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Modulation of basal and stress-induced amygdaloid substance P release by the potent and selective NK1 receptor antagonist L-822429

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Abstract

It has been shown that anxiety and stress responses are modulated by substance P (SP) released within the amygdala. However, there is an important gap in our knowledge concerning the mechanisms regulating extracellular SP in this brain region. To study a possible self-regulating role of SP, we used a selective neurokinin-1 (NK1) receptor antagonist to investigate whether blockade of NK1 receptors results in altered basal and/or stress-evoked SP release in the medial amygdala (MeA), a critical brain area for a functional involvement of SP transmission in enhanced anxiety responses induced by stressor exposure. *In vitro* binding and functional receptor assays revealed that L-822429 represents a potent and selective rat NK1 receptor antagonist. Intra-amygdaloid administration of L-822429 via inverse microdialysis enhanced basal, but attenuated swim stress-induced SP release, while the low-affinity enantiomer of L-822429 had no effect. Using light and electron microscopy, synaptic contacts between SP-containing fibres and dendrites expressing NK1 receptors was demonstrated in the medial amygdala. Our findings suggest self-regulatory capacity of SP-mediated neurotransmission that differs in the effect on basal and stress-induced release of SP. Under basal conditions endogenous SP can serve as a signal that tonically inhibits its own release via a NK1 receptor-mediated negative feedback action, while under stress conditions SP release is further facilitated by activation of NK1 receptors, likely leading to high local levels of SP and activation of receptors to which SP binds with lower affinity.

Keywords: aggression, amygdala, microdialysis, neurokinin autoreceptor, neuropeptide substance P, swim stress.

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Substance P (SP) and its preferred target, the neurokinin-1 (NK1) receptor, play an important role in the modulation of stress and anxiety responses (Culman and Unger 1995; Rupniak 2002; Adell 2004; Ebner and Singewald 2006). Previous studies have demonstrated that SP administered centrally elicits physiological and behavioural responses similar to those exhibited in response to stressors (Unger *et al.* 1988; Culman *et al.* 1997). Although SP may induce anxiolytic-like effects in specific brain areas including the nucleus basalis magnocellularis or the ventral pallidum (Hasenohrl *et al.* 1998; Nikolaus *et al.* 1999), activation of

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Abbreviations used: aCSF, artificial CSF; BSA, bovine serum albumin; CHO, Chinese hamster ovary; hERG, human ether-à-go-go-related gene; LI, like immunoreactivity; MeA, medial amygdala; NGS, normal goat serum; NK1, neurokinin-1; NKA, neurokinin-A; NKB, neurokinin-B; PB, phosphate buffer; SP, substance P; TBS, Tris-buffered saline.

NK1 receptors by SP has been found to increase anxiety-related behaviours in a variety of brain areas including the amygdala (Aguiar and Brandao 1996; Teixeira *et al.* 1996; Gavioli *et al.* 1999, 2002; Duarte *et al.* 2004; Ebner *et al.* 2004). Conversely, pharmacological blockade or genetic deletion of NK1 receptors decreases anxiety-related behaviours (Santarelli *et al.* 2001; Rupniak *et al.* 2000; for review see Ebner and Singewald 2006). Based on these findings, SP has been suggested to play a role in the pathophysiology of stress-related psychiatric disorders including anxiety disorders and depression, opening up the possibility of a therapeutic effect for NK1 receptor antagonists in the treatment of these disorders (Rupniak 2002; Holmes *et al.* 2003; Herpfer and Lieb 2005; Ebner and Singewald 2006; Millan 2006; Rosenkranz 2007; Mathew *et al.* 2008). Indeed, the NK1 receptor antagonist GR205171 was shown to relieve symptoms of social phobia and to attenuate regional cerebral blood flow responses to the Trier Social Stress Test in the medial temporal lobe including the amygdala (Furmark *et al.* 2005). Very recently beneficial effects of NK1 receptor antagonism in alcohol dependent subjects with high trait anxiety (> 39 on the Spielberger Trait Anxiety Inventory) were reported (George *et al.* 2008). Although NK1 receptor antagonists have been shown to relieve the symptoms of depression (Kramer *et al.* 1998, 2004), conflicting results have been obtained more recently from depression trials (Keller *et al.* 2006; Hafizi *et al.* 2007). These variable drug effects are calling for a better characterization of the mechanisms involved. For example, the brain sites and mechanisms mediating the behavioural effects of these drugs are poorly understood. Previous studies have identified the amygdala as one of the potential sites where NK1 receptor antagonists affect anxiety and stress reactions (for review see McLean 2005; Ebner and Singewald 2006). The density of SP-immunoreactive fibres (Roberts *et al.* 1982; Hokfelt *et al.* 2004) as well as of NK1 receptors was shown to be particularly prominent within the medial amygdala (MeA) and to a lower extent in other amygdaloid subregions such as the central amygdala and cortical areas (Maeno *et al.* 1993; Nakaya *et al.* 1994). Increased SP mRNA levels in the MeA and parts of the hypothalamus have been demonstrated in response to chronic mild stress (Sergeyev *et al.* 2005). In an earlier study, we demonstrated that emotional stress increases the *in vivo* release of SP in the MeA of rats and that the released SP in this brain area elicits an anxiogenic-like effect (Ebner *et al.* 2004). Fear provocation in phobic patients was also shown to reduce NK1 receptor availability in the amygdala, indicating increased release of endogenous SP (Michelgard *et al.* 2007). Thus, these data indicate an up-regulation of intra-amygdala SP transmission in response to stressful and anxiety-provoking stimuli both in animals and humans.

However, there is still an important gap in our understanding of the mechanisms regulating SP release in the amygdala. As there is evidence, in particular in the somatosensory system (for review see Malcangio and Bowery 1999), for autoregulation of SP release, the aim of the present study was to study whether a possible self-regulating role of SP can be observed in the amygdala. By using a highly selective and potent NK1 receptor antagonist we, therefore, tested the hypothesis that blockade of NK1 receptors results in altered basal and/or stress-evoked SP release in the MeA. Furthermore, we wanted to gain anatomical support for a potential self-regulation using light and electron microscopy.

Material and methods

Drugs

(2*S*,3*S*)-*N*-{[2-cyclopropoxy-5-(5-trifluoromethyl)tetrazol-1-yl]benzyl}-2-phenylpiperidin-3-amine dihydrochloride (L-822429) and its enantiomer (2*R*,3*R*)-*N*-{[2-cyclopropoxy-5-(5-trifluoromethyl)tetrazol-1-yl]benzyl}-2-phenylpiperidin-3-amine dihydrochloride were synthesized in house via reductive amination of 2-(cyclopropoxy)-5-[5-(trifluoromethyl)tetrazol-1-yl]benzaldehyde (Elliott 1999) with (+)-(2*S*,3*S*)-2-phenylpiperidin-3-amine (Burns *et al.* 2000; Fujino *et al.* 2004) and (−)-(2*R*,3*R*)-2-phenylpiperidin-3-amine, respectively. For *in vivo* experiments (see below), all compounds were reconstituted in distilled water and dissolved in artificial CSF (aCSF).

