

Behavioral and Physiologic Effects of Genetic or Pharmacologic Inactivation of the Substance P Receptor (NK₁)

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Depression and anxiety are among the most common diseases in the United States, thus constituting a substantial financial burden for the health care system. Experimental studies of these affective disorders to date have largely focused on the neurotransmitter pathways with well-established pathophysiological roles, such as serotonergic, noradrenergic, and γ -aminobutyric acid (GABA)-ergic systems; agents modulating the activity of these pathways are known to be clinically effective. More recently, the neuropeptide substance P (SP) and its receptor (the neurokinin-1 receptor [NK1R]) have been implicated in the pathophysiology of affective disorders, including depression. Earlier preclinical and clinical studies, though, did not provide a clear consensus on the role of SP in the regulation of affective behavior and related pathologic conditions. Recent studies in mice clearly demonstrate that both the genetic disruption and acute pharmacologic blockade of the NK1R result in marked reduction in anxiety-like behavior and stress-related responses. In parallel with these behavioral effects, physiologic changes, such as an increased firing rate of 5-hydroxytryptamine (5-HT) neurons in the dorsal raphe nuclei and a desensitization of presynaptic 5-HT_{1A} inhibitory autoreceptors, were observed. These findings provide further evidence for the regulatory role of the SP-NK1R system in modulation of affective behavior and indicate that its effects are mediated, at least in part, via the serotonergic system. Future studies will attempt to delineate the interaction between the SP-NK1R system and various neurotransmitter pathways in greater detail and to address the specific role(s) of this system in different brain regions.

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Neuropeptide substance P (SP) and its receptor, the neurokinin-1 receptor (NK1R), play an important role in the perception of and response to a broad range of noxious and stressful stimuli. In the brain stem, the SP-NK1R system is involved in the regulation of emesis, and antagonists of the NK1R (substance P antagonists [SPAs]) were recently shown to have antiemetic effects.^{1,2} SP and NK1R are also expressed in brain regions implicated in the control of affective behavior, such as amygdala, hypothalamus, hippocampus, frontal cortex, and the midbrain monoaminergic nuclei, such as the dorsal raphe (DR) and the locus ceruleus (LC).^{3–5} This observation, along with the fact that localization of the SP-NK1R system overlaps with that of neurotransmitter pathways known to be involved in the regulation of mood (e.g., those involving

serotonin [5-hydroxytryptamine (5-HT)] and norepinephrine [NE]), has led to the suggestion that the SP-NK1R pathway may also modulate affective behavior. These findings have further indicated the clinical potential of the SP-NK1R system as a novel therapeutic target in affective disorders, most notably depression and anxiety.

To explore the role of the SP-NK1R pathway in affective behavior, early preclinical studies evaluated the effects of central infusion of SP or related peptide agonists. These studies showed that activation of the SP-NK1R pathway results in defensive behavioral and other physiologic changes, such as conditioned place aversion,⁶ an anxiogenic effect in an elevated plus-maze (EPM) paradigm,^{7,8} enhancement of acoustic startle response,⁹ distress vocalizations and escape,¹⁰ and cardiovascular changes similar to those seen in response to threatening stimuli.¹¹ Additionally, exposure of animals to stress has been associated with changes in endogenous SP,^{12,13} and antidepressant and anxiolytic agents have been shown to reduce SP synthesis.^{14,15} In the clinical setting, 1 study has reported elevated levels of SP in the cerebrospinal fluid of depressed patients,¹⁶ although this result could not be reproduced in a subsequent study.¹⁷ Taken together, these findings support the concept that the SP-NK1R pathway may be an important modulator of affective behavior.

Until recently, the evidence for the role of the SP-NK1R system in stress responses from studies with SPAs

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has been less conclusive, largely because of the pharmacologic properties of individual agents used in early studies. First, there is marked species variation in the affinity of different SPAs for NK1R, such that compounds active against the human NK1R had considerably lower affinity for the rat NK1R.¹⁸ Additionally, early SPAs were characterized by nonspecific effects at high doses¹⁹ and some of them penetrated the brain poorly, thus limiting their practical utility.²⁰ These pharmacologic issues have been recently overcome with the development of alternative animal models (e.g., gerbils, guinea pigs, cats) and the use of SPAs with high brain penetration and without nonspecific effects at high doses.^{10,21}

