Substance P in Stress and Anxiety

NK-1 Receptor Antagonism Interacts with Key Brain Areas of the Stress Circuitry

Karl Ebner, Patrik Muigg, Georg Singewald, and Nicolas Singewald

Department of Pharmacology and Toxicology, Institute of Pharmacy and Center for Molecular Biosciences Innsbruck (CMBI), University of Innsbruck, Innsbruck, Austria

In a previous work it was demonstrated that emotional stressors trigger the *in vivo* release of the neuropeptide substance P (SP) in brain areas known to be implicated in stress and anxiety mechanisms, such as the amygdala, lateral septum, nucleus accumbens, and locus coeruleus. However, the specific role of SP within the hypothalamic paraventricular nucleus (PVN), the critical site of the neuroendocrine stress axis, is unclear. Studies performed in neurokinin-1-receptor (NK-1R) knockout mice have provided conflicting results. Therefore, the aim of the present study was to use a pharmacological approach and examine whether intracerebroventricular NK-1R-antagonist treatment modulates stress-induced neuronal activity in key brain areas of the stress circuitry, including the PVN. The elevated plus maze test was used as a mild stressor known to stimulate stress hormone secretion and c-Fos-expression in the PVN and simultaneously to obtain behavioral readout for anxiety-like behavior. Results demonstrate an anxiolytic-like effect of intracerebral NK-1R antagonism that is associated with an attenuation of the stress-induced c-Fos expression in the PVN and lateral septum. In the amygdala and the bed nucleus of stria terminalis, c-Fos induction by elevated plus maze exposure was much lower and was not influenced by NK-1R-antagonist treatment. Thus, our findings provide clear evidence that central NK-1R-blockade reduces neuronal activity in key brain areas of the stress circuitry, which is thought to be associated with attenuation of the neuroendocrine stress response. These findings support the idea that a stress-sensitive subset of the human psychiatric patients may particularly benefit from a pharmacological approach that interfers with SP transmission.

Key words: **substance P; tachykinin; neurokinin; *in vivo* release; anxiety; depression; antidepressant; anxiolytic; stress; HPA axis; NK-1 receptor; NK-1 receptor antagonist; emotional behavior; microdialysis; stress-related disorders**

Introduction

Distribution of Substance P and NK-1 Receptors

The undecapeptide substance P (SP) belongs to a family of neuropeptides known as tachykinins. Together with neurokinin-A, SP is derived from the preprotachykinin-A (PPT-A or Tac1) gene,[1] which produces four splice variants; α- and δ-PPT-A yield SP alone, whereas β- and γ-PPT-A produce both SP and neurokinin-A.[2,3] Within the brain it is generally accepted that tachykinins act as neurotransmitters and/or neuromodulators.[4] Neurokinins exert their effects by binding to G protein–coupled receptors. Bioassay and radioligand binding studies using natural and synthetic agonistic or antagonist ligands led to the detection of three types of tachykinin receptors in mammals. Although all

Address for correspondence: Dr. Karl Ebner, Leopold-Franzens-University of Innsbruck, Department of Pharmacology and Toxicology, Institute of Pharmacy, Center for Molecular Biosciences Innsbruck (CMBI), Peter Mayr-Str. 1, A-6020 Innsbruck, Austria. karl.ebner@uibk.ac.at

Ann. N.Y. Acad. Sci. 1144: 61–73 (2008). © 2008 New York Academy of Sciences.
doi: 10.1196/annals.1418.018

endogenous neurokinins can interact with all three receptor types, SP exhibits high affinity to the neurokinin-1 (NK-1) receptor.[5,6] The distribution of SP and its preferred receptor in mammalian brains has been reviewed in detail previously.[7,8] Notably, it was described that both SP and NK-1 receptors are highly distributed in many forebrain, midbrain, and brain-stem areas, including areas implicated in the modulation of stress, anxiety, and mood responses, such as the cingulate cortex, caudate putamen, nucleus accumbens, septum, hippocampus, amygdala, various hypothalamic areas, as well as periaqueductal gray, dorsal raphe nucleus, and locus coeruleus. In these regions, SP frequently coexists in the same neuron with other neurokinins and with "classic" neurotransmitters, such as dopamine, acetylcholine, serotonin, noradrenaline, GABA, and glutamate,[9,10] suggesting functional interactions with other neurotransmitter systems.

Substance P in Stress Regulation

There is evidence that SP modulates physiological and behavioral stress responses in the brain. In conscious rats, SP administered centrally induces a pattern of cardiovascular and behavioral responses that closely resemble the responses to stressful stimuli.[11,12] This idea was further based on the finding that the exposure to aversive and/or stressful situations induces changes in SP contents and/or receptor binding in brain areas known to be implicated in processing emotions and stress reactions.[13] Moreover, there is also evidence from receptor endocytosis studies for an increase in NK-1 receptor internalization reflecting an increased local SP release in discrete brain areas such as amygdala in response to stressful situations.[14–16] However, although these methods have provided important preliminary evidence, in which brain areas SP transmission may be important in response to stressful situations, this kind of assessment is only an indirect and very crude measure of SP neurotransmission, which can give no information on temporal dynamics of release.

Stress-Induced Substance P Release in Distinct Brain Areas

Since neuropeptides such as SP only become biologically active after their release into the extracellular space, attempts to measure intracerebral release have to focus on approaches that are able to reflect concentrations and their fluctuations in the extracellular fluid.[17,18] Therefore, *in vivo* sampling methods such as push-pull superfusion and microdialysis technique in conjunction with highly sensitive radioimmunoassays have been successfully used to investigate the effects of acute stressful life situations on the *in vivo* efflux of SP in discrete brain areas.[19,20] We could demonstrate for the first time that various emotional stressors increase SP efflux in discrete forebrain areas implicated in the regulation of stress and anxiety reactions such as amygdala and septum.[21,22] By using specific small-sized microdialysis probes and micropush-pull cannulae systems constructed in our laboratory that allowed us to perfuse even subregions of the amygdala complex, we found a stress-induced increase of SP release in the medial but not in the central amygdala.[21] Interestingly, the release of SP in the medial amygdala varies depending on the nature and/or severity of the stressor, as the release seems considerably more pronounced and prolonged after a severe emotional stressor than in response to a rather mild stressor.[21] Our finding of high extracellular SP levels in the medial amygdala is consistent with immunohistochemical studies demonstrating a dense plexus of SP-containing cell bodies and terminals in this brain area.[8,23] Postsynaptically, it has been shown that immobilization stress also leads to a decrease in the number of NK-1 receptors in the amygdala,[24] which may represent endocytosis of somatodendritic receptors as the NK-1 receptor has been shown to undergo internalization following receptor stimulation.[25] More recently, we also found an increase of SP release after swim stress exposure in additional areas such as lateral septum, nucleus accumbens, and locus coeruleus.[22,26,27] Interestingly, in the lateral septum and locus