Binding affinity determinations for the cloned rat NK1–3 receptors and native rat NK1 receptor

Neurokinin receptor binding assays were performed using intact Chinese hamster ovary (CHO) cells or plasma membranes thereof, expressing recombinant rat NK1, NK2 or NK3 receptors, with a modification of the assay conditions previously described (Cascieri *et al.* 1992; Hale *et al.* 1998). NK1 receptor binding assay was also performed using rat brain membranes according to the method previously described (Tousignant *et al.* 1991). Binding affinities of test compounds were determined by the displacement of [^{125}I]-Tyr^{8}-SP (NEX-1520; Perkin Elmer, Waltham, MA, USA) from the rat cloned NK1 receptors, [^{125}I]-neurokinin-A (NKA) (NEX-2520; Perkin Elmer) from the rat cloned NK2 receptors or [^{125}I]-His, MePhe^{7}-neurokinin-B (NKB) (NEX-2850; Perkin Elmer) from the rat cloned NK3 receptors. [^{3}H][Sar^{9},Met(O$_2$)^{11}]SP (NET1025; Perkin Elmer) was used in the NK1 receptor binding assay performed using rat brain membranes. Briefly, dilutions of test compounds in 5 μL dimethylsulphoxide were incubated with cells in 0.25 mL of 50 mM Tris–HCl, pH 7.5, containing 5 mM $MnCl_2$, 150 mM NaCl, 0.02% bovine serum albumin (BSA), 40 μg/mL bacitracin, 0.01 mM phosphoramidon and 4 μg/mL leupeptin at 25°C for 60 min. The receptor–ligand complexes were harvested by filtration over GF/B filters pre-soaked in 0.25% polyethylenimine for NK1 and NK2 or GF/C filters pre-soaked in 0.5% BSA for NK3 using a Tomtec 96-well harvester (Hamden, CT, USA). A similar procedure as above described was used for NK1 receptor assays with rat brain membranes. Membranes were incubated in 50 mM Tris–HCl buffer containing 3 mM $MnCl_2$, 2 mg/mL BSA, 40 μg/mL bacitracin, 2 μg/mL chymostatin and 4 μg/mL leupeptin for 30 min at 25°C and the test ligand in a final volume of 1 mL. Reaction was terminated by rapid filtration through Whatman GF/B

glass filters, pre-soaked with 0.3% polyethylenimine, using a Brandel M24R Cell Harvester. Non-specific binding was determined using excess SP (1 or 10 μM), NKA or NKB (1 μM) for the three receptor subtypes respectively. Studies with membrane preparations of CHO cells expressing the cloned rat receptors were conducted essentially as the whole cell assay, except NaCl was excluded from the assay buffer. The concentration necessary to produce 50% inhibition (IC_{50}) was determined using the Prism software package (Graph Pad, San Diego, CA, USA).

Inhibition of substance P-induced inositol phosphate synthesis

Functional antagonism can be assayed by determining the ability of compounds to inhibit SP-induced inositol phosphate formation in CHO cells expressing rat NK1 receptors by a modification of Brandish *et al.* (2003). All steps were conducted in a 37°C incubator with 5% CO_2 unless otherwise noted. Cells (2.5×10^4/well) were plated overnight in 150 μL of growth medium in 96-well plates. The following day, the cells were washed once with loading medium [inositol-free medium (MP Biomedicals, Solon, OH, USA #1642954) supplemented with 0.02% BSA, 2 mM L-glutamine, 70 mM HEPES, pH 7.5 and 1x hypoxantine-thymidine supplement (100x; Gibco #11067-030, Grand Island, NY, USA)], followed by the addition of 1 μCi of ^{3}H-myo-inositol (NET114A; Perkin Elmer) in 150 μL loading medium. Inositol phosphate generation was measured the following day as follows. LiCl (10 mM) was added to each well for 15 min. The monolayers were then pre-incubated with antagonist (0.03–10 μM) for 30 min followed by a 30 min challenge with agonist (SP) after which the medium was removed and the cells were lysed for 1 h in 60 μL 10 mM formic acid at 25°C. A 25 μL aliquot of each lysate was incubated with 1 mg RNA Binding Y Si (2–5 μm) SPA beads (RPNQ0013; GE Healthcare, Buckinghamshire, UK) in Optiplates for 2 h with shaking at 25°C. The resulting ^{3}H-inositol phosphates were quantitated using a Packard Topcount (Perkin-Elmer) and data were analysed using the Prism software package (Graph Pad).

Affinity for selected ion channels

Affinity for L-type calcium channels was measured by displacement of *cis*-(+)-[*n*-methyl-^{3}H]diltiazem (Perkin Elmer) binding using the modified procedures of Schoemaker and Langer (1985) and membranes prepared from human skeletal muscle (Himmel *et al.* 1991). Affinity for the channel responsible for the rapid delayed rectifier K^+ current of human ventricular myocardium (I_{Kr}) was assessed by the displacement of ^{3}H-MK-0499 binding from human ether-à-go-go-related gene (hERG) K^+ channel protein expressed in human embryonic kidney 293 cells as described (Greengrass 2003). Affinity for the cloned human cardiac sodium channel ($Na_V1.5$) expressed in human embryonic kidney 293 cells was assessed by displacement of ^{3}H-BPBTS (*N*-{[2′-(aminosulphonyl)biphenyl-4-yl]methyl}-*N*′-(2,2′-bithien-5-ylmethyl)succinamide) binding as described by previously (Priest *et al.* 2004).

Immunocytochemistry

For immunocytochemical studies, four adult male Sprague–Dawley rats were deeply anaesthetized with thiopental (thiopentone sodium 100 mg/kg, i.p) and perfused transcardially with saline for 15 min by a fixative composed of 4% *p*-formaldehyde, 0.2% picric acid, made up in 0.1 M phosphate buffer (PB), pH 7.2–7.4. For electron microscopy studies, 0.05% glutaraldehyde was included in the fixative. The brains were removed quickly, rinsed extensively in PB, and sectioned in the coronal plane on a vibratome at 40 μm thickness for immunofluorescence studies and at 70 μm thickness for electron microscopy.

Immunofluorescent experiments were performed as described previously (Ferraguti *et al.* 2004). Briefly, free-floating sections were treated with 1% $NaBH_4$ (Sigma, St Louis, MO, USA) in PB for 20 min and extensively washed in PB. After a further wash in Tris-buffered saline (TBS), pH 7.2–7.4, non-specific binding was blocked by 20% normal goat serum (NGS) incubated for 2 h at 23°C. Sections were then incubated for 72 h (4°C) in primary antibodies alone or in combination: rabbit polyclonal anti-SP (1 : 10 000; Chemicon, Temecula, CA, USA) and guinea pig polyclonal SP receptor (1 : 3000; Biotrend, Köln, Germany) made up in 1% NGS, 0.1% Triton X-100 and TBS (TBS-Triton X). The following day, after extensive washes with TBS, sections were incubated overnight (4°C) in secondary antibodies: donkey anti-KABBIT Cy3 (1 : 400; Jackson ImmunoResearch, West Grove, PA, USA) and donkey anti-guinea pig Alexa (1 : 1000; Molecular Probes, Eugene, OR, USA). After three washes in TBS, sections were then mounted onto gelatine-coated slides and coverslipped with Vectashield (Vector Laboratories, Burlingame, CA, USA). To control for a possible cross-reactivity between IgGs in double immunolabelling experiments, some sections were processed through the same immunocytochemical sequence except that only one primary antibody was applied; but the full complement of secondary antibodies was maintained. In addition, the secondary antibodies utilized were highly pre-adsorbed to the IgGs of numerous species. All these control reactions always resulted in a lack of labelling of the species-unrelated secondary antibodies, confirming the specificity of the immunosignals.

