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Neurokinin 1 receptor antagonism requires norepinephrine to increase serotonin function

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Abstract The present studies examined the role of norepinephrine (NE) system in mediating the enhancement of 5-HT function produced by neurokinin (NK)1 receptor antagonism. Dorsal raphe 5-HT and locus coeruleus NE neurons were recorded *in vivo* in mice lacking NK1 receptors in wildtype mice pretreated with the NK1 antagonist RP67580 and its inactive enantiomer RP 68651. RP67580 and RP68651 were also tested on 5-HT neurons of mice lacking the 5-HT_{1A} receptor. RP67580 increased the firing rate of 5-HT neurons in wildtype mice and in 5-HT_{1A} null mutant mice to the same degree, thus indicating that the mechanism by which NK1 antagonists enhances 5-HT firing is independent of 5-HT_{1A} receptors. NE neuronal burst activity was increased in NK1 null mutant and wildtype mice given RP67580, but not with RP68651. After NE depletion, RP67580 was ineffective in increasing 5-HT neuronal firing activity in NK1 wildtype mice, and the enhancement of 5-HT neuronal firing observed in NK1 null mutant mice was abolished. In conclusion, NE neurons are essential for the action of NK1 antagonists on 5-HT neurons. In addition, the desensitization of 5-HT_{1A} autoreceptors produced by NK1 receptor antagonism is not critical for enhancing 5-HT neuronal firing.
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1. Introduction

Tricyclic antidepressant drugs (TCAs), selective serotonin (5-hydroxytryptamine; 5-HT) reuptake inhibitors (SSRIs) and/or norepinephrine (NE) reuptake inhibitors (SNRIs) and mono-amine oxidase inhibitors (MAOIs), used in the treatment of both mood and anxiety disorders, modulate the function of these two monoamine systems by different mechanisms. Their net long-term effect is an enhancement of 5-HT neurotransmission (Blier and de Montigny, 1999) and a modification of locus coeruleus firing activity (Szabo and Blier, 2001). Nevertheless, the use of these drugs is limited by their side-effect profiles, their delayed onset of action and their partial effectiveness. Indeed, approximately 50% of depressed patients do not show an acceptable response to an initial antidepressant trial (Thase and Rush, 1997). SSRIs require prolonged administration before a significant clinical improvement occurs because the rapid increase of synaptic 5-HT availability caused by reuptake inhibition in the raphe nuclei stimulates cell body 5-HT$_{1A}$ autoreceptors, which in turn exerts a negative feedback on 5-HT neuronal firing and release in projection areas. Only when 5-HT$_{1A}$ autoreceptors are desensitized can SSRIs, SNRIs and MAOIs markedly enhance 5-HT neurotransmission (Blier and de Montigny, 1999). In addition, the firing activity of locus coeruleus NE neurons is reduced after long-term administration of SSRIs, that is, when the anxiolytic action of SSRIs also becomes evident (Szabo et al., 2000; Seager et al., 2004).

The observation that antagonists of Substance P (SP; neurokinin 1; NK1) receptors can be effective in the treatment of depression in patients with significant anxiety symptoms has generated research endeavors in a potential therapeutic strategy for the treatment of depression and anxiety (Kramer et al., 1998, 2004; Ranga and Krishnan, 2002). Subsequently, several studies did not replicate such earlier findings (Keller et al., 2006), although clinical trials are still ongoing with the possibility that even if NK1 antagonists are not eventually used as a monotherapy, they could still be prescribed as an augmentation strategy (see Blier et al., 2004). The mechanism of action of NK1 antagonists is presently not fully understood. Nevertheless, the presence of NK1 receptors and SP on some 5-HT neurons of dorsal raphe, on NE neurons of locus coeruleus, and on mesolimbic dopamine neurons lead to the hypothesis that NK1 receptor antagonists may exert, at least in part, their clinical actions by modulating monoaminergic transmissions (for a review see Gobbi and Blier, 2005).

Genetic deletion, as well as the pharmacological block-ade of NK1 receptors, decreases anxiety-related behaviors, possibly by increasing the spontaneous firing rate of 5-HT neurons and desensitizing 5-HT$_{1A}$ autoreceptors (Santarelli et al., 2001; Haddjeri and Blier, 2001a). As for other types of antidepressant treatments, long-term but not subacute administration of a non-peptidic NK1 antagonist enhances the degree of tonic activation of postsynaptic 5-HT$_{1A}$ receptors in the hippocampus (Haddjeri et al., 1998; Haddjeri and Blier, 2001a). This was attributed to a greater increase in 5-HT neuronal firing achieved after 14 than after 2 days of administration, possibly resulting from a more pronounced desensitization of the 5-HT$_{1A}$ autoreceptors (Haddjeri and Blier, 2001a). Prior investigations have reported that NK1 receptor antagonism enhances the

activity of NE neurons (Millan et al., 2001), promotes their burst firing pattern (Maubach et al., 2002), and attenuates the responsiveness of the α_2-adrenergic autoreceptor on the cell body of locus coeruleus NE neurons (Haddjeri and Blier, 2000, 2001b).

In the light of these results, the goals of the present study were to investigate: 1) whether, as for SSRIs, 5-HT$_{1A}$ autoreceptor desensitization represents an essential condition for the pharmacological action of the NK1 antagonists on 5-HT firing activity; 2) whether spontaneous NE neuronal firing activity is modified by the pharmacological and genetic blockage of NK1 receptors; 3) and whether the pharmacological activity of NK1 receptor antagonism on 5-HT neurotransmission is mediated by the NE system. Electrophysiological experiments were thus performed using wildtype mice and mice lacking NK1 receptors (NK1 $-/-$) and mice lacking 5-HT$_{1A}$ receptors (5-HT$_{1A}-/-$). The NK1 antagonist RP67580, as well as its inactive enantiomer RP68651, was used to ascertain the pharmacological NK1 receptor selectivity of the active compound (Garret et al., 1991).

2. Experimental procedures

2.1. Animals

NK1 and 5-HT$_{1A}$ receptor knockouts were generated as previously described (Santarelli et al., 2001; Ramboz et al., 1998). Knockout and wildtype 129/SvEv age-matched adult male mice (12—20 weeks) derived from heterozygote crossings (Phillips et al., 1999) were used in electrophysiological experiments. Male Swiss and 129/SvEv mice were used for neurochemical analyses. Mice were housed 4—5 per cage in a colony room at 22 °C with food and water *ad libitum*. The animals were maintained on a 12:12 h cycle (lights on 06:00—18:00). All procedures were approved by local institutional care and use committees and followed guidelines released by the Canadian Institutes of Health Research, National Institutes of Health and the Italian Ministry of Health (D.L. 116/92).