coeruleus we could show functional, significant interactions of stress-induced SP release with that of monoamines (e.g., serotonin and noradrenaline), which are known to be implicated in regulation of mood and affective behavior.[28,29] In a very recent study we obtained evidence for autoregulatory properties of SP within the amygdala, which may have functional consequences in particular during stress.[30] Finally, we found differences in stress-related SP neurotransmission in rats bred for extremes in high anxiety–related behavior (HAB), an established psychopathological animal model of trait anxiety and comorbid depression.[31] Compared to their low anxiety (LAB) counterparts, HAB rats show a higher increase in the stress-induced SP release within the medial amygdala, whereas basal release of the neuropeptide within this area was not different between the two lines.[32] This suggests a hyperactive SP neurotransmission in the medial amygdala in these high-anxiety rats after exposure to an aversive stimulus and confirms clinical findings of a disturbed SP neurotransmission in patients with stress-related diseases such as anxiety disorders and depression.[33,34]

Further evidence corroborating the role of SP in stress mechanisms comes from studies investigating the effects of NK-1 receptor activation and/or blockade on neuronal activity in brain areas known to be important in stress reactions in response to aversive stimuli. A key area of the stress circuitry is the hypothalamic paraventricular nucleus (PVN), since this structure regulates the hypothalamic–pituitary–adrenal (HPA) axis by a select population of neurosecretory neurons that release the main stress hormone secretagogues corticotropin releasing factor and arginine vasopressin.[35,36] While pharmacological blockade of NK-1 receptors has been found to attenuate stress-induced c-Fos expression (as marker for neuronal activation) in brain areas such as prefrontal cortex, periaqueductal gray, and locus coeruleus[37,38] (but see also Ref. 39), information on the PVN or other key areas of the stress circuitry are lacking in rats. In mice with a genetic deletion of the NK-1 receptor (NK-1 receptor knockout mice), this issue has been studied; however, inconsistent findings were obtained. Both attenuated stress-induced neuronal activation in the PVN of NK-1 receptor knockout mice[40] or no differences between knockout and wild-type mice[41] were reported. Genetic background-specificity resulting from strain differences in HPA axis activity may have contributed to this discrepancy.[41] Furthermore, it is not entirely clear whether neuronal and behavioral changes found in conventional NK-1 receptor knockout mice are a result of NK-1 receptor deficiency itself or a consequence of functional adaptation during ontogenesis. Thus, the aim of the present study was to use a pharmacological approach in rats to examine the effects of global receptor blockade within the brain on neuronal processing in core stress-related brain areas. We chose a mild stressful challenge, exposure to the elevated plus maze, which activates stress pathways,[42,43] increases stress hormones such as corticosterone,[44,45] and provides behavioral readout (i.e., anxiety-related behavior, locomotor activity).

Experimental Procedures

Animals

Experiments were carried out on adult male Sprague-Dawley rats (250–350 g). Prior to use, the animals were housed in groups of 4–6 under controlled laboratory conditions of a 12-h light–dark cycle (lights on from 7 AM to 7 PM) at 21 ± 1°C and 60% humidity with food and water *ad libitum* for at least one 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 guide cannula (21 gauge or G, length 12 mm) was stereotaxically implanted under sodium pentobarbital (40 mg/kg, i.p.) and ketamine (50 mg/kg, i.p.) anaesthesia according to the stereotaxic atlas of Paxinos and Watson.[46] The tip of the guide cannula was positioned 1 mm above the right lateral ventricle (0.6 mm caudal to bregma, 1.6 mm lateral to the midline, 1.8 mm below the surface of the skull). The cannula was fixed to the skull with two stainless steel screws and dental cement. After surgery, a stylet was inserted into the guide cannula to prevent obstruction, and rats were housed individually in transparent Plexiglas cages until testing. They were handled for 3 min twice daily to familiarize them with the experimental procedure and to minimize nonspecific stress responses during the experiments. At least 24 h before the experiment, animals were kept in the experimental room and allowed to habituate.

Intracerebroventricular Injections

Seven days after surgery, the stylet of the guide cannula was replaced by an infusion cannula (25 G) which was 2 mm longer than the guide cannula, thus reaching into the lateral ventricle. The injection cannula was connected to a Hamilton microsyringe by a polyethylene tubing and for injections, the prehandled animals were gently held, and a volume of 5 μL was injected over a period of 60 s. Animals received either vehicle (artificial cerebrospinal fluid [aCSF]) or the potent and selective rat NK-1 receptor antagonist L822429 (5 nmol/5 μL, for pharmacological characterization, see Singewald *et al.*[30]). L822429 was reconstituted in a small amount of distilled water and dissolved in aCSF. The injection cannula remained in the guide cannula for an additional 30-s period after infusion. After removing of the injection cannula, rats were left undisturbed for the next 10 min in their home cages before behavioral testing.