Immunofluorescence was studied using a Zeiss Axioplan 2 microscope with epifluorescence illumination equipped with band-path filter blocks. Images were recorded through an Axiocam (Carl Zeiss GmbH, Vienna, Australia) camera and were analysed and displayed using the OPENLAB software (verson 3.1.5; Improvision, Coventry, UK). Contrast and brightness were adjusted for the entire frame and no part of a frame was modified in any way.

Immunocytochemistry for electron microscopy was performed according to previously published procedures (Ferraguti *et al.* 2005). Free-floating sections were incubated for 2 h in 20% NGS diluted in TBS and then incubated for 72 h (4°C) in primary antibodies: rabbit polyclonal anti-SP (1 : 5000; Chemicon) and guinea pig polyclonal anti-SP receptor (1 : 1500; Biotrend) made up in TBS containing 1% NGS. After the primary antibody, the sections were extensively washed with TBS and then incubated overnight (4°C) in secondary antibodies: goat anti-rabbit nanogold (1 : 100; Nanoprobes, Yaphank, NY, USA) and goat anti-guinea pig biotinylated (1 : 100; Vector Laboratories). In double labelling studies, SP was visualized by silver-intensified immunogold reaction and SP receptor by immunoperoxidase reaction. After three washes in TBS, the sections were washed in double-distilled water, followed by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes) for 8–15 min. Sections were subsequently incubated in ABC complex (diluted 1 : 100; Vector Laboratories) in TBS overnight at 4°C. Peroxidase was visualized with 3-3′-diaminobenzidine (0.5 mg/mL) using 0.01% H_2O_2 as substrate for 5–10 min. After several washes in 0.1 M PB, sections

were then treated with 2% OsO_4, contrasted with 1% uranyl-acetate, dehydrated and embedded in epoxy resin (Durcupan ACM, Fluca; Sigma-Aldrich, Gillingham, UK). Serial ultrathin (70- to 80-nm-thick) sections were collected on pioloform-coated copper grids and analysed in a transmission electron microscope (Philips CM120, Eindhoven, Germany). Micrographs were recorded using a 11 Megapixel side-mounted TEM CCD-camera (Morada; SIS, Olympus, Münster, Germany). Samples were obtained from two different rat brains, and two blocks of each animal were cut for electron microscopy.

Pharmacokinetic profiling

Male Sprague–Dawley rats were dosed with L-822429 either i.v. or orally (p.o.) at 1 mg/kg of body weight and pharmacokinetic parameters were calculated by established equations after measuring L-822429 levels in plasma by liquid chromatography-tandem mass spectrometry.

Microdialysis

Experiments were carried out on adult male Sprague–Dawley rats (250–350 g). Prior to 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 07:00 h, 21 ± 1°C, 60% humidity, pelleted food and water *ad libitum*) for at least 3 weeks after delivery from the supplier. All procedures were approved by the Austrian Animal Experimentation Ethics Board (Bundesministerium für Wissenschaft und Verkehr, Kommission für Tierversuchsangelegenheiten, Austria) in compliance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes ETS no.: 123. Every effort was taken to minimize animal suffering and the number of animals used.

Surgery

A microdialysis probe (U-shaped Hemophan® membrane molecular cut-off of 18 kDa, Wuppertal, Germany) was stereotaxically implanted under sodium pentobarbital (40 mg/kg, i.p.) and ketamine (50 mg/kg, i.p.) anaesthesia according to a stereotaxic atlas (Paxinos and Watson 1998). The probe was positioned with its U-shaped tip reaching the MeA (implantation coordinates: 2.8 mm caudal to bregma, 3.4 mm lateral to the midline, 9.0 mm below the surface of the skull). The probes were fixed to the skull with two jeweller's screws and dental cement. The two endings of each probe were connected to approximately 5 cm long pieces of PE-20 polyethylene tubing and fixed with dental cement. After surgery animals received Buprenorphine injections (0.5 mg/kg) as analgesic treatment.

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 non-specific stress responses during the experiments; 24 h before the experiment, animals were kept in the experimental room and allowed to habituate. Experiments were performed between 07:00 and 15:00 h.

Effects of intra-amygdaloid administration of a NK1 receptor antagonist on the release of SP in the MeA

Two days after surgery, the microdialysis probe was connected to a syringe mounted onto a microinfusion pump and superfused with 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, at a rate of 6.5 µL/min. To minimize the binding of SP to the plastic surfaces and to reduce peptidase activity, BSA (0.2%) and bacitracin (0.03%) were included in the perfusion medium. After an equilibration period of 120 min, nine consecutive 30-min dialysates were collected directly into Eppendorf vials, which were stored at −80°C until assay. Starting from dialysis period 3, animals were dialysed either with vehicle (aCSF; $n = 9$) or aCSF containing the NK1 receptor antagonist (L-822429; 1, 10 or 50 µM; $n = 8$) or its low-affinity 2*R*,3*R* enantiomer (1 or 10 µM; $n = 10$ or 8, respectively). At the beginning of dialysis period 7, each animal was transferred from their home cage into a square plastic tank (35 × 35 × 41 cm) filled with water (20°C) up to a level of 30 cm and forced to swim for 10 min with ongoing microdialysis. After swim stress exposure, rats were gently dried using a towel, returned to their home cages with ongoing microdialysis for further 80 min.

Histology

After the experiment, animals were killed by an overdose of pentobarbital and their brains removed. For histological verification of the localization of the microdialysis probes, brains were sectioned and 60 µm coronal sections were stained with cresyl violet. Successful location of the probe in the MeA (Fig. 2) were determined on the basis of previous definitions of a brain atlas (Paxinos and Watson 1998).

Quantification of SP in microdialysates

The content of SP in microdialysates was measured by a highly sensitive and selective radioimmunoassay (detection limit: 0.3 fmol/sample; cross-reactivity of the antiserum RD2 with other related peptides, like NKA and NKB, was < 0.01 and 0 respectively) using synthetic SP (Sigma) as a standard and iodinated peptide (approximate specific activity 74 TBq/mmol = 2000 Ci/mmol; Amersham) as a tracer (for a detailed description see Ebner *et al.* 2004).

Statistical analysis

Experimental subjects were included in the statistical analysis only if the microdialysis probes were localized in the target brain area (Fig. 2). Statistical analysis was performed using a computer software package (GB-Stat 6.0; Dynamic Microsystems, Silver Springs, MD, USA). Data obtained from microdialysis experiments were expressed as a percentage of averaged baseline values and analysed by a two-way ANOVA with repeated measures (treatment × time) followed by Fisher's Least Significant Difference *post hoc* analysis. Data were presented as mean ± SEM. Significance was accepted if $p < 0.05$.

