-containing fibers innervate calbindin-containing neurons in the LS (Szeidemann *et al*, 1995). Thus, a postsynaptic mechanism through an inhibitory relay between SP and 5-HT release seems likely. Similar to evidence from other brain areas (eg Maubach *et al*, 2001),

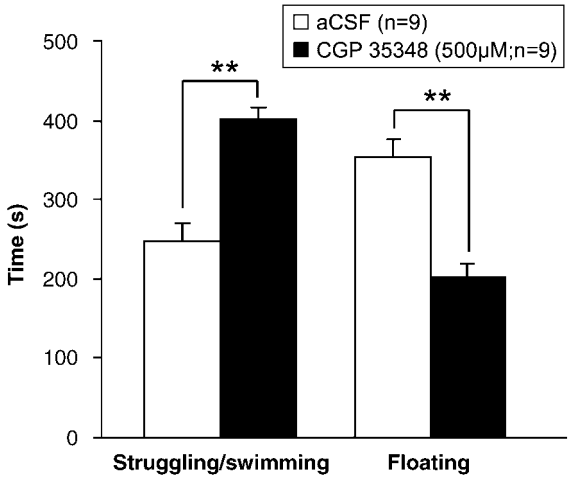

Figure 6 Effect of the selective $GABA_B$ receptor antagonist CGP 35348 applied into the LS on the time animals spent struggling/swimming (active coping) and floating (passive coping) during 10 min of forced swimming. Data are presented as means + SEM. $^{**}p < 0.01$ *vs* vehicle-treated controls (Student's *t*-test).

SP might promote the release of an inhibitory transmitter such as GABA from interneurones that would, in turn, inhibit 5-HT release (Figure 7). Since $GABA_B$ receptors are highly expressed on nerve terminals and activation of presynaptic $GABA_B$ receptors inhibits the release of various transmitters including monoamines such as 5-HT (Misgeld *et al*, 1995), we examined whether septal $GABA_B$ receptors are involved in this mechanism. Our data suggest an involvement of septal $GABA_B$ receptors as the administration of a selective $GABA_B$ receptor antagonist into the LS modulated stress coping in a similar way as antidepressant drugs (Figure 6). However, whether septal $GABA_B$ receptor blockade increases extracellular 5-HT levels within the LS is currently under investigation. An alternative possibility would be that SP modulates 5-HT release via an inhibitory effect involving presynaptically localized NK1Rs on 5-HT terminals. A presynaptic inhibitory mechanism of SP has already been postulated for the release of glutamate (Malcangio and Bowery, 1999; Sekizawa *et al*, 2003). Although we observed sporadic NK1R immunoreactive presynaptic boutons forming mostly symmetric but also asymmetric synapses, no detectable levels of NK1Rs on serotonergic afferents to the LS were observed. Hence, it can be excluded that presynaptic NK1Rs exert a significant modulation of 5-HT release from the same terminal endings in the LS (Figure 7).

Taken together, the results of the present study demonstrate that exposure to swim stress increases SP release within the LS. The released endogenous SP in this brain area is involved in the modulation of behavioral responses to this stressor since the stress-coping style was switched to a more active one by intraseptal administration of a selective NK1R antagonist. This behavioral effect seems to be mediated by enhanced septal 5-HT transmission via 5-HT1A receptors since (i) it can be blocked by intraseptal 5-HT1A receptor antagonism and (ii) intraseptal NK1R antagonist administration was shown to facilitate the attenuated local release of 5-HT during stress. Notably, the latter finding indicates that such a stimulatory effect on