Behavioral Testing on the Elevated Plus Maze

Ten minutes after drug injection, animals were tested on the elevated plus maze for 5 min, as described previously.[21] The behavioral parameters scored were (1) entries into open arms (ratio of open arm entries to total number of entries into all arms), (2) time spent on the open arms (ratio of time spent on open arms to total time spent on all arms), and (3) overall activity (total number of entries into closed arms and total distance traveled). After plus maze exposure, rats were returned to their home cages.

c-Fos Immunohistochemistry

Two hours after a 5-min exposure to the elevated plus maze, c-Fos immunohistochemistry was performed, as described previously.[47] Briefly, animals were deeply anesthetized with sodium pentobarbital (200 mg/kg) and transcardially perfused with 100 mL of 0.9% saline, followed by 100 mL of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS, pH 7.4). The brains were removed and postfixed in the same solution at 4°C overnight. Coronal forebrain sections (100 μm) were cut with a vibratome (Ted Pella Inc., Redding, California) and collected freely floating in immunobuffer. Sections were incubated for 48 h in a polyclonal primary anti-Fos antibody (diluted 1:20,000 in immunobuffer comprising 0.1 M NaCl, 5 mM KCl, 8 mM Na_2HPO_4, 15 mM NaH_2PO_4, 10 mM Tris-HCl, 0.3% Triton X-100, and 0.04% thimerosal), followed by a 24-h incubation in a biotinylated goat antirabbit secondary antibody (1:200). An avidin–biotin–horseradish peroxidase procedure with 3,3′−diaminobenzidine as the chromogen was used to visualize the immunoreactivity. Cells containing a nuclear brown-black reaction product were considered positive for c-Fos immunoreactivity, and are referred to hereafter as c-Fos positive cells. The anatomical localization of c-Fos positive cells was aided by using the

illustrations in a stereotaxic atlas.[46] For quantitative analysis, the number of c-Fos positive cells was counted bilaterally in a tissue area of 0.01 mm^2 of investigated brain regions.

For histological control of placement of i.c.v. injection cannula, 100 μm serial coronal sections were stained with cresyl violet.

Statistical Analysis

Experimental subjects were included in the statistical analysis only if the i.c.v. injection cannulae were confirmed to be localized within the lateral ventricle. Data are presented as means + SEM. Differences between groups were determined by nonparametric Mann–Whitney U test.

Results and Discussion

Effects of Intracerebroventricular Administration of a Neurokinin-1 Receptor Antagonist on Anxiety-Related Behavior

Based on the findings presented earlier we investigated the effects of intracerebroventricular NK-1 receptor antagonist administration on emotional or anxiety-related behavior. Therefore, we infused the selective NK-1 receptor antagonist L822429 into the brain by microinjections into the ventricular system and analyzed the behavior of rats in the elevated plus maze test, a well-established ethological animal model of anxiety.[48] This potent and selective NK-1 receptor antagonist has previously been shown to be a useful tool to investigate the behavioral impact of endogenously released SP within the rat amygdala and septum.[21,22] The studied behavioral parameters in the elevated plus maze test can be divided into those related to anxiety (e.g., time spent in open arms, number of entries in open arms) and those associated with locomotor activity (e.g., total number of entries into closed arms, total distance traveled). In the present experiments, we found that NK-1 receptor antagonist–treated animals had a significantly higher percentage of entries into open arms (Fig. 1) and tended to spend more time in open arms (data not shown) than controls, indicating an anxiolytic-like effect. To exclude that these behavioral effects are due to nonspecific motor effects of the drug, we also measured parameters related to locomotor activity, such as closed arm entries and the total path length animals traveled on the maze. If anxiolytic effects of a drug are indeed related to increased locomotor activity, one would expect increased closed arm entries or greater distances traveled on the maze of drug-treated animals. However, in our study there was no significant difference either in the number of closed arm entries (Fig. 1, right panel) or in the total distance traveled on the maze (data not shown) between NK-1 receptor antagonist–treated animals and controls. Accordingly, we concluded that the observed behavioral effects of NK-1 receptor blockade are related to changes in emotionality and not to changes in motor activity. Thus, our findings are consistent with previous studies demonstrating that anxiety levels in mice and rats can be modulated by interfering with central NK-1 receptor function.[20] Most of these results show that NK-1 receptor activation (e.g., by administration of SP) induces anxiogenic-like responses, while NK-1 receptor blockade induces the opposite (anxiolytic-like) effects in different behavioral tasks, including the elevated plus maze test. In a previous study we could show that SP infusions into the medial amygdala produce an anxiogenic-like effect in this test similar to that induced by immobilization stress.[21] Interestingly, although a NK-1 receptor blockade within the medial amygdala was able to induce anxiolytic-like effects in the plus maze test, the same treatment had no effects in tests that are more related to depression-like behaviors such as the forced swim test (Ebner *et al.*, unpublished data). This is also interesting because swim stress triggers SP release within the medial amygdala.[30] Very recently we could show that the lateral septum might be a critical brain area

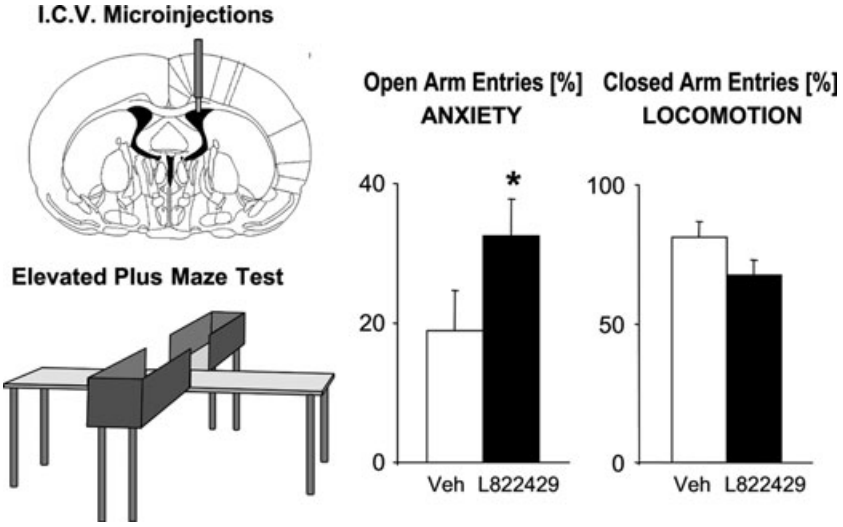

Figure 1. Effects of intracerebroventricular administration of the neurokinin-1 (NK-1) receptor antagonist L822429 on anxiety-related behavior in rats tested on the elevated plus maze test. NK-1 receptor antagonist–injected animals (5 nmol/5μL) had a significantly higher percentage of entries into open arms (*left panel*) than vehicle-treated (artificial cerebrospinal fluid [aCSF]) controls, indicating an anxiolytic-like effect. The percentage of closed arm entries (*right panel*) was not different between groups, indicating similar locomotor activity. Means + SEM. *, $P < .05$ vs. vehicle-treated control group (Mann-Whitney U test).