Results

Selectivity of L-822429 for the cloned rat NK1–3 receptors and native NK1 receptor

In vitro binding studies revealed that L-822429 binds with high affinity to the rat cloned NK1 receptor (IC_{50} = 1.1 nM)

Table 1 Binding affinity (IC_{50}, nM): comparison of binding affinities of L-822429 and its low-affinity enantiomer for the cloned rat NK1–3 receptors and native NK1 receptor

Compound	Rat NK1	Rat NK2	Rat NK3
L-822429 (2*S*,3*S*)	Cloned: 1.1 ± 0.2 (10) Native: 1.6 ± 0.6 (3)	> 10 000 (4)	3200 ± 100 (4)
Enantiomer (2*R*,3*R*)	Cloned: > 10 000 (3) Native: > 10 000 (3)	> 10 000 (4)	> 10 000 (4)

The IC_{50} values are represented as mean ± SEM (number of experiments).

and with similar affinity to the native NK1 receptor from rat brain membranes (IC_{50} = 1.6 nM; Table 1). L-822429 was > 9000- and 2900-fold selective for the rat NK1 receptor over the rat NK2 and NK3 receptors, respectively (Table 1). In contrast, the less active (2*R*,3*R*) enantiomer demonstrated little to no binding affinity for any of the three rat cloned NK1–3 receptors and native NK1 receptor from rat brain (IC_{50} values > 10 μM, Table 1).

Affinities for selected ion channels

L-822429 demonstrated weak affinities of 1.4–6.1 μM for three ion channels (L-type calcium channel, hERG and cardiac sodium channel) therefore, demonstrating excellent selectivity (1200- to 5500-fold) for rat NK1 receptor relative to these ion channels (data not shown). The (2*R*,3*R*) enantiomer demonstrated similar weak affinities to that of L-822429 for the hERG and sodium channels.

Inhibition of substance P-induced inositol phosphate synthesis

In cells expressing the cloned rat NK_1 receptor, SP stimulated inositol phosphate accumulation seven- to eight-fold in a dose-dependent manner with an EC_{50} of 0.50 nM. Pre-treatment with L-822429 (0.03–10 μM), prior to SP stimulation, increased the EC_{50} of SP in a dose-dependent manner without any effect on the maximal SP response (Fig. 1a). A Schild plot transformation of those data yielded a linear curve with a calculated K_b of 7.3 nM and a slope approaching 1.0, indicating the compound is a potent competitive-like NK_1 receptor antagonist (Fig. 1b). Furthermore, L-822429 demonstrated no agonist activity (inositol phosphate generation) in these cells when tested up to 3 μM in the absence of SP (data not shown). In contrast, the low-affinity enantiomer had no effect on inositol phosphate generation in these cells at up to 10 μM in the absence or presence of SP, in agreement with its poor rat NK_1 receptor binding affinity (data not shown).

Pharmacokinetic profiling of L-822429

L-822429 was shown to be bioavailable in rats with plasma half-lives of 2 h by both routes of administration, either i.v. or orally (p.o.) at 1 mg/kg of body weight. Following i.v. dosing, a clearance rate of 58 mL/min/kg and volume of distribution of 8.5 L/kg was observed and a maximal plasma concentration of 9 nM was achieved at 1.2 h post-oral dosing.

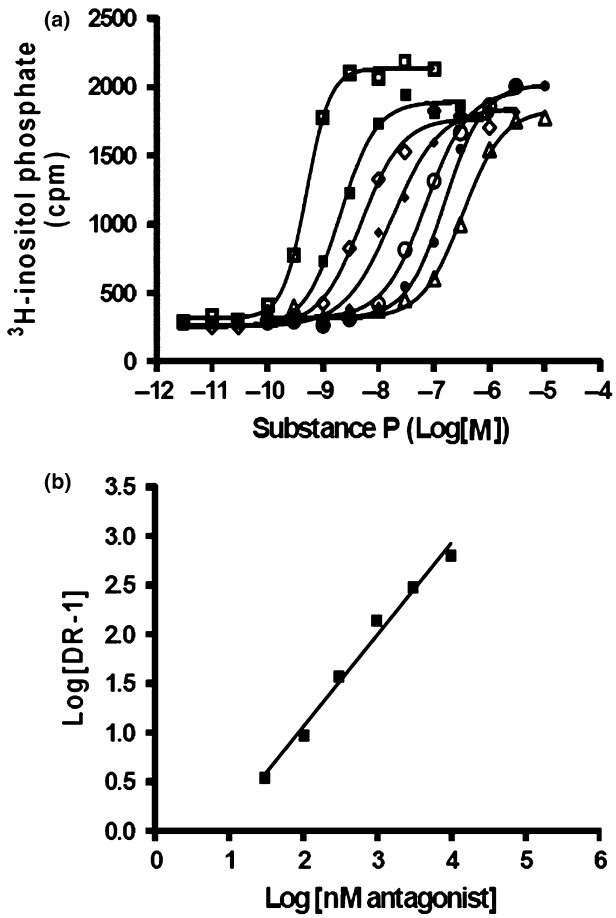

Fig. 1 (a) Antagonism of substance P (SP)-stimulated inositol phosphate generation by L-822429 in CHO cells expressing rat NK_1 receptors. Cells, pre-loaded with ^{3}H-myoinositol, were pre-treated for 30 min with dimethylsulphoxide (□) or L-822429 [0.03 μM (■); 0.1 μM (◇); 0.3 μM (◆); 1 μM (○); 3 μM (●); 10 μM (Δ)] prior to a 30 min challenge with increasing concentrations of SP. Intracellular ^{3}H-inositol phosphate generation was quantitated as indicated in Materials and methods. Data points represent mean duplicate determinations. (b) Schild Plot of antagonism of SP-stimulated inositol phosphate generation by L-822429 in CHO cells expressing rat NK_1 receptors. Dose ratio (DR) = EC_{50} with antagonist dose/EC_{50} without antagonist. K_b is the anti-log of the *x*-axis intercept.

Microdialysis

Effects of intra-amygdaloid administration of a NK1 receptor antagonist on the release of SP in the MeA

The SP content in microdialysates collected in the MeA (Fig. 2) under basal conditions reached an average of 3.4 ± 0.3 fmol/dialysate and was thus more than one order of magnitude above the detection limit of the radioimmunoassay. Intra-amygdaloid infusion of the NK1 receptor antagonist L-822429 via retrodialysis significantly increased SP release within the MeA (Fig. 3). Two-way ANOVA with repeated measurements of the second factor revealed significant main effects for drug treatment ($F_{4,38} = 5.05$, $p = 0.0023$), time ($F_{5,190} = 5.45$, $p = 0.0001$) and a time by treatment interaction ($F_{20,190} = 2.20$, $p = 0.0033$). *Post hoc* test revealed that animals that received 10 or 50 μM of the NK1 receptor antagonist showed a significant increase in SP release in the MeA compared with basal levels and compared with the respective values of the vehicle treated control rats. At the highest concentration tested, SP release remained high during the whole period of retrodialysis. In contrast, retrodialysis with the low-affinity (2*R*,3*R*) enantiomer of the antagonist had no significant effect on the SP release within the MeA (Fig. 3).