These breakthroughs have allowed more accurate assessment of the effects of SPAs on stress-induced responses, providing additional evidence for the important role of the SP-NK1R pathway in the regulation of affective behavior. In gerbils, the anxiety-like behavior of foot tapping induced by central infusion of the NK1R agonist GR73632 was strongly inhibited by several SPAs (aprepitant [MK-0869], its analog L-760,735, and a structurally different agent, L-733,060).¹⁰ Additional studies in guinea pigs further demonstrated that systemic pretreatment with the SPAs substantially attenuated audible, long-lasting vocalizations induced by intracerebroventricular administration of GR73632 or transient maternal separation.^{10,21} Importantly, the inhibition of separation-induced vocalizations seen with SPAs was also observed with antidepressants (phenelzine, imipramine, fluoxetine, and venlafaxine) and anxiolytics (diazepam, chlordiazepoxide, and buspirone),^{10,21} underscoring the therapeutic potential of SPAs in depression and anxiety.

A phase 2 trial with aprepitant in patients with major depressive disorder, which included both an active control (paroxetine, 20 mg/day) and placebo, demonstrated that treatment with either aprepitant or paroxetine resulted in statistically significant improvements in depression symptom scores versus the placebo, thus providing support for the concept that SPAs may be clinically useful antidepressants.¹⁰ This concept remains valid despite the lack of significant effect with aprepitant observed in the subsequent dose-finding study, because the active control used in this trial (fluoxetine, 20 mg/day) was also therapeutically ineffective.²²

Collective evidence from preclinical and clinical studies thus supports the idea that the SP-NK1R system is an important regulator of behavioral responses to stressful stimuli. To gain further insight into the role of the SP-NK1R pathway in the control of affective behavior, we evaluated the effects of genetic or pharmacologic (using SPAs) inactivation of the NK1R in mice in a series of behavioral and physiologic assays.²³ Genetic disruption has been used previously to assess the role of other neurotransmitter pathways (e.g., serotonergic) in affective behavior.²⁴

BEHAVIORAL ASSAYS

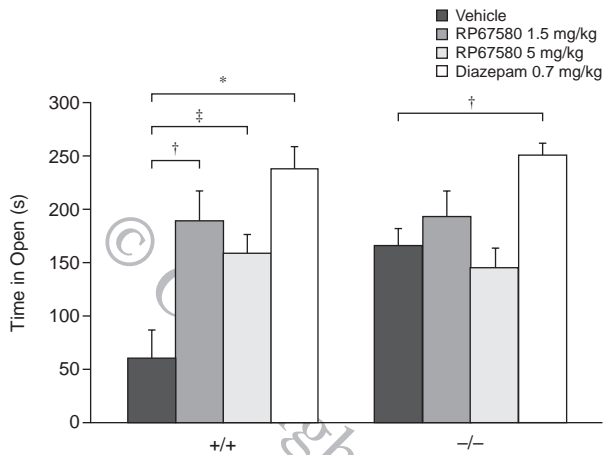
The behavioral analysis employed to establish the effect of NK1R disruption in preclinical models included paradigms based on the choice between opposite drives: active exploration/hunger satisfaction, which is associated with danger, and avoidance, which is associated with safety. The behavioral tests used included the EPM, novelty suppressed feeding (NSF), ultrasonic vocalization (USV), forced swimming (FS), and the open field (OF). We sought to determine whether genetic or pharmacologic disruption of the NK1R had anxiolytic-like effects in these behavioral tests. It should be noted that NK1R knockout (–/–) mice have no obvious abnormalities and that their behavior and locomotory activity under normal conditions are indistinguishable from those of wild-type animals (+/+).

Elevated Plus Maze

Mice placed in the EPM are faced with a choice between exploration of a novel environment and a fear of heights and open spaces (represented by the open arms of the maze). The behavioral parameters assessed in this test can be divided into those related to anxiety (e.g., number of entries in open arms as a percentage of total entries, number of head dips in open arms, time spent in open arms) and those associated with activity (e.g., total number of entries and rearings). In these experiments, NK1R –/– mice, as well as wild-type mice treated with a mouse- and rat-specific SPA, RP67580,²⁵ displayed significantly less anxiety-like behavior than placebo-treated wild-type mice.²³ Mice with a genetic or pharmacologic (Figure 1) disruption of the NK1R had a significantly higher percentage of entries in open arms, a significantly greater number of head dips in open arms, and also spent significantly more time in open arms, despite the fact that their locomotor activity was similar to that of wild-type animals.²³ Notably, the anxiolytic effect of RP67580 (1.5-mg/kg and 5-mg/kg doses, administered by subcutaneous injection 30 minutes before the assay) was comparable to that of the anxiolytic agent diazepam (0.7 mg/kg dose) in NK1R +/+ mice, whereas only diazepam was effective in NK1R –/– mice.²³ This finding demonstrates that the NK1R –/– mice have an intact γ -aminobutyric acid (GABA)-A system and that the anxiolytic action of SPAs is likely independent of that of benzodiazepines, such as diazepam. The consistency of results obtained in mice with different modes of NK1R inactivation strongly implies that the SP-NK1R system regulates the behavioral response to stressful stimuli.