2.2. Drugs

RP67580 (Aventis, Bridgewater, NJ, USA) and its inactive enantiomer devoid of activity at NK1 receptor, RP68651 (Garret et al., 1991), were dissolved in 0.1% HCl diluted in saline (0.9% NaCl); 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) was dissolved in saline. *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) was diluted in saline.

2.3. Preparation of the electrophysiological experiments

Mice were anaesthetised with chloral hydrate (400 mg kg^{-1}, i.p., using a 2% solution) and placed in a stereotaxic frame (using the Kopf mouse adaptor) with the skull positioned horizontally. In order to maintain a full anaesthetic state in which there was no reaction to a tail or paw pinch, chloral hydrate supplements of 100 mg kg^{-1} were given as needed. The extracellular recordings were carried out using single or double-barreled glass micropipettes (R and D Scientific Glass, Spencer-ville, MD, USA). Single and double-barreled glass micropipettes were preloaded with fiberglass strands in order to promote capillary filling with, respectively, a 1 M and 1.5 M NaCl solution. The single micropipettes were used for recording both dorsal raphe 5-HT and locus coeruleus NE neurons and their tips were of 1—3 μm in diameter. Double-barreled micropipettes were used to assess the responsiveness of 5-HT neurons to the 50-sec microiontophoretic applications of 8-OH-

DPAT. The impedance of the central barrel used for unitary typically ranged between 5 and 7 MΩ and for the 8-OH-DPAT-containing barrel between 15 and 20 MΩ.

2.4. Recording of dorsal raphe 5-HT neurons

The single- or double-barreled glass micropipettes were positioned 0.5—1 mm posterior to the interaural line on the midline and lowered into the dorsal raphe nucleus, usually attained at a depth of between 2.5 and 3.5 mm from the brain surface (Fig. 1; Franklin and Paxinos, 1997). The dorsal raphe 5-HT neurons were identified according to the following criteria: a slow (0.5—2.5 Hz) and regular firing rate and a long-duration (0.8—1.2 ms) positive action potential. The total number of spontaneously active 5-HT neurons was recorded and their average firing rate was assessed for a minimal period of 1 min.

2.5. Recording of locus coeruleus NE neurons

The single-barreled glass micropipette was positioned 1.5 mm posterior to the interaural line and 0.2—1.1 mm from the midline and lowered into the locus coeruleus usually encountered at a depth of between 2.7 and 4.0 mm from the brain surface (Fig. 2). NE neurons in mice were identified by their regular firing rate (0.5—5 Hz), long duration (0.8—1.2 ms) positive action potentials and their brief but brisk increase of firing in response to nociceptive stimulation of the contralateral hind paw, as previously observed in rats (Cedarbaum and Aghajanian, 1976). The total number of spontaneously active NE neurons was recorded and their average firing rate was assessed for a minimal period of 1 min.

2.6. Lesioning of NE neurons

Lesions of NE neurons were carried out with the selective NE neuron neurotoxin DSP-4 injected intraperitoneally in a single dose
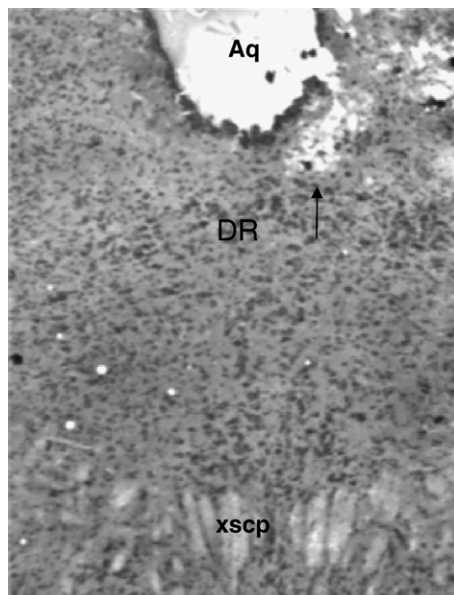

Figure 1 Identification of an electrolytic lesion in the dorsal raphe (DR) area. Thionin-stained coloration slice through the dorsal (X5), at the level of −4.60 mm from bregma and −0.80 mm from interaural. The Sylvius aqueduct (Aq) is indicated at the top of the micrograph. The lesion left at the end of the experiment is marked by the arrow. xscp stands for decussation of the superior peduncles.
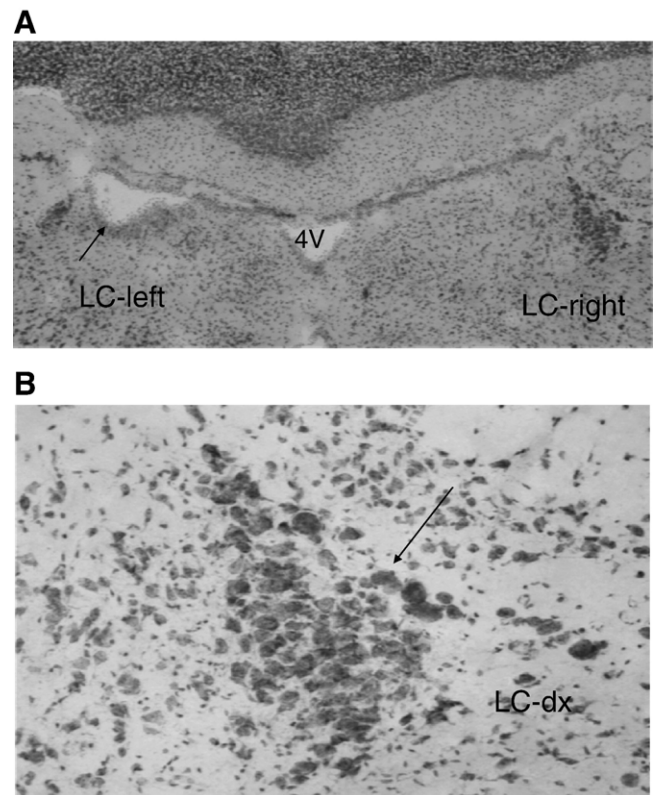

Figure 2 (A) Identification of the electrolytic lesion in the locus coeruleus (LC) area. Thionin-stained coloration slice through the LC (×5), at the level of −5.52 mm from bregma and −1.72 mm from interaural. The recording and electrolytic lesion were done in the left locus LC. The right LC appears intact. The lesion is marked by the arrow. 4V = fourth ventricle. (B). The intact right LC (×20) with the typical norepinephrine LC neurons are indicated by the arrow.