mediating antidepressant-like effects of NK-1 receptor antagonism, as intraseptal NK-1 receptor blockade reduced immobility time in the forced swim test.[22] Based on these data, it may be speculated that different aspects of affective behaviors are regulated in different brain areas via SP/NK-1 receptor pathways (see also Adell *et al.*[49]). Accordingly, it is conceivable that the amygdala especially the medial amygdala, is important in mediating anxiolytic effects of NK-1 receptor antagonists, while antidepressant effects of these drugs may be processed in some other limbic areas, such as the lateral septum.[20]

Effects of Intracerebroventricular Administration of a Neurokinin-1 Receptor Antagonist on Stress-Induced c-Fos Expression

In additional experiments, we identified neuronal populations that may be involved in the processing of the NK-1 receptor-mediated behavioral effects. Based on the findings in NK-1 receptor knockout mice, we investigated whether central blockade of NK-1 receptors in rats also affects neuronal activity in key areas of stress regulation such as PVN and lateral septum, as well as in brain structures that are more related to anxiety, such as amygdala and bed nucleus of the stria terminalis. Therefore, we infused a selective NK-1 receptor antagonist intracerebrally (as described earlier in the chapter) and analyzed neuronal activity of various brain areas by immunohistochemical detection of c-Fos expression. The expression of the immediate early gene c-Fos was chosen as an established method for detecting neuronal activation after exposure to aversive and stressful situations, with high (cellular) spatial resolution in widespread regions of the brain.[50–52] We used the elevated plus maze test as an anxiogenic and therefore mildly stressful environment to increase neuronal activation in stress- and anxiety-related areas (see later in the article) for the identification of neuronal correlates underlying the anxiolytic-like effects of NK-1 receptor blockade. Previous studies

indicated that elevated plus maze exposure induced significant c-Fos-expression of the stress and anxiety circuit in selected brain areas, including the cingulate cortex, PVN, amygdala, and lateral septum.[42,43] Interestingly, we found that intracerebroventricular administration of a selective NK-1 receptor antagonist attenuated stress-induced c-Fos expression in the PVN (Fig. 2A). Thus, our data confirm previous findings in mice demonstrating reduced c-Fos response to elevated plus maze exposure in the PVN of NK-1 receptor knockout and antagonist pretreated wild-type mice.[40] As the PVN is a key area for the integration of the neuroendocrine stress response,[53] which contains a moderate density of SP binding sites,[54] and because increased c-Fos expression within the PVN can be taken as an index of activated HPA axis,[55,56] these data indicate that NK-1 receptor blockade within the PVN attenuates neuroendocrine stress responses. Hence, a facilitatory role of endogenous SP within the PVN on the HPA axis stress responses is suggested, which is consistent with previous findings demonstrating a potentiation of stress-induced corticosterone secretion after central injections of SP.[57] This effect seems to be mediated by NK-1 receptors, since pretreatment with a NK-1 receptor antagonist prevented the SP effects on stress-induced corticosterone levels. In line with this facilitatory role of SP found in rats are further results from NK-1 receptor knockout mice that showed lower stress-induced corticosterone secretion compared to wild types.[58,59] However, in another study no differences in the stress-induced corticosterone levels or hypothalamic c-fos expression were found between NK-1 receptor knockout and wild type mice.[41] Beside genetic background specificity resulting from strain differences in HPA axis activity this discrepancy might be related to the use of different stressors. Since differences between knockout and wild-type mice were detectable only in studies using a rather mild emotional stressor (e.g., exposure to a novel environment), it is conceivable that a ceiling effect precluded detection of differences between groups in HPA axis responses to a more severe and physical stressor such as restraint used in the study of McCutcheon and coworkers. Interestingly, also other anxiolytic drugs such as benzodiazepines exert similar suppressing effects on stress-induced HPA axis excitability,[60,61] which may be an important component associated with anxiolysis under specific conditions.

In the lateral septum, a key area in the processing of stress and affective responses,[62] we also found a reduced c-Fos response to elevated plus maze exposure in NK-1 receptor antagonist–treated animals compared to controls (Fig. 2B). Interestingly, this effect was observed especially in the ventral part, and to a lesser extent in the dorsal part of the lateral septum, while in the intermediate part this effect did not reach statistical significance. There is a functional distinction between different subnuclei within the lateral septum, which are known to differ in their cytoarchitecture, neuroanatomical connections, and neurochemical makeup.[63–65] In particular, varied or potentially opposing affective functions of lateral septum subregions have been reported.[62] For example, there is some evidence that different behavioral stress responses are mediated by different subregions; the dorsal part of the lateral septum promotes active, whereas the ventral part promotes passive coping responses. Interestingly, in a previous study it was shown that enhanced freezing behavior in stress-sensitized animals is associated with a massive c-Fos induction in the ventral part of the lateral septum.[66] Hence, the reduction of stress-induced activity in the ventral part of the lateral septum may contribute to the anxiolytic effect of NK-1 receptor blockade. Moreover, since the ventral part of the lateral septum directly projects to the periventricular zone of the hypothalamus (including the PVN),[65,67] this subarea might also be involved in the control of neuroendocrine and autonomic stress responses. Indeed, previous studies indicate that the lateral septum plays an important role in the regulation of HPA axis activity.[68] However, although the lateral septum has been proposed as a primarily inhibitory site