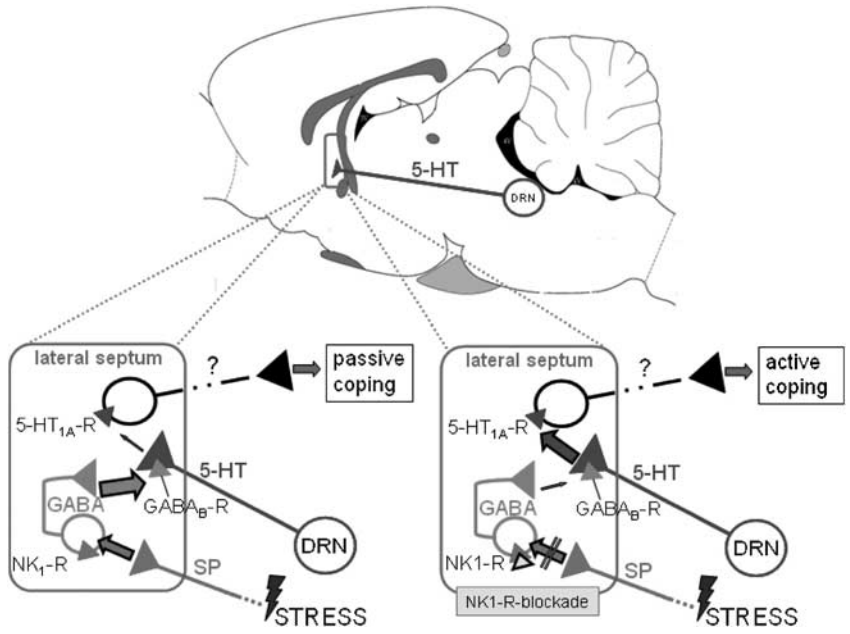

Figure 7 Proposed neurochemical mechanisms of how activation (left) or blockade (right) of NK1Rs within the LS modulate septal 5-HT release and coping behavior during stress exposure. Our data indicate an inhibitory effect of endogenous SP on septal 5-HT released from 5-HT-containing afferents via an indirect mechanism. Enhanced SP release during stress is proposed to promote the release of GABA from interneurons that inhibits 5-HT release via activation of presynaptic $GABA_B$ receptors. Reduced 5-HT transmission in the septum is associated with a passive stress-coping style. By blocking NK1Rs, the inhibitory effect on septal 5-HT transmission is counteracted resulting in enhanced 5-HT release and a facilitation of active stress-coping strategies.

5-HT transmission can be elicited locally in a terminal region of 5-HT neurons and does not necessarily involve interaction with NK1Rs in raphe nuclei. Until now it was generally assumed that NK1R-mediated effects on 5-HT transmission involved interactions with neuronal firing at the cell body level of 5-HT neurons via indirect (eg glutamate-mediated) alteration of local 5-HT levels and modulation of 5-HT1A autoreceptors (Blier *et al*, 2004; Gobbi and Blier, 2005; Guiard *et al*, 2006). Consequently, increased 5-HT function was thought to result after downregulation or functional desensitization of somatodendritic 5-HT1A-inhibitory autoreceptors, requiring chronic treatment (Froger *et al*, 2001; Adell, 2004; Guiard *et al*, 2005; but see Conley *et al*, 2002). Our data demonstrate for the first time immediate NK1R antagonist-induced elevation of extracellular 5-HT levels in the LS, a dorsal raphe terminal field that is known to play a crucial role in processing stress and anxiety reactions. It seems that the observed effect is regionally very discrete, since no facilitatory effects of NK1R antagonist on 5-HT efflux could be demonstrated in other 5-HT target areas such as the frontal cortex and hippocampus (Froger *et al*, 2001; Millan *et al*, 2001; Lejeune *et al*, 2002; Zocchi *et al*, 2003; Guiard *et al*, 2004, 2006). The now observed action may be of relevance for the clinical evidence that NK1R antagonists exert therapeutic effects also after acute or short-time treatment (McLean, 2005). Furthermore, by combining SSRIs with a NK1R antagonist, a faster onset in addition to the observed potentiation of neurochemical (Lejeune *et al*, 2003; Guiard *et al*, 2004, 2005, 2006) and behavioral (Chenu *et al*, 2006) effects of SSRIs may be expected, thus improving the existing therapeutic possibilities to modulate affective and behavioral responses to stress in stress-related disorders (Millan, 2006).

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DISCLOSURE/CONFLICT OF INTEREST

The author(s) declare that, except for income received from my primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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