(50 mg/kg; Cheetham et al., 1996). The lesioned mice were allowed to recover for 7 days before carrying out electrophysiological recordings and neurochemical measurements.

2.7. Burst activity and spontaneous active cells count

Firing frequency and burst firing were analyzed for each cell during each experimental series. A burst was defined according to Gartside et al. (2000) as a train of at least 2 spikes with the first interspike interval of 20 ms or less, and a termination interval greater than or equal to 160 ms. Burst activity was analyzed according to the total percent of firing occurring in bursts called percentage of bursts (Gobbi et al., 2001). The analysis of these two parameters (spikes/sec and % burst firing) is an important index for the activity of NE and 5-HT cells and allows one to evaluate the influence that a putative drug exerts on the cell firing pattern. The number of spontaneously active 5-HT and NE neurons was determined by passing the electrode five to eight times through four different areas within the dorsal raphe or locus coeruleus, according to the method described by Blier and de Montigny (1985a). The total number of active cells were counted and divided by the number of passes to obtain the number of cells/track.

2.8. Histology

At the end of each experiment, an electrical lesion was made, using a current of +10—15 μA for 15 min, to identify the area of the

recordings. The brains were then removed and coronal cryotome sections (20 μm thick) were cut through the dorsal raphe or the locus coeruleus. Sections were mounted onto gelatinized slides, thionin stained, and used to localize the electrical lesion (Figs. 1, 2).

2.9. Neurochemical determinations

Control and DSP-4 lesioned wildtype mice were killed by cervical dislocation. The brain was rapidly removed from the cranium and immediately frozen on dry ice. 1 mm coronal sections were sliced using a cooled rodent brain matrix and a cooled single-edge blade. Dorsal raphe and locus coeruleus were dissected from the slices formed, under a stereomicroscope. Tissue samples were weighted into a 2.0 ml tube containing 100 μl of perchloric acid 0.1 M, disrupted by sonication and then centrifuged at 10.000 ×g for 8 min at 4 °C. Supernatants were analyzed by HPLC for quantification of the NE, 5-HT and 5-hydroxyindolacetic acid (5-HIAA) levels. Neurotransmitter concentrations were determined by microbore HPLC using a SphereClone 150-mm × 2-mm column (3-μm packing). The detection was accomplished with a Unijet cell (BAS) with a 6-mm-diameter glassy carbon electrode at +650 mV, connected to an electrochemical detector (INTRO). The chromatographic conditions were: (i) a mobile phase composed of 85 mM of sodium acetate, 0.34 mM EDTA, 15 mM sodium chloride, 0.81 mM of octanesulphonic acid sodium salt, 5% methanol (v/v), pH=4.85 (ii) a rate flow of 220 μl/min (iii) a total runtime of 65 min.

2.10. Statistical analyses

The data are expressed throughout as means ± SEM; the sample size (n) represents the number of recorded neurons in electrophysiological experiments or neurochemical experiments. When two means were compared, statistical significance of their difference was assessed using the two-tailed paired or non-paired Student's t test as indicated. For multiple comparisons, one way or two-way ANOVA was used for independent and paired samples, respectively, followed by Tuckey's tests. Chi-square analysis of contingency tables or Fisher's exact test were used to compare the distributions of cells discharging in bursts pattern.

3. Results

3.1. Effects of the NK1 receptor deletion and of NK1 antagonist RP67580, RP68651 on 5-HT neuronal firing activity

The mean spontaneous firing rate of electrophysiologically-identified dorsal raphe nucleus 5-HT neurons in the NK1 +/+ mice group was 1.72 ± 0.2 Hz ($n = 19$). The mean firing frequency of 5-HT neurons was significantly increased by 86% in NK1 −/− mice, and by 97% in the NK1 +/+ animals pretreated with RP67580 (1.5 mg/kg, i.p., injected 30 min injected before initiating recording), when compared to control mice (Fig. 3). The non-active enantiomer RP 68580 (1.5 mg/kg, i.p., injected 30 min before cell recording) did not increase 5-HT firing activity (1.89 ± 0.3 Hz; Fig. 3).

3.2. Alteration of the responsiveness of 5-HT$_{1A}$ autoreceptors by the NK1 antagonist RP67580

It has previously been shown that presynaptic 5-HT$_{1A}$ autoreceptors on 5-HT neurons in the dorsal raphe are
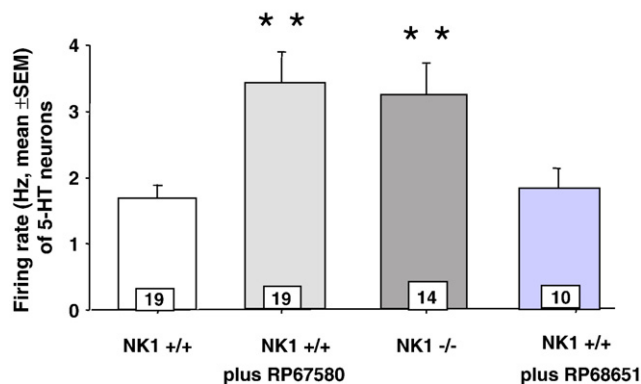

Figure 3   Effects of NK1 antagonism and genetic deletion on 5-HT neuron activity. Mean frequency of firing of 5-HT neurons in the dorsal raphe of NK1 +/+ mice, NK1 −/− mice, NK1 +/+ mice pretreated with RP67580 and RP68651. The number of recorded neurons is indicated in the boxes at the bottom of each column. Data were analysed using a one way Analysis of Variance ($p < 0.001$); a multiple comparison showed statistically significant differences between NK1 +/+ vs NK1 +/+ pretreated with RP67580 and vs NK1 −/− mice (**, $P < 0.001$).

desensitized in NK1 −/− mice, as demonstrated by a reduced response to the 5-HT$_{1A}$ agonist 8-OH-DPAT using *in vivo* microiontophoretic applications and its *in vitro* perfusion (Santarelli et al., 2001; Froger et al., 2001). In order to determine the sensitivity of 5-HT$_{1A}$ autoreceptors 30 min after the administration of RP67580, electrode trajectories were carried out through the dorsal raphe using two-barreled micropipettes. Small ejection currents (+2 to +8 nA) of 8-OH-DPAT consistently decreased the firing activity of 5-HT neurons in NK1 +/+ mice in a current-dependent manner (Fig. 4). In NK1 −/− mice, 5-HT neurons showed a marked attenuation of their inhibitory response to the ejection of 8-OH-DPAT. Similarly, the NK1 +/+ mice pretreated with RP67580 showed a decreased responsiveness to 8-OH-DPAT, although less pronounced than the NK1 −/− mice.