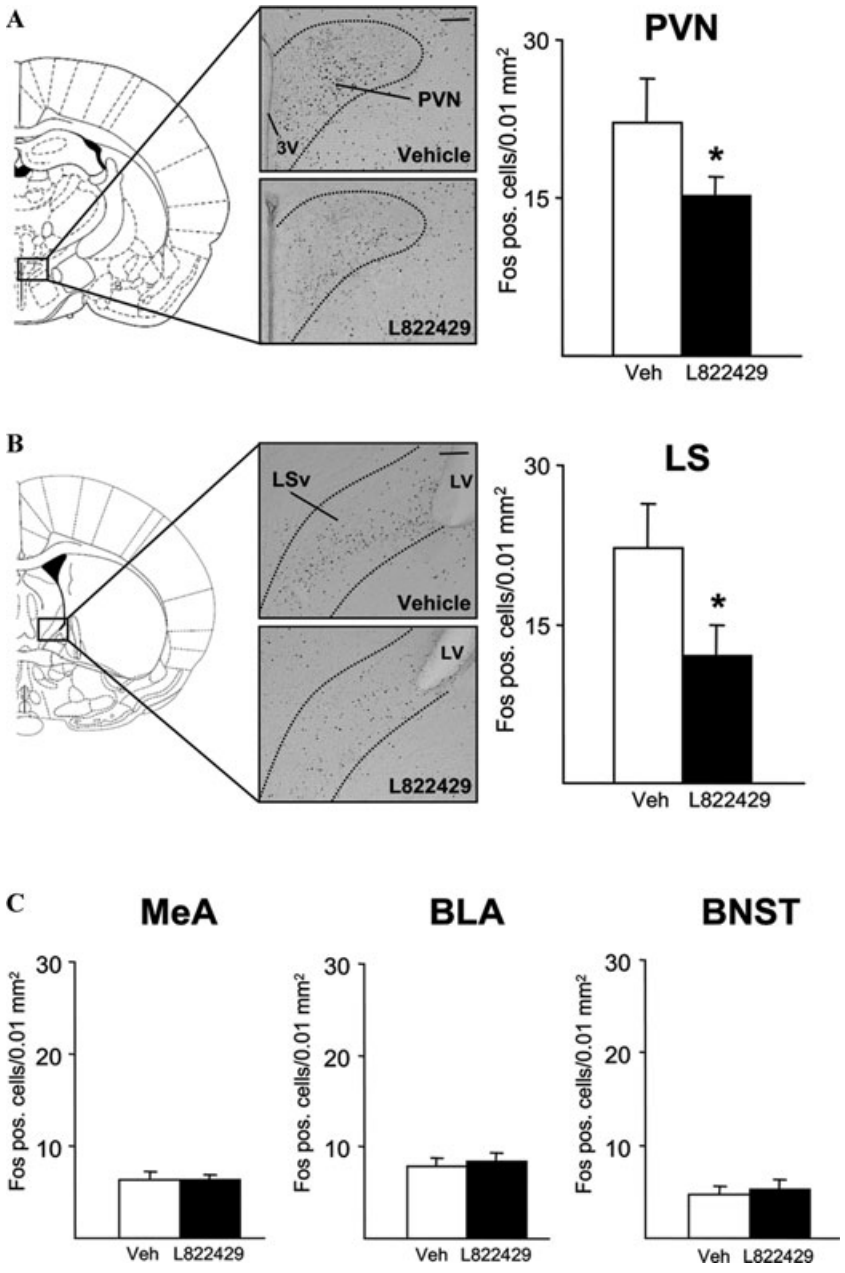

Figure 2. Effects of intracerebroventricular administration of the neurokinin-1 (NK-1) receptor antagonist L822429 on stress-induced c-Fos expression in brain areas of the stress and anxiety circuitry of rats. NK-1 receptor antagonist injections attenuated c-Fos responses to elevated plus maze exposure in the (**A**) hypothalamic paraventricular nucleus (PVN) and (**B**) the lateral septum (LS, ventral part). Depicted are schematic diagrams adapted from the atlas of Paxinos and Watson[46] (*left panel*), representative bright-field photomicrographs (*middle panel*) and c-Fos quantification presented as bar graphs (*right panel*). (**C**) The NK-1 receptor antagonist did not modulate stress-induced c-Fos expression in the medial (MeA) and basolateral nucleus of the amygdala (BLA), or in the bed nucleus of the stria terminalis (BNST). $^{*}P < .05$ vs. vehicle-treated control group (Mann–Whitney U text). Abbreviations: 3V, third ventricle; LV, lateral ventricle; Scale bar = 100 μm.

on the HPA axis function, the possibility of regional specialization might also be relevant for neuroendocrine functions. Accordingly, since in the present study NK-1 receptor antagonism attenuates PVN activity, we postulate that the ventral part of the lateral septum is involved in HPA axis excitation rather than inhibition. However, further studies should clarify whether distinct subregions of the lateral septum do indeed regulate HPA axis activity differentially.

Since we observed a clear anxiolytic effect of NK-1 receptor antagonist treatment, we also investigated neuronal activity in key areas of proposed anxiety networks, the amygdala and the bed nucleus of the stria terminalis.[69,70] Previous studies from our group and others have shown differential involvement of various amygdaloid nuclei in SP-mediated affective behaviors.[21,71–73] Moreover, elevated plus maze exposure induced induction of c-Fos expression in several subregions of the amygdala such as medial and basolateral amygdala, as well as in the bed nucleus of the stria terminalis.[42,43,74] Compared to the PVN or lateral septum, we found a much lower c-Fos expression after a 5-min elevated plus maze exposure in these amygdaloid areas (only about one-third of labeled cells, Fig. 2). No difference in the c-Fos response was observed between NK-1 receptor antagonist–treated and –untreated rats in these regions (Fig. 2C). Especially in the medial amygdala it was surprising to find no significant differences in the c-Fos expression between antagonist-treated animals and controls (Fig. 2C; left panel), because emotional stressors such as the exposure to an elevated platform increases SP release within the medial amygdala.[21] Possible explanations for this discrepancy may be (1) that the activation pattern after elevated plus maze exposure is too low in this area, and thus the sensitivity of the method is not sufficient to reveal significant differences in c-Fos response between treated and untreated rats, or (2) that under low-stress conditions anxiolytic-like effects of NK-1 receptor blockade are not primarily mediated via the amygdala. This assumption would be supported by our previous finding indicating that unstressed rats do not respond to NK-1 receptor antagonist injections into the medial amygdala.[21] Another but more unlikely explanation might be (3) that cells presumably mediating anxiolysis within the medial amygdala are not c-Fos responsive. In this context it should be born in mind, that, although c-Fos expression is a widely established marker of neuronal activation, neuronal firing might not always be associated with c-Fos expression.[52] The use of additional markers of neuronal activation and different stimuli will help to clarify this issue.