Next, we examined the effects of local NK1 receptor blockade on stress-evoked intra-amygdaloid SP release. Forced swim stress caused a 200% increase in SP efflux in the MeA. SP levels remained significantly elevated up to 60 min after onset of the stressor and gradually declined to pre-stress values within the next 90 min (Fig. 4). Local administration of the NK1 receptor antagonist L-822429 attenuated the swim stress-induced SP release within the MeA. Statistical analysis revealed significant main effects for drug treatment ($F_{4,36} = 2.87$, $p = 0.036$) and time ($F_{4,144} = 6.70$, $p < 0.0001$). The *post hoc* test revealed a significant

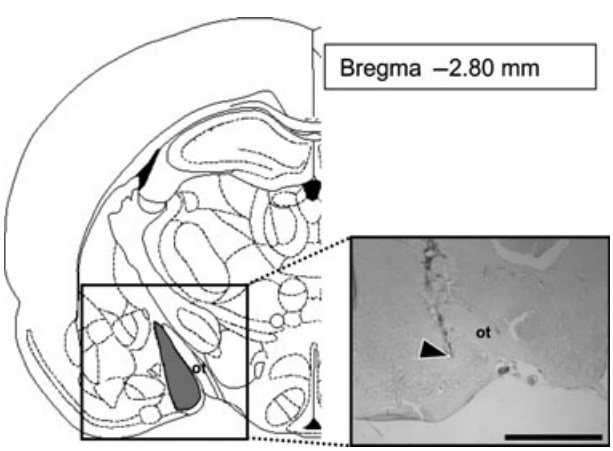

Fig. 2 A schematic drawing and a representative microphotograph of a cresyl-stained coronal brain section showing the localization of the tip of the membrane of a microdialysis probe (arrowhead) within the medial amygdala (MeA). ot, optic tract.

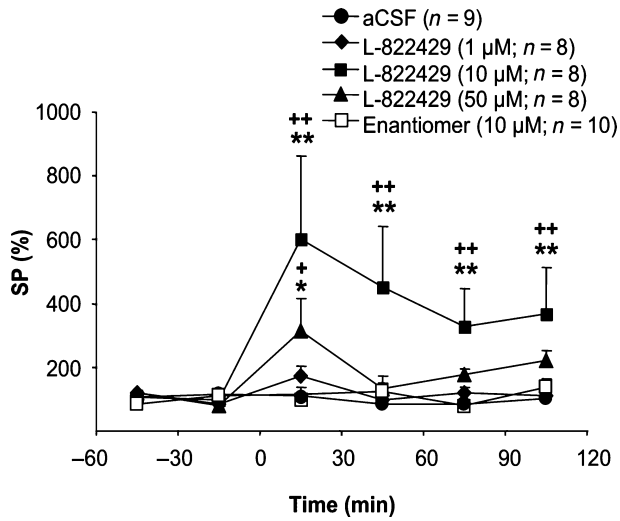

Fig. 3 Unstressed (resting) condition: effects of intra-amygdaloid NK1 receptor antagonist administration on SP release in the MeA. From time period 0, either vehicle (aCSF, black circles), aCSF containing the NK1 receptor antagonist (L-822429; 1, 10 or 50 μM) or its low-affinity enantiomer (10 μM) was applied via inverse microdialysis into the MeA. Data are expressed as a percentage of baseline + SEM; $n = 6$–10 per group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus basal; $^{+}p < 0.05$ and $^{++}p < 0.01$ versus respective value in the vehicle treated controls (two-way ANOVA followed by Fisher's LSD *post hoc* test).

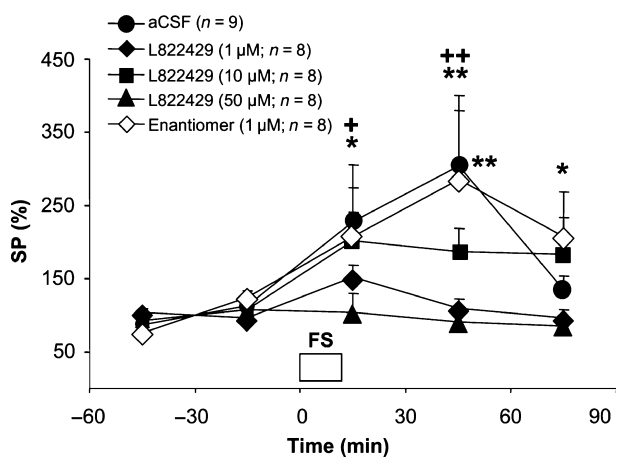

Fig. 4 Effects of intra-amygdaloid NK1 receptor antagonist administration on the stress-induced SP release in the MeA measured in 30-min microdialysates. Animals were administered either vehicle (aCSF, black circles), aCSF containing the NK1 receptor antagonist (L-822429; 1, 10 or 50 μM) or its low-affinity enantiomer (1 μM) via inverse microdialysis into the MeA. From time period 0, animals were forced to swim (FS, grey shaded bars) for 10 min (20°C). Data are expressed as a percentage of baseline + SEM; $n = 6$–10 per group; $^{*}p < 0.05$, $^{**}p < 0.01$ versus basal; $^{\dagger}p < 0.05$ versus respective value in L-822429 (50 μM), $^{\dagger\dagger}p < 0.01$ versus respective values in the antagonist treated groups (1 and 50 μM) and $p < 0.05$ versus respective value in the L-822429 (10 μM) (two-way ANOVA followed by Fisher's LSD *post hoc* test).

reduction of the stress-induced SP release within the MeA in animals treated with the NK1 receptor antagonist (1, 10 and 50 μM) compared with vehicle-treated control rats. In contrast, SP release in animals treated with the low-affinity enantiomer of the antagonist (1 μM) did not differ from controls (Fig. 4).

Immunocytochemistry

Immunolabelling for NK1 receptors in the amygdaloid complex was mostly localized to neuronal cell bodies and dendrites although intense neuropil staining was observed in a few regions such as the anterior cortical nucleus, amygdalo-hippocampal area and the intermediate division of the central nucleus (Fig. 5). The MeA contained numerous medium sized neurones with moderate to low NK1 receptor-like immunoreactivity (LI), whereas in the intercalated cell masses and in the basolateral and lateral amydgaloid nuclei NK1-LI was restricted only to few morphologically heterogeneous non-principal neurones, although the immunostaining of these neurones was relatively intense. These