A two-way ANOVA (NK1 antagonism × current) revealed a significant effect of the NK1 blockade ($F_{2,61} = 3.95$, $p = 0.024$), but not a significant effect of 8-OH-DPAT currents ($F_{2,61} = 0.5$, $p = 0.59$), and no significant interaction effect ($p = 0.59$). Multiple comparison procedures showed a significant difference between the NK1 +/+ and NK1 −/− mice and between NK1 +/+ vs NK1 +/+ pretreated mice with RP67580 (Fig. 4).

3.3. Effects of the NK1 antagonist RP67580 on 5-HT neuronal firing activity in 5-HT$_{1A}$−/− mice

The subsequent series of experiments were conducted to assess whether NK1 receptor antagonism could modify the activity of 5-HT neurons in the absence of 5-HT$_{1A}$ receptors. To this end, the firing activity of 5-HT neurons in 5-HT$_{1A}$−/− mice was examined prior to and after the injection of RP67580. The mean firing rate of 5-HT neurons in 5-HT$_{1A}$−/− mice was about 100% higher than in their wildtype littermates (Figs. 6A, B, 7), as previously reported (Richer et al., 2002). This is due to the lack of 5-HT$_{1A}$ autoreceptors normally mediating the presynaptic inhibition.
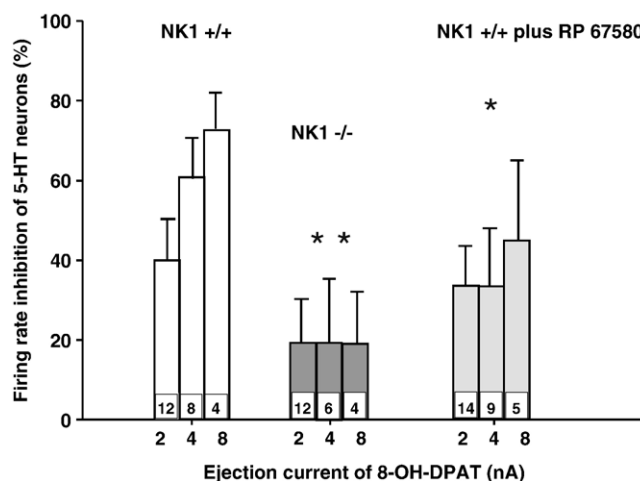
Figure 4 Reduced responsiveness of 5-HT$_{1A}$ autoreceptors to the 5-HT$_{1A}$ agonist 8-OH-DPAT after NK1 receptor blockade. Responsiveness, assessed in percentage inhibition (mean $\pm$ SEM), of 5-HT neurons to the microiontophoretic application of the 5-HT$_{1A}$ agonist 8-OH-DPAT (2, 4 and 8 nA). The number of neurons tested is given at the bottom of each column. (**) $p<0.01$, (*) $p<0.05$ using a two-way Analysis of Variance, and a multiple comparison procedure (Tukey's test).

Remarkably, the systemic injection of RP67580 caused a further increase of the mean firing rate of 5-HT neurons in 5-HT$_{1A}$−/− animals, indicating that 5-HT neuronal firing can be enhanced by NK1 receptor antagonism despite the absence of 5-HT$_{1A}$ autoreceptors (Figs. 5C, 6C, 7). Importantly, the number of neurons firing in doublets and/or displaying burst
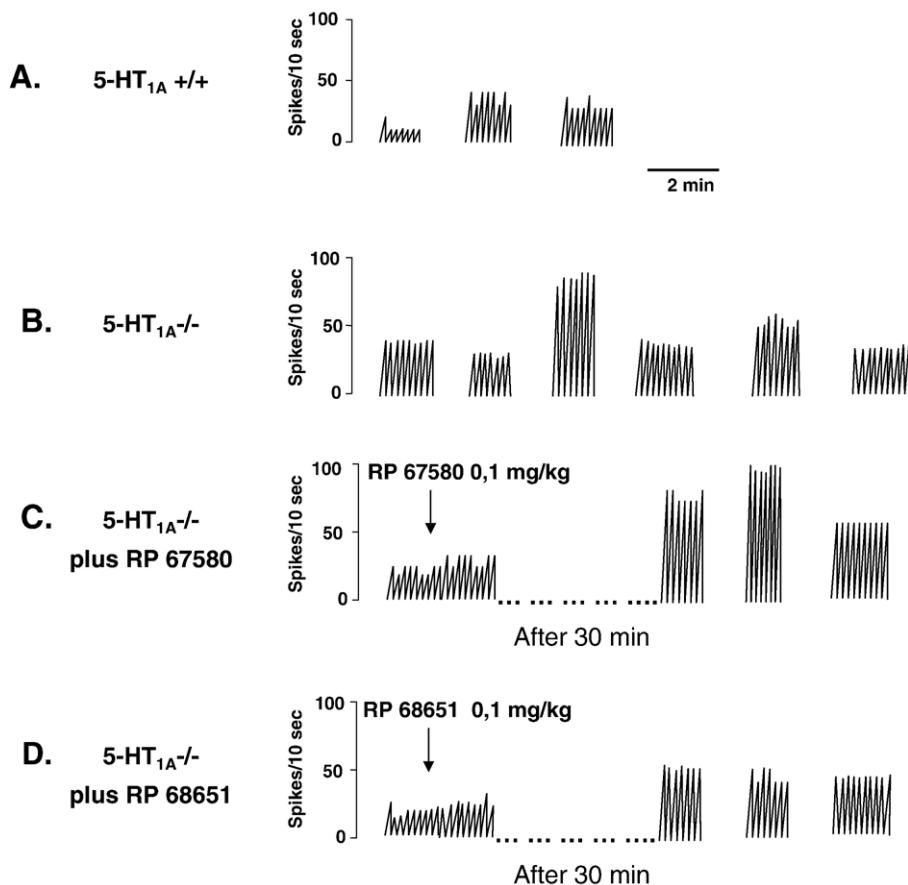