The EPM is a stressful task known to stimulate the hypothalamic-pituitary-adrenal (HPA) axis, and additional experiments were performed to assess whether genetic disruption of NK1R modulates HPA axis activity. Serum corticosterone levels prior to the EPM were not signifi-

Figure 1. Effect of Substance P Antagonist RP67580 and Anxiolytic Diazepam on Anxiety-Related Behavior Evaluated by the Elevated Plus-Maze Paradigm^a



^aReproduced with permission from Santarelli et al.²³ The substance P antagonist RP67580 and anxiolytic diazepam significantly decreased anxiety-related parameters evaluated in the elevated plus-maze paradigm in wild-type (+/+) mice, whereas only diazepam was effective in neurokinin-1 receptor knockout (-/-) mice (N = 9–10/group). Error bars indicate standard error of the mean. * $p < .001$; † $p < .01$; ‡ $p < .05$.

cantly different between the wild-type and NK1R $-/-$ mice, but the increase in response to 10-minute exposure to EPM was significantly lower in NK1R $-/-$ animals (250% vs. 700% increase in wild-type mice, $p < .05$).²³ These results indicate that the SP-NK1R pathway is an important mediator of stress-induced activation of the HPA axis.

Novelty Suppressed Feeding

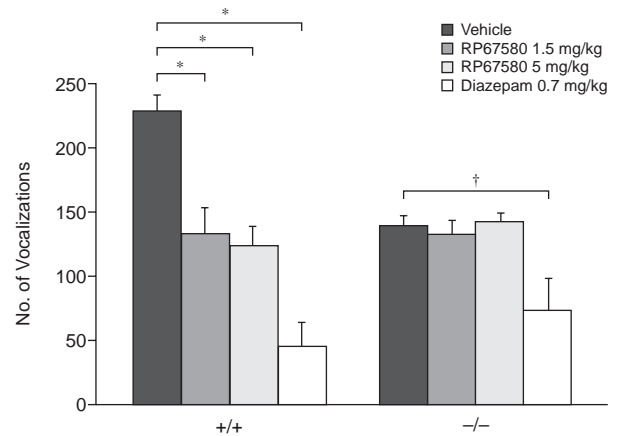
In order to extend the findings from the EPM to other models of anxiety-like behavior, wild-type and NK1R $-/-$ mice were subjected to the NSF test.

The NSF is a conflict test, in which food-deprived animals are presented with a food pellet placed in the center of a brightly lit open field. This paradigm elicits competing motivations: the drive to eat the food pellet and the fear of venturing into the center of the arena.²³ The latency to start eating is an index of anxiety-related behaviors and is shortened by anxiolytic and chronic antidepressant drug treatments.²⁶ Compared with wild-type mice, those lacking the NK1R displayed significantly reduced latency to feeding (175 seconds vs. 100 seconds, respectively, $p < .05$),²³ further supporting the idea that the SP-NK1R pathway modulates anxiety-like behavior.

Ultrasonic Vocalization

The anxiolytic-like effect of genetic and pharmacologic inactivation of the NK1R was also confirmed in the separation-induced USV paradigm, which measures anxiety-related behavior resulting from the separation of

Figure 2. Effect of Substance P Antagonist RP67580 and Anxiolytic Diazepam on Separation-Induced Ultrasound Vocalizations^a