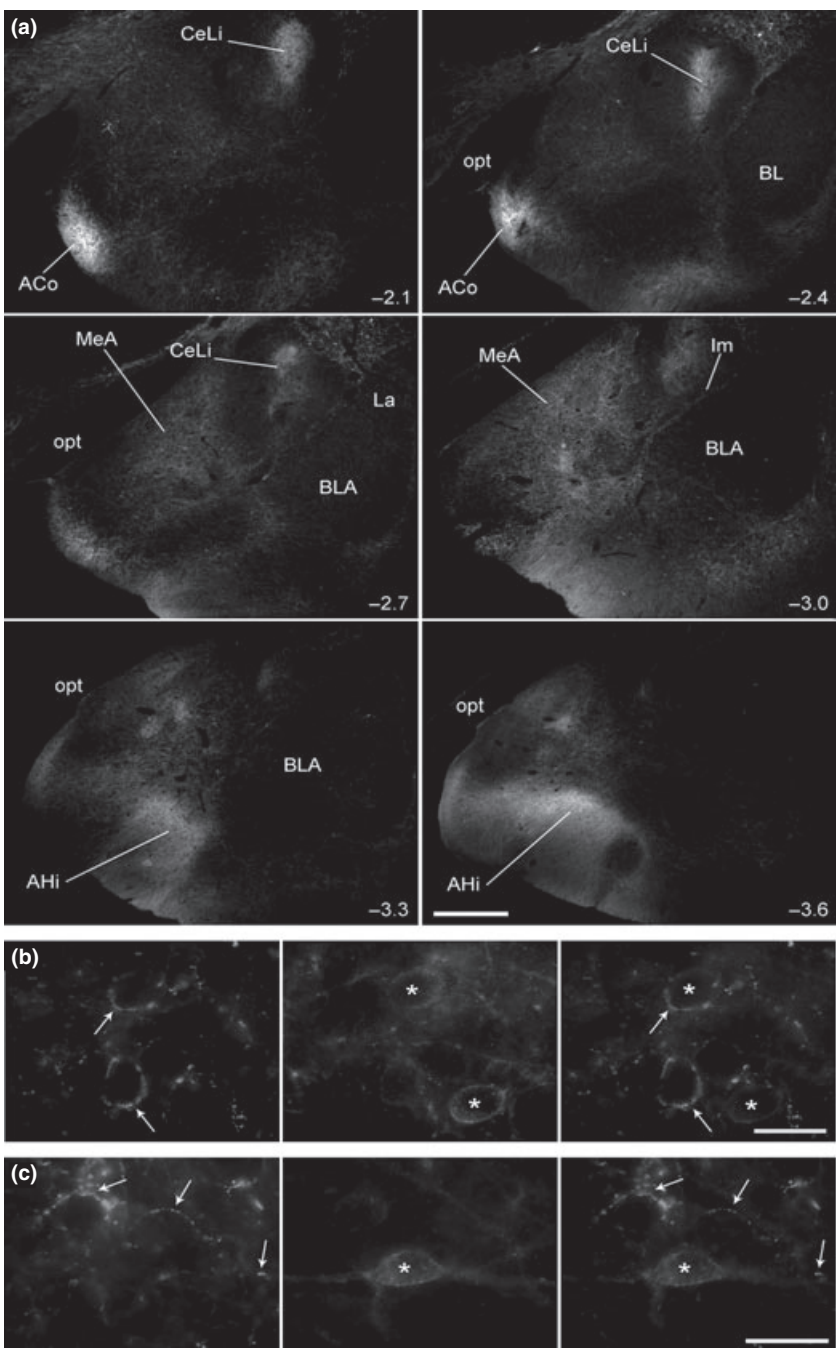

Fig. 5 NK1-receptor-like immunoreactivity in the rat amygdala. (a) Rostro-caudal distribution of NK1 receptor-LI in the rat amygdala; approximate bregma levels are indicated in the bottom right corner of each panel. Densest NK1-LI was observed in the anterior cortical nucleus (ACo), in the intermediate subdivision of the central nucleus (CeLi), in the medial amygdala (MeA) and in the amygdalo-hippocampal area (AHi). Several strongly and moderately immunoreactive neurons were observed within the intercalated cell masses (Im) and in the basolateral (BLA) and lateral (La) amygdaloid nuclei. Opt = optic tract. (b and c) Fluorescence micrographs taken at the same focal depth and showing SP-containing fibres and nerve terminals decorating NK1-immunolabelled neurons (indicated by asterisks) in the MeA. Arrows indicate SP-LI axon terminals making putative synaptic contacts with the soma or proximal dendrite of NK1-immunopositive neurons. Calibration bars: (a) = 500 μm; (b and c) = 20 μm.

findings are consistent with previous autoradiographic (Shults *et al.* 1984) and immunocytochemical (Nakaya *et al.* 1994) studies.

Next, we have compared by double immunofluorescence experiments the anatomical distribution of NK1 receptor- and SP-LI in the MeA. In this amygdaloid nucleus the density of SP-labelled fibres was found moderate to high, in agreement with previous reports (Emson *et al.* 1978; Roberts *et al.* 1982; Hokfelt *et al.* 2004; Shigematsu *et al.* 2008). Some somata and dendrites immunolabelled for NK1 receptor in the MeA appeared decorated by SP-immunopositive fibres (Fig. 5), unlike what was observed in the basal and lateral nuclei, where no anatomical correlation between SP-containing fibres and neurons immunostained for NK1 could be detected (data not shown). A few somata and dendrites in the MeA also displayed SP-LI.

We further investigated the subcellular distribution and possible synaptic relationship of NK1 receptors and SP release sites in the MeA by means of pre-embedding electron microscopy. SP-LI was revealed by silver-intensified gold particles whereas NK1 receptor-LI was demonstrated by peroxidase reaction. Labelling for SP was observed in axon terminals forming mostly symmetric synapses with dendritic shafts (93 out of 102 SP-LI boutons analysed), although a few (9 out of 102) made asymmetric synapses also on dendrites (Fig. 6). At times, immunometal particles for SP were found intracytoplasmic in both somata and dendrites (Fig. 6). NK1 receptor-LI was detected in the somatoden-