Figure 5 NK1 receptor antagonism increases spontaneous firing activity of 5-HT neurons in 5-HT$_{1A}$ −/− mice. Integrated firing rate histograms of 5-HT neurons illustrating the activity of all 5-HT neurons recorded in single trajectories through the dorsal raphe, respectively, of a 5-HT$_{1A}$ +/+ mouse (A), a 5-HT$_{1A}$ −/− mouse (B), and a 5-HT$_{1A}$ +/+ mouse pretreated with the NK1 antagonist RP67580 (C) and RP68651 (D). The dots at the bottom of the traces (in C) represent interruptions of the physiograph recording. The time base applies to all traces.
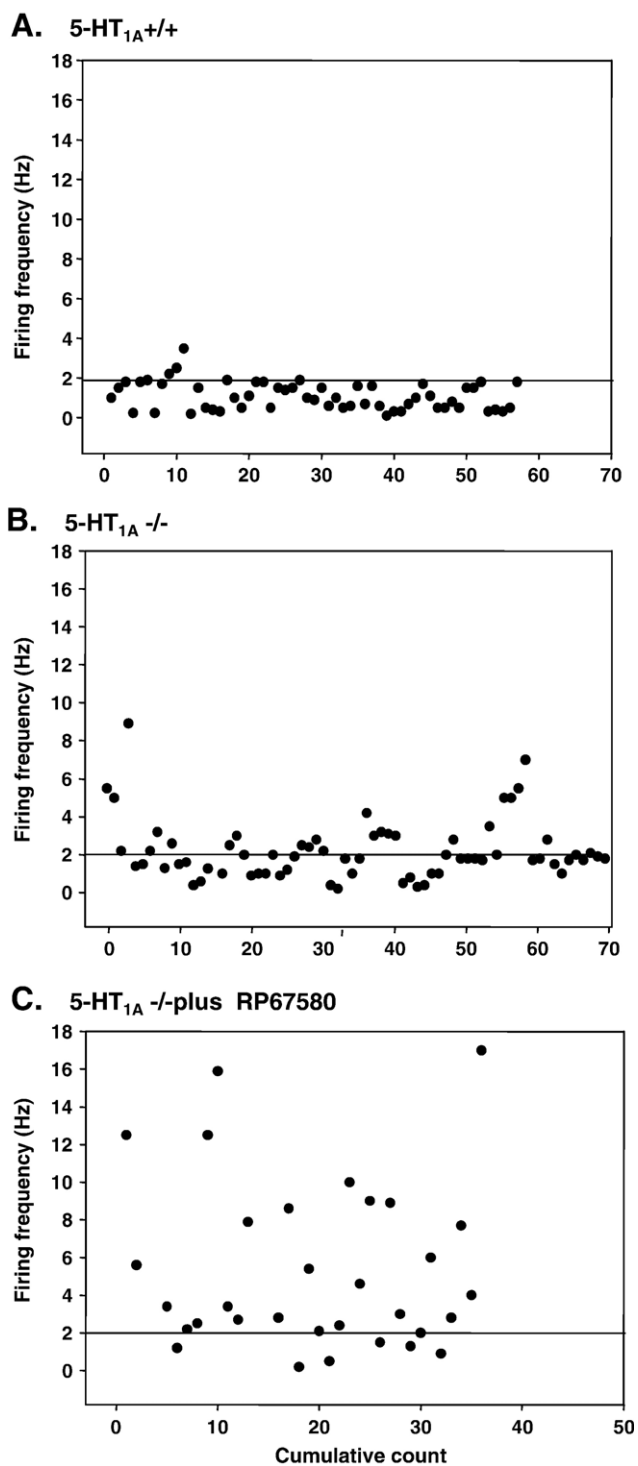
A. 5-HT$_{1A}$+/+

B. 5-HT$_{1A}$ -/-

C. 5-HT$_{1A}$ -/-plus RP67580

Figure 6 Scattergrams depicting the firing frequency of all spontaneously active 5-HT neurons encountered during systematic electrode descents through the dorsal raphe of wildtype mice (A-5-HT$_{1A}$), of null mutant mice for the 5-HT$_{1A}$ receptor (B-5-HT$_{1A}$ −/−), and of 5-HT$_{1A}$ −/− null mutant mice following the injection of the NK1 antagonist RP67580.

activity was significantly increased (control: 12%; RP67580: 35%, $p = 0.05$, using Fisher's test) 30 min after the administration of RP67580. The inactive enantiomer RP68651 did not

increase the 5-HT neuronal firing and burst activity after its injection, as determined by systemic electrode descents, in 5-HT$_{1A}$−/− as well as in 5-HT$_{1A}$+/+ mice (Figs. 5, 7).

Interestingly, a significant increase in the average number of cells per track was found in the 5-HT1A−/− mice, when compared with 5-HT1A+/+ mice (4.6 ± 0.9, $n = 12$ tracks; 1.9 ± 0.3, $n = 11$ tracks, respectively, $p < 0.05$). The increased number of spontaneously active 5-HT neurons persisted after the injection of RP67580 (3.8 ± 0.8, $n = 8$ tracks), and of its inactive enantiomer RP68651 (5 ± 3, $n = 3$ tracks), indicating that 5-HT1A−/− mice have more spontaneously active neurons than control mice, independent of the treatment.

3.4. Spontaneous firing activity of locus coeruleus NE neurons in NK1 +/+ and NK1 −/− mice and effects of RP67580 on the activity of NE in NK1 +/+ mice

Locus coeruleus NE neurons recorded from NK1 wildtype mice displayed the same wave characteristics and patterns of activity as those previously described in rats (Cedarbaum and Aghajanian, 1976). They also responded in mice to a pinch of the contralateral paw with a brisk increase in firing rate followed by a short pause.

No differences between genotypes were observed in the frequency of such electrophysiologically-identified NE-neurons (Fig. 8A, B), or in the number of neurons/track (data not shown). Injection of RP67580 (1.5 mg/kg, $n = 8$ neurons), significantly enhanced the firing rate of NE neurons in NK1 +/+ mice ($p < 0.01$, Fig. 8A, B). Since the same effect was observed also after the injection of the inactive enantiomer RP68651 (1.5 mg/kg, $n = 8$), it was concluded that this effect was not NK1 receptor related.