The effect of NK-1 receptor blockade on stress-induced c-Fos expression in the central amygdala has not been investigated since NK-1 receptor antagonist administration increases c-Fos expression in this subregion already under basal conditions.[38,75]

Concluding Remarks

In a series of studies our group and others have assessed changes in SP transmission in response to different stimuli. Considerable evidence now suggests that stressful challenges lead to the activation of circuits utilizing SP signaling in brain regions that play a major role in anxiety and depression (such as the amygdala, septum, hypothalamus, hippocampus, striatum, nucleus accumbens, periaqueductal gray, raphe nucleus, and locus coeruleus). On the other hand, a vast body of evidence has demonstrated that blocking SP transmission either by selective antagonists or genetic disruption of binding sites attenuates the effects of stress and induces anxiolytic-like and/or antidepressant effects in several animal models. Similarly, in the present study we could show anxiolytic-like efficacy after intracerebroventricular administration of a selective NK-1 receptor antagonist which is associated with reduced neuronal activity in key brain areas of the stress circuitry, such as PVN and lateral septum. In contrast, activity in brain areas of proposed anxiety networks such as medial and basolateral amygdala

was not affected by NK-1 receptor blockade, suggesting that the amygdala is not a primary target of NK-1 receptor antagonists to induce anxiolysis under low stress conditions of elevated plus maze exposure. Indeed, our previous work demonstrates that microinjections of a NK-1 receptor antagonist into the amygdala do not exert anxiolytic-like effects on the elevated plus maze in unstressed rats. However, after inducing stress either by microinjection of SP or immobilization, NK-1 receptor blockade within the amygdala subsequently produces anxiolysis.[21] Along similar lines it has been shown in patients with social anxiety disorders that the public speaking–induced increase in regional blood flow was attenuated by NK-1 receptor antagonist treatment.[76] Thus, together with other studies in NK-1 receptor knockout mice, these data suggest that interference with NK-1 receptor function is especially effective in high-stressed, deranged, or pathophysiological systems. Similarly, in humans patients with clinical syndromes of a hyperactivated stress axis should also be especially sensitive to the therapeutic benefit of NK-1 receptor antagonism in the treatment of psychiatric illness.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Krase, W., M. Koch & H.U. Schnitzler. 1994. Substance P is involved in the sensitization of the acoustic startle response by footshocks in rats. Behav. Brain Res. **63:** 81–88.
2. Severini, C., G. Improta, G. Falconieri-erspamer, *et al*. 2002. The tachykinin peptide family. Pharmacol. Rev. **54:** 285–322.
3. Nawa, H., T. Hirose, H. Takashima, *et al*. 1983. Nucleotide sequences of cloned cDNAs for two types of bovine brain substance P precursor. Nature **306:** 32–36.
4. Otsuka, M. & K. Yoshioka. 1993. Neurotransmitter functions of mammalian tachykinins. Physiol. Rev. **73:** 229–308.
5. Regoli, D., A. Boudon & J.L. Fauchere. 1994. Receptors and antagonists for substance P and related peptides. Pharmacol. Rev. **46:** 551–599.
6. Maggi, C.A. 1995. The mammalian tachykinin receptors. Gen. Pharmacol. **26:** 911–944.
7. Ribeiro-da-Silva, A. & T. Hökfelt. 2000. Neuroanatomical localisation of substance P in the CNS and sensory neurons. Neuropeptides **34:** 256–271.
8. Hökfelt, T., E. Kuteeva, D. Stanic & A. Ljungdahl. 2004. The histochemistry of tachykinin systems in the brain. *In* Tachykinins. P. Holzer, Ed.: 63–120. Springer. Heidelberg, Germany.
9. Hökfelt, T., D. Millhorn, K. Seroogy, *et al*. 1987. Coexistence of peptides with classical neurotransmitters. Experientia **43:** 768–780.
10. Merighi, A. 2002. Costorage and coexistence of neuropeptides in the mammalian CNS. Prog. Neurobiol. **66:** 161–190.
11. Unger, T., S. Carolus, G. Demmert, D. Ganten, *et al*. 1988. Substance P induces a cardiovascular defense reaction in the rat: pharmacological characterization. Circ. Res. **63:** 812–820.
12. Culman, J. & T. Unger. 1995. Central tachykinins: mediators of defence reaction and stress reactions. Can. J. Physiol. Pharmacol. **73:** 885–891.
13. Herpfer, I. & K. Lieb. 2005. Substance P receptor antagonists in psychiatry: rationale for development and therapeutic potential. CNS Drugs **19:** 275–293.
14. Kramer, M.S., N. Cutler, J. Feighner, *et al*. 1998. Distinct mechanism for antidepressant activity by blockade of central substance P receptors. Science **281:** 1640–1645.
15. Smith, D.W., L. Hewson, P. Fuller, *et al*. 1999. The substance P antagonist L-760,735 inhibits stress-induced NK(1) receptor internalisation in the basolateral amygdala. Brain Res. **848:** 90–95.
16. Steinberg, R., R. Alonso, L. Rouquier, *et al*. 2002. SSR240600 (R)-2-(1-[2-[4-[2-[3,5-bis(trifluoromethyl)phenyl acetyl]-2-(3,4-dichloro phenyl)-2-morpholinyl]ethyl]-4-piperidinyl)-2-methylpropanamide], a centrally active nonpeptide antagonist of the tachykinin neurokinin 1 receptor: II. Neurochemical and behavioral characterization. J. Pharmacol. Exp. Ther. **303:** 1180–1188.
17. Landgraf, R. & I.D. Neumann. 2004. Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication. Front. Neuroendocrinol. **25:** 150–176.
18. Wotjak, C.T., R. Landgraf & M. Engelmann. 2008. Listening to neuropeptides by microdialysis: Echoes and new sounds? Pharmacol. Biochem. Behav. **90:** 125–134.
19. Brodin, E., N. Lindefors & U. Ungerstedt. 1983. Potassium evoked in vivo release of substance P in