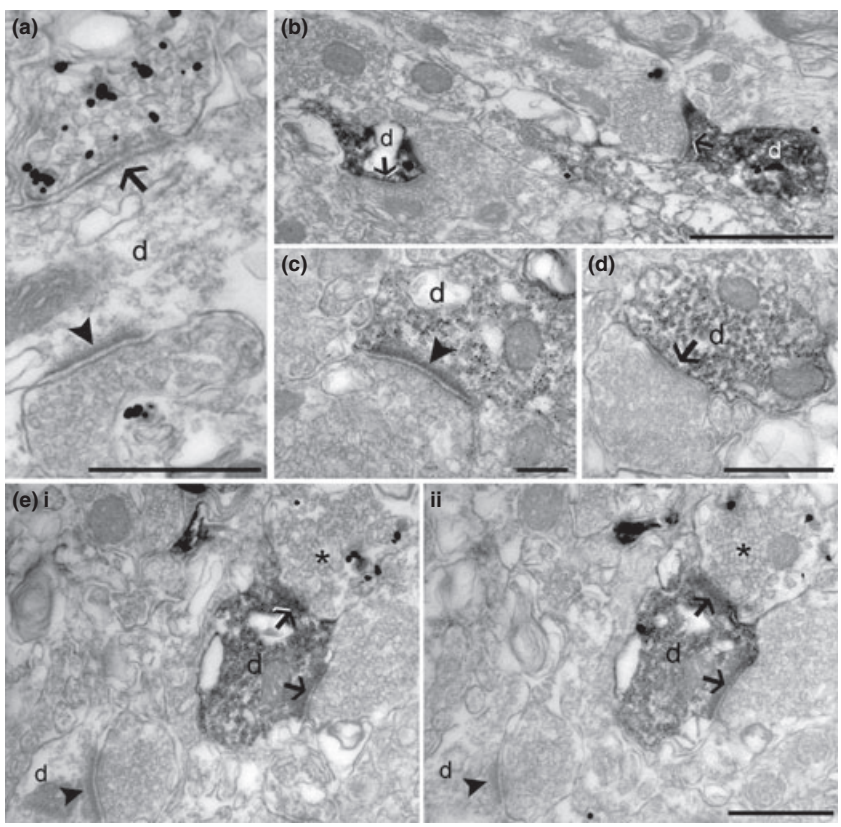

Fig. 6 Electron microscopic images showing SP and NK1 receptor like-immunoreactivity (LI) in the rat MeA. (a) Immunoreactivity for SP is demonstrated by silver-intensified immunogold reaction (electron dense particles). Immunometal particles can be observed in an axonal nerve terminal forming a symmetric synapse (indicated by the arrow) with a dendritic shaft (d), which also receives an unlabelled terminal making an asymmetric synapse (indicated by the arrowhead) almost opposed to the symmetric one. (b) Immunoreactivity for NK1 receptors is demonstrated by immunoperoxidase end-product, a homogeneuous electron-opaque deposit that diffuses throughout the labelled dendrites. The immunoperoxidase reaction being an enzyme-based reaction is more sensitive than the immunogold; it was therefore used for the visualization of the NK1 receptor whose amount is supposed to be much lower compared with SP. Two small dendrites (d) receiving symmetric synapses (arrows) display intense NK1-LI and contain several immunometal particles indicating the concurrent expression of SP. (c) Dendrites immunolabelled for NK1 were found to receive both asymmetric (arrowhead) and (d) symmetric synapses (arrow). (e) Consecutive sections of a small NK1-immunopositive dendrite (d) receiving two nerve terminals forming symmetric synapses (arrows). The bouton indicated by the asterisk (*) shows immunoreactivity for SP; the immunometal particles appear distributed near dense core vesicles where the neuropeptide is accumulated and from where it might have leaked out upon freeze-thawing of the tissue. Calibration bars: (a, d and e) = 500 nm; (b) = 1 μm; (c) = 200 nm.

dritic domain of neurons, but not in axon terminals. NK1 receptor immunolabelled dendrites received both symmetric and asymmetric synapses and often the peroxidase reaction end product filled the entire dendrite (Fig. 6). Double immunoelectron microscopy demonstrated the presence of symmetric synapses between SP-containing fibres and dendrites immunolabelled for NK1 receptors. However, this occurred only rarely as only three out of the 102 SP-LI boutons analysed formed a synapse with dendrites containing NK1 receptors. More frequently, we observed SP-containing fibres and boutons adjacent to NK1-immunolabelled dendrites with which they however did not form synapses.

Discussion

Characterization of L-822429 as a potent and selective rat NK1 receptor antagonist

We demonstrated here that L-822429 represents a potent rat NK1 receptor antagonist with nanomolar affinity at both cloned and native NK1 receptors. L-822429 displayed excellent selectivity over the other two rat NK receptor subtypes, NK2 and NK3, whereas its (2*R*,3*R*) enantiomer demonstrated no binding for any of these NK receptor subtypes. Pre-clinical investigation of NK1 receptor antagonists in rat and mouse models of anxiety and affective behaviour have been complicated by the rather low NK1 receptor affinity of most available compounds in rodents versus gerbils, guinea pigs and humans (for a discussion of species differences see Rupniak 2002). For example, the occurrence of 'non-specific' pharmacological effects attributed e.g. to calcium channel blockade have been described, partly because of necessary high dosage of these antagonists in rodents (Rupniak 2002). In contrast, L-822429 possesses high affinity to rat (and mouse) NK1 receptors and showed only micromolar binding affinity for L-type calcium channels, which are involved in affective behaviour (Striessnig *et al.* 2006), as well as for hERG and cardiac sodium channels. Hence, the affinity to these sites is more than 1200-fold lower relative to the NK1 receptor affinity. L-822429 potently antagonized agonist-driven NK1 receptor-mediated responses both *in vitro* and *in vivo*. Pre-treatment of CHO cells expressing rat NK1 receptors with L-822429 resulted in a significant reduction in the potency of SP to induce intracellular inositol phosphate accumulation. L-822429 demonstrated acceptable bioavailability when administered p.o. or i.v. in rats and has been shown previously to be brain penetrant when administered i.v. to gerbils with good potency and durability of action in inhibiting hind foot drumming taken as a measure of CNS NK1 receptor blockade (Chicchi *et al.*, unpublished).

Inhibitory SP autoregulation in unstressed condition

Substance P-containing fibres and terminals within the MeA (Roberts *et al.* 1982; Shults *et al.* 1984; Nakaya *et al.* 1994) have been reported to originate from intrinsic neurones or from fibres originating from distant brain areas outside the amygdaloid complex (Emson *et al.* 1978; Ottersen 1980, 1981). These include stress-responsive areas such as the periaqueductal grey (Li *et al.* 1990) that has been characterized previously as an important neural substrate for influencing stress-coping behavioural strategies (Bandler and Shipley 1994).

Furthermore, NK1 receptors are densely distributed in the MeA (Shults *et al.* 1984; Nakaya *et al.* 1994). We chose an *in vivo* approach, microdialysis, to study NK1-mediated regulation of SP release. *In vivo* approaches with intact afferent and efferent networks are more likely to reveal the 'real life regulation' and thus have advantages over *in vitro* approaches such as investigation of release from a slice or a synaptosomal preparation. The finding that NK1 receptor blockade increases SP release under basal/resting conditions in a dose-dependent, stereoselective way indicates that NK1 receptors within the MeA are tonically activated by the endogenous ligand and mediate an inhibitory autoregulatory capacity of this neuropeptide system. It should be noted that the response was attenuated at the highest concentration used, suggesting an inverted U-shape relationship with a maximum response at around 10 μM. The exact reason for this response (e.g. diffusion of higher concentrations to more distant counter-regulatory receptors) is uncertain at present, but such inverted U-shape responses indicating more complex regulation are common in neuropeptide research, including SP (Kalivas and Miller 1984; Khan *et al.* 2000). Pre-synaptic modulation of SP transmission by a NK1 autoreceptor on SP terminals is one possible explanation for this observation. Indeed, pre-synaptic autoreceptors have been documented for many classical neurotransmitters, including dopamine, noradrenaline, serotonin, histamine, acetylcholine, GABA and glutamate (Starke *et al.* 1989; Langer 1997). The existence of NK1 autoreceptors was proposed in enteric neurons of both rat and guinea-pig (Patacchini *et al.* 2000) and in the somatosensory system (Tang *et al.* 2007; Hu *et al.* 1997 for review see Malcangio and Bowery 1999). In the spinal cord, it has been shown that the NK1 receptor antagonists RP67580 and SR140333 could increase electrically evoked SP release from slices (Malcangio and Bowery 1994), which was taken as evidence for the existence of an inhibitory NK1 autoreceptor, as NK1 receptors are not present on inhibitory neurons such as GABAergic neurons in the investigated part of the spinal cord (Malcangio and Bowery 1999). Evidence for pre-synaptic localization of NK1 receptors on SP-containing terminals has been gained neuroanatomically in distinct areas of the brain such as nucleus accumbens, periaqueductal grey and striatum (Jakab and Goldman-Rakic 1996; Barbaresi 1998; Pickel *et al.* 2000; Levesque *et al.* 2006), but the amygdala has not been studied in this respect. Hence, an important aim was to clarify whether NK1 receptors can be

found on SP-containing terminals in the amygdala. In our detailed electron microscopic investigation we could not detect a pre-synaptic localization of NK1 receptors in the MeA. Although false negative labelling for NK1 receptors in axon terminals because of the lack of antibody penetration into the tissue cannot be totally excluded with the pre-embedding immunoelectron microscopy method, the consistent presence of well-labelled dendritic shafts for NK1 and axon terminals for SP suggest a solely post-synaptic location of this receptor in the MeA.