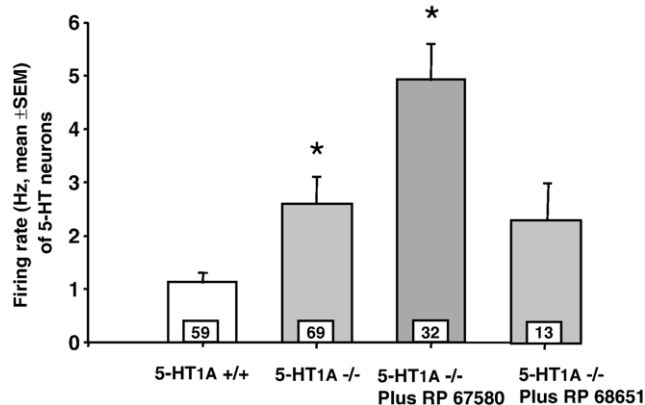Nevertheless, the NK1 −/− mice showed an increased percentage of neurons discharging in bursts (44% of total

Figure 7 Mean frequency of firing of dorsal raphe 5-HT neurons recorded in 5-HT$_{1A}$ +/+, 5-HT$_{1A}$ −/−, and 5-HT$_{1A}$ −/− mice pretreated with RP67580 and RP68651 30 min prior to recording. The number of neurons recorded and the number of mice used are indicated in the boxes at the bottom of each column. Data were analyzed using a one way Analysis of Variance ($p < 0.05$); a multiple comparison showed statistically significant differences between 5-HT$_{1A}$ +/+ vs 5-HT$_{1A}$ −/−, and 5-HT$_{1A}$ −/− vs 5-HT$_{1A}$ −/− mice plus RP67580 (*, $P < 0.001$).
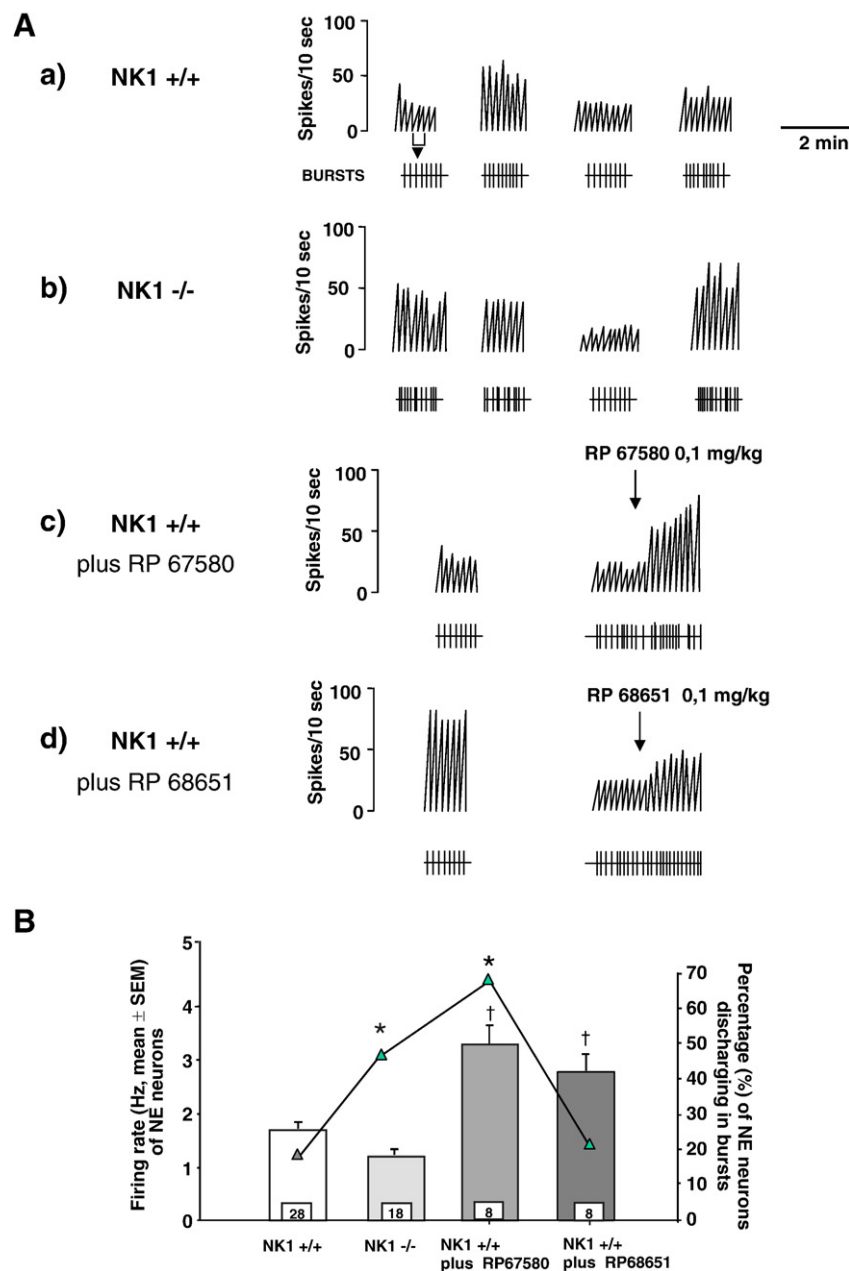
Figure 8 (A) Acute effects of the NK1 antagonist RP67580 on NE firing activity. Integrated firing rate histograms of NE neurons illustrating the activity of all NE neurons recorded in the locus coeruleus, respectively, of a NK1 +/+ mouse (a), a NK1 −/− mouse (b), and a NK1 +/+ mouse pretreated with the NK1 antagonist RP67580 (c) and with RP68651 (d). The time base applies to all traces. Events/sec indicates the burst activity, that is when at least two action potentials occurred with an interval <20 ms. (B). On the left and bars: mean frequency of firing of NE neurons in the locus coeruleus of NK1 +/+, NK1 −/− mice, NK1 +/+ pretreated with RP67580 and RP68651. The number of recorded neurons is indicated in the boxes at the bottom of each column. Data were analyzed using one way Analysis of Variance ($p<0.001$; a multiple comparison showed statistically significant differences between NK1 +/+ treated with RP67580 or RP68651 vs NK1 +/+ (†, $P<0.01$). On the right and lines: percentage of NE recorded cells showing a burst pattern activity. Chi-square test showed a significant difference between the NK1 −/− mice vs NK1 +/+ mice, and between NK1 +/+ mice pretreated with RP67580 vs NK1 +/+ mice (*, $P<0.05$).

recorded cells) compared to NK1 +/+ mice (14%, ($\chi^2(1)=3.7$, $p=0.05$, Fig. 6B). RP67580 (1.5—3 mg/kg), but not RP68651 (1.5 mg—3 mg/kg, i.p.), robustly enhanced the burst firing pattern (68% of recorded cells showing burst activity with RP67580 vs 25% with RP68651 ($\chi^2(1)=2.8$, $p=0.05$, Fig. 8B, right).