rat caudate nucleus measured using a new technique of brain dialysis and an improved substance P-radioimmunoassay. Acta Physiol. Scand. Suppl. **515:** 17–20.
20. Ebner, K. & N. Singewald. 2006. The role of substance P in stress and anxiety responses. Amino Acids **31:** 251–272.
21. Ebner, K., N.M. Rupniak, A. Saria & N. Singewald. 2004. Substance P in the medial amygdala: emotional stress-sensitive release and modulation of anxiety-related behavior in rats. Proc. Natl. Acad. Sci. USA **101:** 4280–4285.
22. Ebner, K., G.M. Singewald, N. Whittle, *et al*. 2008. Neurokinin 1 receptor antagonism promotes active stress coping via enhanced septal 5-HT transmission. Neuropsychopharmacology **33:** 1929–1941.
23. Roberts, G.W., P.L. Woodhams, J.M. Polak & T.J. Crow. 1982. Distribution of neuropeptides in the limbic system of the rat: the amygdaloid complex. Neuroscience **7:** 99–131.
24. Takayama, H., Z. Ota & N. Ogawa. 1986. Effect of immobilization stress on neuropeptides and their receptors in rat central nervous system. Regul. Pept. **15:** 239–248.
25. Mantyh, P.W., C.J. Allen, J.R. Ghilardi, *et al*. 1995. Rapid endocytosis of a G protein-coupled receptor: substance P evoked internalization of its receptor in the rat striatum in vivo. Proc. Natl. Acad. Sci. USA **92:** 2622–2626.
26. Ebner, K. & N. Singewald. 2007. Stress-induced release of substance P in the locus coeruleus modulates cortical noradrenaline release. Naunyn-Schmiedebergs. Arch. Pharmacol. **376:** 73–82.
27. Berton, O., H.E. Covington 3rd, K. Ebner, *et al*. 2007. Induction of deltaFosB in the periaqueductal gray by stress promotes active coping responses. Neuron **55:** 289–300.
28. Nestler, E.J., M. Barrot, R.J. Dileone, *et al*. 2002. Neurobiology of depression. Neuron **34:** 13–25.
29. Slattery, D.A., A.L. Hudson & D.J. Nutt. 2004. Invited review: the evolution of antidepressant mechanisms. Fundam. Clin. Pharmacol. **18:** 1–21.
30. Singewald, N., G.G. Chicchi, C.C. Thurner, *et al*. 2008. Modulation of basal and stress-induced amygdaloid substance P release by the potent and selective NK1 receptor antagonist L-822429. J. Neurochem. **106:** 2476–2488.
31. Landgraf, R. & A. Wigger. 2002. High vs low anxiety-related behavior rats: an animal model of extremes in trait anxiety. Behav. Genet. **32:** 301–314.
32. Sartori, S.B., K. Ebner, P. Muigg, *et al*. 2005. Differences in substance P neurotransmission between rats with high vs. low trait anxiety/depression [abstract]. J. Neurochem. **94:** 229.
33. Geracioti, T.D. Jr., L.L. Carpenter, M.J. Owens, *et al*. 2006. Elevated cerebrospinal fluid substance P concentrations in posttraumatic stress disorder and major depression. Am. J. Psychiatry **163:** 637–643.
34. Michelgard, A., L. Appel, A. Pissiota, *et al*. 2007. Symptom provocation in specific phobia affects the substance P neurokinin-1 receptor system. Biol. Psychiatry **61:** 1002–1006.
35. Whitnall, M.H. 1993. Regulation of the hypothalamic corticotropin-releasing hormone neurosecretory system. Prog. Neurobiol. **40:** 573–629.
36. Antoni, F.A. 1986. Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. Endocr. Rev. **7:** 351–378.
37. Hahn, M.K. & M.J. Bannon. 1999. Stress-induced c-fos expression in the rat locus coeruleus is dependent on neurokinin 1 receptor activation. Neuroscience **94:** 1183–1188.
38. Muigg, P., S.B. Sartori, T. Sparber, *et al*. 2005. Reduced depression-like behaviour in HAB rats after NK1-receptor antagonist treatment is associated with an altered neuronal activation pattern in specific brain areas [abstract]. Abstract at the Meeting of the Austrian Neuroscience Association.
39. Hahn, M.K. & M.J. Bannon. 1998. Tachykinin NK1 receptor antagonists enhance stress-induced c-fos in rat locus coeruleus. Eur. J. Pharmacol. **348:** 155–160.
40. Santarelli, L., G. Gobbi, P. Blier & R. Hen. 2002. Behavioral and physiologic effects of genetic or pharmacologic inactivation of the substance P receptor (NK1). J. Clin. Psychiatry **63**(Suppl. 11): 11–17.
41. McCutcheon, J.E., A.S. Fisher, E. Guzdar, *et al*. 2008. Genetic background influences the behavioural and molecular consequences of neurokinin-1 receptor knockout. Eur. J. Neurosci. **27:** 683–690.
42. Duncan, G.E., D.J. Knapp & G.R. Breese. 1996. Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. Brain Res. **713:** 79–91.
43. Silveira, M.C., G. Sandner & F.G. Graeff. 1993. Induction of Fos immunoreactivity in the brain by exposure to the elevated plus-maze. Behav. Brain Res. **56:** 115–118.
44. File, S.E., H. Zangrossi, Jr., F.L. Sanders & P.S. Mabbutt. 1994. Raised corticosterone in the rat after exposure to the elevated plus-maze. Psychopharmacology **113:** 543–546.
45. Rodgers, R.J., J. Haller, A. Holmes, *et al*. 1999. Corticosterone response to the plus-maze: high correlation with risk assessment in rats and mice. Physiol. Behav. **68:** 47–53.
46. Paxinos, G. & C. Watson. 1998. The Rat Brain in Stereotaxic Coordinates. Academic Press. New York.
47. Singewald, N., P. Salchner & T. Sharp. 2003. Induction of c-Fos expression in specific areas of the fear