As we observed NK1 receptors on SP-containing neurons, modulation of somatodendritc autoreceptors and recurrent collaterals is another possibility of automodulation. However, the most frequently reported post-synaptic action of SP seems to be excitatory (Otsuka and Konishi 1976; Collingridge and Davies 1982) including the observation of slow onset excitation of neurons in the MeA of anaesthetized rats (Le Gal La Salle and Ben-Ari 1977). Hence, the observed inhibitory feedback regulation in the resting/unstressed condition most likely involves interaction with inhibitory neurons and neurotransmitter systems. GABAergic interneurons seem to be likely candidates. NK1 receptors located on GABAergic neurons have been indeed demonstrated in other parts of the amygdala (Maubach *et al.* 2001).

Facilitatory SP autoregulation in stress condition

As we observed inhibitory autoregulation of SP under resting conditions, we hypothesized that such an effect will be also evident during stress exposure. It has been proposed previously that the self-inhibitory effect of SP via NK1 receptors may represent a safety response aimed at preventing overstimulation (Malcangio and Bowery 1999). However, we observed that NK1 receptor blockade reduced swim stress-induced SP release in the MeA in a stereoselective way, indicating that NK1 receptor activation by the endogenous neuropeptide *facilitates* SP release under stress conditions. Thus, this modulatory regulation is opposite to the inhibitory effect observed under resting conditions. How can this difference be explained? It is possible that different intracellular effectors get activated during SP-induced NK1 receptor stimulation in different states (stress vs. resting conditions) of our experiment. Apart from activating the phospholipase C–inositol triphosphate–calcium signal transduction cascade (see also present study), NK1 receptor stimulation can trigger the activation of the mitogen-activated protein kinase, protein kinase Cs and cyclooxygenase-2, which have been shown to promote SP release, while c-Jun *N*-terminal kinase activation was found to suppress SP release in cultured rat dorsal root ganglion neurons (Tang *et al.* 2007). More likely, the observed inhibitory and excitatory autoregulation under resting and stress conditions, respectively, may result from different interneuronal signalling (wired vs. volume transmission) within the MeA area similar as proposed recently for the locus coeruleus (Ebner and Singewald, 2007). It is well known that neuropeptides are released non-synaptically from multiple sites of the neuronal membrane. Thus, it is conceivable that under stress conditions signalling via volume transmission predominates over synaptic transmission in the MeA and additional targets are reached, which contribute to further excitation of SP transmission. Indeed, in preliminary experiments we observed attenuation of NMDA-induced SP release at high doses of L-822429 (Ebner *et al.*, unpublished) suggesting interaction with glutamatergic mechanisms (see also Kangrga and Randic 1990; Liu *et al.* 1997). Finally, it may be speculated that different receptor internalization in the two conditions contributes to differential self-regulation. Evidence for NK1 receptor internalization induced by high SP concentrations has been gained both *in vitro* and *in vivo* in different brain areas including the amygdala (Takayama *et al.* 1986; Mantyh *et al.* 1995; Smith *et al.* 1999; Tang *et al.* 2007). Considering the proposed interaction with GABAergic interneurons (see above), it is conceivable, that an initial massive increase in SP release during forced swimming triggers internalization of NK1 receptors located on these GABAergic interneurons resulting in subsequently reduced inhibitory GABAergic action and elevated stress-induced SP levels. Further studies will be necessary to clarify the exact mechanisms underlying this differential self-modulatory effect of SP.

Possible functional consequences of SP self-regulation in the MeA

While the MeA has previously been suggested to be mainly involved in processing of social olfactory signals, there is growing evidence that the MeA is of importance also in processing of other aspects of emotion, including stress-, fear- and anxiety-related behaviour. Immediate early gene mapping revealed strong neuronal activation in the MeA after emotional stress exposure which has been found to be more pronounced than in other parts of the amygdala such as the central nucleus (Cullinan *et al.* 1995; Beckett *et al.* 1997; Dayas *et al.* 2001; Salchner *et al.* 2004). Stress responsiveness of MeA neurons was also demonstrated by increased oxidative activity after chronic stress in this brain area (Kanarik *et al.* 2008) and reduced dendritic spine density following 1 h of restraint stress (Marcuzzo *et al.* 2007). Higher c-Fos response to social defeat was found in the MeA of high anxiety behaviour rats, which also displayed greater ultrasonic vocalization and freezing responses when compared with low anxiety behaviour rats (Frank *et al.* 2006). Along these lines, inactivation of the MeA reduced defensiveness in wild rodents (Kemble *et al.* 1984), blocked trimethylthiazoline-induced freezing (Muller and Fendt 2006) and inhibited escape behaviour in the elevated plus maze (Herdade *et al.* 2006), underlining a role of the MeA in defensive behaviour (Blanchard *et al.* 2005). Furthermore, the MeA was identified to be an important site of therapeutic drug

action, as injections of antidepressants such as imipramine into the MeA inhibit behavioural despair and also aggression (Horovitz *et al.* 1966; Duncan *et al.* 1986; Smith *et al.* 1999).

Interestingly, SP has been demonstrated to be an important mediator of these stress- and anxiety-related behavioural changes elicited via the MeA (Ebner *et al.* 2004). Hence, the proposed self-facilitation of SP release under stress conditions may have important functional consequences, as it is expected to lead to very high levels of SP and thus to activation of additional receptors, to which SP binds with lower affinity, such as NK2 and NK3 receptors. Indeed, while NK2 receptor binding sites seem very low or almost absent in the amygdala (Saffroy *et al.* 2003), a moderate amount of NK3 receptors has been identified in the MeA (Ding *et al.* 1996; Shughrue *et al.* 1996) and modulation of anxiety- and depression-like behaviour has been proposed for NK3 receptors (Massi *et al.* 2000; Dableh *et al.* 2005; Chahl 2006; Salome *et al.* 2006). It will be interesting for future studies to investigate occupancy and functional involvement of SP-sensitive receptors in addition to NK1 in stress mechanisms.

Taken together, our data demonstrate that L-822429 is a selective NK1 receptor antagonist with high affinity to the rat NK1 receptor. Using this antagonist and its low-affinity enantiomer as tools, we provide evidence of NK1 receptor-mediated autoregulatory properties of SP released within the MeA of freely moving rats. It appears that the effect of SP on its release is inhibitory in unstressed (resting) conditions and facilitatory during stress. This further facilitation of stress-induced SP release may have functional consequences via the involvement of other receptors in addition to NK1, such as the NK3 receptor.

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