3.5. Effects of DSP-4 on norepinephrine and serotonin levels

To assess whether NE neurons contribute to the alteration of 5-HT neuronal firing after NK1 receptor antagonist, the concentrations of NE were determined in various brain

Table 1 Effect of DSP-4 (50 mg/kg i.p.) on NE, 5-HT and 5-HIAA levels in dorsal raphe and locus coeruleus

Brain areas	NE		5-HT		5-HIAA	
	Control	DSP-4	Control	DSP-4	Control	DSP-4
Dorsal raphe	9.6 ± 1.3	2.7 ± 0.5***	7.3 ± 2.0	6.6 ± 1.6	2.2 ± 0.3	2.0 ± 0.5
Locus coeruleus	13.2 ± 2.7	6.0 ± 1.2*	2.5 ± 0.3	4.7 ± 0.8*	1.5 ± 0.2	2.8 ± 0.6*

Values are expressed as means $\pm$ S.E.M in pmol/mg of tissue. Differences between groups were analysed by unpaired t test. *$p < 0.05$; ***$p < 0.001$ in comparison with the control groups.

structures following the lesion of NE neurons. Noradrenergic neurons were lesioned using the selective toxin DSP-4. Seven days after DSP-4 administration (50 mg/kg i.p.), NE levels were significantly altered in all target areas. In particular, NE levels were decreased by 72% in dorsal raphe ($t_{17} = 4.717$, $p < 0.001$, $n = 9-10$) and by 55% in locus coeruleus ($t_{17} = 2.372$, $p < 0.05$, $n = 10$, Table 1).

Noradrenergic lesion altered 5-HT levels in the locus coeruleus but not in the dorsal raphe ($t_{17} = 0.2783$, n.s., $n = 9-10$). Serotonin concentrations were significantly increased (91%) in locus coeruleus following noradrenergic lesion ($t_{17} = 2.751$, $p < 0.05$, $n = 9-10$). 5-HIAA levels paralleled 5-HT alterations observed in lesioned mice, remaining unchanged in dorsal raphe ($t_{17} = 0.3561$, n.s., $n = 9-10$) and significantly increased (+89%) in locus coeruleus ($t_{17} = 2.263$, $p < 0.05$, $n = 10$, Table 1).

3.6. Effects of the noradrenergic lesion on 5-HT neuronal firing in NK1 $-/-$ and NK1 $+/+$ mice pretreated with the NK1 antagonist RP67580

Seven days after the DSP-4 lesion, in vivo electrophysiological experiments were conducted to assess the effects of the
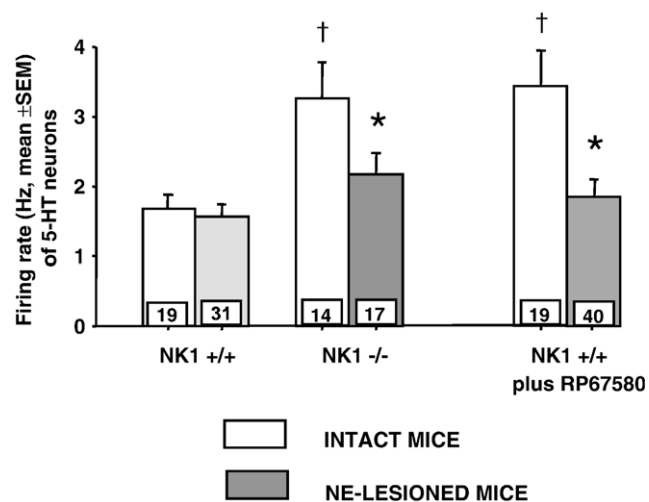

Figure 9 After NE depletion, NK1 receptor antagonism and deletion fail to increase 5-HT activity. Mean frequency of firing of dorsal raphe 5-HT neurons recorded in intact NK1 +/+ mice, NK1 +/+ pretreated with RP67580 and NK1 $-/-$ mice (white) and in NE-lesioned mice using DSP-4 (gray). The number of neurons recorded is indicated in the boxes at the bottom of each column. The asterisk (*$p < 0.001$) indicates the differences obtained between intact mice and NE-lesioned mice; the cross (†), indicates the p value ($p < 0.001$) of non-lesioned groups vs control group using the One Way Analysis of Variance.

NE lesion and of NK1 receptor antagonism on the spontaneous firing rate of 5-HT neurons (Fig. 9). In NE-lesioned wildtype mice, the mean spontaneous firing rate of 5-HT neurons was similar to that of the controls. In NK1 +/+ mice, RP67580 (1.5 mg/kg, i.p.) significantly increased the mean firing rate of 5-HT neurons by 100% when NE neurons were intact, but not in NE-lesioned mice (+13%). The firing frequency of 5-HT neurons was approximately enhanced to the same degree in NK1 +/+ mice (110%) having received RP67580 and in NK1 $-/-$ mice, when compared to controls. However, in intact NK1 $-/-$ mice, the NE-lesion abolished the increase in spontaneous firing rate of 5-HT neurons normally observed in such mutant mice.

4. Discussion

The major finding of the present study was that the increase in the firing rate of 5-HT neurons induced by NK1 receptor antagonism and genetic deletion is dependent on the integrity of NE neurons, but independent of 5-HT$_{1A}$ autoreceptors.

4.1. NK1 receptor antagonism and the 5-HT system

In this study, it was confirmed that both the pharmacological blockage of NK1 receptor and its genetic deletion increase 5-HT neuronal firing activity. It was reported that this enhancement was associated with a desensitization of 5-HT$_{1A}$ autoreceptors resembling that induced by SSRI treatments (Froger et al., 2001). It is proposed herein that 5-HT$_{1A}$ autoreceptor desensitization is not the only mechanism responsible for the increased 5-HT activity by NK1 receptor antagonism. Although NK1 receptor antagonism attenuates the response of 5-HT neurons to a 5-HT$_{1A}$ agonist (Fig. 4), it may still increase 5-HT firing activity in the absence of 5-HT$_{1A}$ autoreceptors, as shown in 5-HT$_{1A}$ null mutant mice (Fig. 5A, B).

Interestingly, 5-HT$_{1A}$ null mutant mice show anxiety-related behaviors compared to wildtype mice in the elevated plus-maze (Ramboz et al., 1998; Zhuang et al., 1999; Gross et al., 2002), that are decreased by RP67580 (Gobbi et al., 2002). Moreover, these mutant mice were still responsive to both NE reuptake inhibitors imipramine and desipramine in the novelty-suppressed feeding paradigm, but not to the SSRIs fluoxetine (Santarelli et al., 2003). These results indicate that some antidepressants, such as SSRIs, require 5-HT$_{1A}$ receptors for their behavioral activity, but not other classes as TCAs or NK1 antagonists, suggesting that the psychotropic action of NK1 antagonists is due in part to a neuronal system other than the 5-HT system.