circuitry in rat forebrain by anxiogenic drugs. Biol. Psychiatry **53:** 275–283.
48. Pellow, S., P. Chopin, S.E. File & M. Briley. 1985. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Methods **14:** 149–167.
49. Adell, A., E. Castro, P. Celada, *et al*. 2005. Strategies for producing faster acting antidepressants. Drug Discov. Today **10:** 578–585.
50. Singewald, N. 2007. Altered brain activity processing in high-anxiety rodents revealed by challenge paradigms and functional mapping. Neurosci. Biobehav. Rev. **31:** 18–40.
51. Kovacs, K.J. 1998. c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem. Int. **33:** 287–297.
52. Hoffman, G.E. & D. Lyo. 2002. Anatomical markers of activity in neuroendocrine systems: Are we all 'fosed out'? J. Neuroendocrinol. **14:** 259–268.
53. Swanson, L.W., P.E. Sawchenko & R.W. Lind. 1986. Regulation of multiple peptides in CRF parvocellular neurosecretory neurons: implications for the stress response. Prog. Brain Res. **68:** 169–190.
54. Mantyh, P.W., T. Gates, C.R. Mantyh & J.E. Maggio. 1989. Autoradiographic localization and characterization of tachykinin receptor binding sites in the rat brain and peripheral tissues. J. Neurosci. **9:** 258–279.
55. Sawchenko, P.E., E.R. Brown, R.K. Chan, *et al*. 1996. The paraventricular nucleus of the hypothalamus and the functional neuroanatomy of visceromotor responses to stress. Prog. Brain Res. **107:** 201–222.
56. Li, H.Y. & P.E. Sawchenko. 1998. Hypothalamic effector neurons and extended circuitries activated in "neurogenic" stress: a comparison of footshock effects exerted acutely, chronically, and in animals with controlled glucocorticoid levels. J. Comp. Neurol. **393:** 244–266.
57. Mello, D.M., D.R. Marcinichen, D. Madruga, *et al*. 2007. Involvement of NK1 receptors in metabolic stress markers after the central administration of substance P. Behav. Brain Res. **181:** 232–238.
58. Santarelli, L., G. Gobbi, P.C. Debs, *et al*. 2001. Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function. Proc. Natl. Acad. Sci. USA **98:** 1912–1917.
59. Rupniak, N.M., E.J. Carlson, J.K. Webb, *et al*. 2001. Comparison of the phenotype of NK1R-/- mice with pharmacological blockade of the substance P (NK1) receptor in assays for antidepressant and anxiolytic drugs. Behav. Pharmacol. **12:** 497–508.
60. Kalman, B.A., P.J. Kim, M.A. Cole, *et al*. 1997. Diazepam attenuation of restraint stress-induced corticosterone levels is enhanced by prior exposure to repeated restraint. Psychoneuroendocrinology **22:** 349–360.
61. Imaki, T., X.Q. Wang, T. Shibasaki, *et al*. 1995. Chlordiazepoxide attenuates stress-induced activation of neurons, corticotropin-releasing factor (CRF) gene transcription and CRF biosynthesis in the paraventricular nucleus (PVN). Brain Res. Mol. Brain Res. **32:** 261–270.
62. Sheehan, T.P., R.A. Chambers & D.S. Russell. 2004. Regulation of affect by the lateral septum: implications for neuropsychiatry. Brain Res. Brain Res. Rev. **46:** 71–117.
63. Risold, P.Y. 2004. The septal region. *In* The Rat Nervous System. G. Paxinos, Ed.: 605–632. Academic Press. New York.
64. Risold, P.Y. & L.W. Swanson. 1997. Chemoarchitecture of the rat lateral septal nucleus. Brain Res. Brain Res. Rev. **24:** 91–113.
65. Risold, P.Y. & L.W. Swanson. 1997. Connections of the rat lateral septal complex. Brain Res. Brain Res. Rev. **24:** 115–195.
66. Mongeau, R., G.A. Miller, E. Chiang & D.J. Anderson. 2003. Neural correlates of competing fear behaviors evoked by an innately aversive stimulus. J. Neurosci. **23:** 3855–3868.
67. Staiger, J.F. & F.G. Wouterlood. 1990. Efferent projections from the lateral septal nucleus to the anterior hypothalamus in the rat: a study combining Phaseolus vulgaris-leucoagglutinin tracing with vasopressin immunocytochemistry. Cell Tissue Res. **261:** 17–23.
68. Herman, J.P., H. Figueiredo, N.K. Mueller, *et al*. 2003. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamopituitary-adrenocortical responsiveness. Front. Neuroendocrinol. **24:** 151–180.
69. Walker, D.L., D.J. Toufexis & M. Davis. 2003. Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. Eur. J. Pharmacol. **463:** 199–216.
70. Davis, M., D.L. Walker & Y. Lee. 1997. Roles of the amygdala and bed nucleus of the stria terminalis in fear and anxiety measured with the acoustic startle reflex. Possible relevance to PTSD. Ann. N.Y. Acad. Sci. **821:** 305–331.
71. Boyce, S., D. Smith, E. Carlson, *et al*. 2001. Intra-amygdala injection of the substance P antagonist L-760735 inhibits neonatal vocalisations in guinea-pigs K(1) receptor. Neuropharmacology **41:** 130–137.
72. Sergeyev, V., S. Fetissov, A.A. Mathe, *et al*. 2005. Neuropeptide expression in rats exposed to chronic mild stresses. Psychopharmacology **178:** 115–124.
73. Zhao, Z., Y. Yang, D.L. Walker & M. Davis. 2008. Effects of Substance P in the amygdala, ventromedial hypothalamus, and periaqueductal gray on

fear \ potentiated startle. Neuropsychopharmacology: In press.
74. Knapska, E., K. Radwanska, T. Werka & L. Kaczmarek. 2007. Functional internal complexity of amygdala: focus on gene activity mapping after behavioral training and drugs of abuse. Physiol. Rev. **87:** 1113–1173.
75. Baulmann, J., H. Spitznagel, T. Herdegen, *et al*. 2000. Tachykinin receptor inhibition and c-Fos expression in the rat brain following formalin-induced pain. Neuroscience **95:** 813–820.
76. Furmark, T., L. Appel, A. Michelgard, *et al*. 2005. Cerebral blood flow changes after treatment of social phobia with the neurokinin-1 antagonist GR205171, citalopram, or placebo. Biol. Psychiatry **58:** 132–142.