It is important to emphasize that an increase in 5-HT neuronal firing may not necessarily lead to an increase in 5-HT transmission. Indeed, a two-day administration of the NK1 antagonist CP-96,365 enhanced 5-HT neuronal firing by 50% but did not alter the tonic activation of 5-HT$_{1A}$ receptors in the rat hippocampus (Haddjeri et al., 2001a). After 14 days of treatment with the same drug, the increase in firing was of 90% which intensified 5-HT$_{1A}$ transmission in the hippocampus. Consequently, it would appear that limited increases in firing of 5-HT neurons can be offset by 5-HT reuptake and/or the negative feedback action of terminal 5-HT autoreceptors. Consistent with this possibility is the observation that a NK1 antagonist, while having no effect on its own on extracellular 5-HT levels measured with microdialysis in the frontal cortex, potentiated the enhancing effect of a SSRI (Millan et al., 2001; Lejeune et al., 2002; Zocchi et al., 2003; Guiard et al., 2004). Another possibility for the lack of effect of NK1 antagonists on 5-HT levels in postsynaptic structures using microdialysis is that intra-synaptic 5-HT concentrations are increased; but that the spillover into the extracellular compartment may not be sufficient to be detected by a microdialysis probe of 0.25 mm in diameter. Consistent with this is the increase in dialysate 5-HT in the rat dorsal raphe after long-term administration of a NK1 antagonist, even after a washout period (Guiard et al., 2005). This significant increase detected in that particular brain region possibly resulted from the fact that in the raphe nuclei 5-HT cell bodies and dendrites are densely intertwined where 5-HT in the dialysate may more adequately reflect synaptic concentrations of 5-HT.

4.2. Neurochemical effects of DSP-4 administration

Although different strains of mice were used for the electrophysiological recordings and the neurochemical determinations, this is not likely to have influenced the effects of DSP-4. Indeed, Fornai et al. (1996) did not observe a differential effect of DSP-4 in depleting NE in different brain areas of inbred C57 black mice and Swiss Webster mice which are not inbred mice. The greater decrease of NE levels in the dorsal raphe, as a projection area of locus coeruleus NE neurons (Anderson et al., 1977), than in the cell body region may appear unusual. These NE depletion ratios are, however, fully consistent with those obtained in a prior study examining locus coeruleus and hippocampus using DSP-4 (Hutter et al., 1996). This pattern of depletion, whereby terminals are more affected than cell bodies, is usually obtained using various monoaminergic neurotoxins (Blier and de Montigny, 1985b). Finally, the increase in 5-HT concentrations in the locus coeruleus is likely the result of decreased NE activating α_2-adrenoceptors on 5-HT terminals which normally exert an inhibitory role on 5-HT release (Mongeau et al., 1997).

4.3. NK1 antagonism and the NE system

In the present experiments, it was observed that the acute administration of the NK1 antagonist RP67580 and the genetic deletion of NK1 receptors increased noradrenergic burst activity. Conversely, the transient increase in overall NE firing activity generated by the i.v. injection of RP67580

does not seem to be specific for two reasons. First, it is mimicked by its inactive enantiomer and second, other NK1 antagonists (CP-96,345 and WIN 51,708) do not modify the firing activity of NE neurons, but desensitize the α_2-adrenergic autoreceptors (Haddjeri and Blier, 2000). A sustained burst firing activity of NE neurons recorded in vitro was also reported after long-term administration of the potent NK1 antagonist L-760735 and imipramine (Maubach et al., 2002). It is known that the burst pattern of firing is linked to an augmentation of monoamine release in terminal areas (Gonon, 1988; Gartside et al., 2000; Florin-Lechner et al., 1996). In support of this notion, an increase in NE release in frontal cortex and dorsal hippocampus was observed in microdialysis experiments following acute administration of the NK1 antagonist GR205171 (Millan et al., 2001). It is unlikely that RP68580 increased NE neuronal firing and burst activity by an interference with α_2-adrenoceptors because the α_2-adrenergic antagonist idazoxan enhances the firing rate of NE neurons without altering burst activity (Dremencov et al., in press). Since it has been observed that NK3 receptor agonism facilitates burst activity in neonatal rat spinal cord (Marchetti and Nistri, 2001), it is also possible that a preferential action of SP at NK3 receptors occurring in the presence of selective NK1 receptor blockage could account for this shift in firing pattern, since a permissive action of NK3 on NK1 receptor modulation in the locus coeruleus has been reported by Bert et al. (2002).

In DSP-4 lesioned mice, both wildtype animals pretreated with the NK1 antagonist RP67580 and NK1 $-/-$ mice no longer displayed an enhanced 5-HT firing activity. These results are analogous to those obtained with the α_2-adrenergic antagonist mirtazapine (Haddjeri et al., 1996) and the NE releaser bupropion (Dong and Blier, 2001) that no longer increase 5-HT neuronal firing activity in the absence of NE neurons, indicating that the integrity of the NE neurons is also required for the effect of these two antidepressant drugs on 5-HT neuronal activity. Cryan et al. (2004) reported on the importance of the NE system for the antidepressant-like activity of various classes of antidepressants. They observed that NE-deficient mice failed to exhibit behavioral effects of various antidepressants, including SNRIs, MAOIs, bupropion and SSRIs; the ability of fluoxetine to increase 5-HT levels in hippocampus was also blocked.

It has been postulated that NK1 antagonists activate dorsal raphe 5-HT neurons via the lateral habenula, a cerebral area involved in the mechanism of stress responses that has a high density of NK1 receptors (Conley et al., 2002). The amygdala, which feedbacks to the raphe (Bosker et al., 1997), also appears to be a leading structure for the anxiolytic effects of NK1 antagonists (Ebner et al., 2004). While the habenula and the amygdala may play a significant role in the effects of NK1 antagonists on 5-HT neurons, the results presented herein indicate that the impact of NK1 receptor antagonism on 5-HT neurons is also influenced to a major extent by NE neurons.

In conclusion, substance P receptor antagonists, because of their modulatory effects on monoamine systems that resemble the actions of antidepressant treatments, may well become a new class of therapeutic agents for the treatment of mood and anxiety disorders used alone or to potentiate antidepressant therapies.

Acknowledgement

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