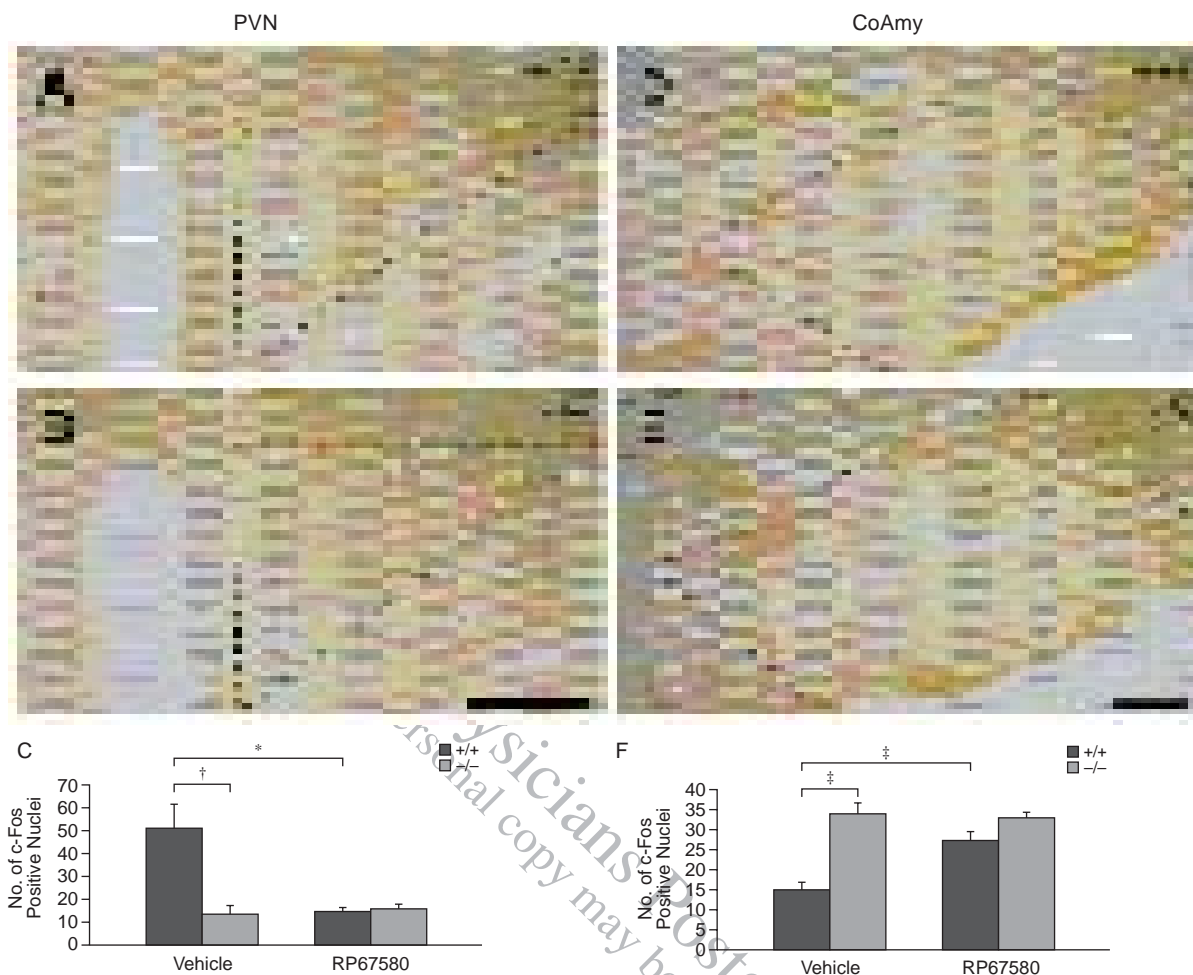
^aReproduced with permission from Santarelli et al.²³ The substance P antagonist RP67580 and anxiolytic diazepam significantly reduced the number of separation-induced ultrasound vocalizations in wild-type (+/+) mice, whereas only diazepam had this effect in neurokinin-1 receptor knockout (-/-) mice. Error bars indicate standard error of the mean. * $p < .001$; † $p < .01$.

8-day-old pups from their mother. Previous studies have shown that the number of ultrasonic vocalizations is decreased by the administration of various drugs that have anxiolytic or antidepressant effects in humans.²⁷

A significantly lower number of USVs were observed in NK1R $-/-$ mice (75 vs. 237 in wild-type mice, $p < .001$).²³ A similar reduction was also seen in wild-type mice treated with RP67580 (1.5 mg and 5 mg) or diazepam (0.7 mg), whereas only diazepam reduced the number of USVs in NK1R $-/-$ mice (Figure 2).²³ These findings are consistent with those reported in the EPM paradigm, suggesting that the SP-NK1R pathway modulates anxiety-like behavior during a young age as well.

Forced Swimming

In the FS paradigm,^{28,29} animals are forced to swim in a narrow cylinder with no escape option; an initial period of vigorous activity is followed by a relatively prolonged period of immobility. The immobility is thought to reflect a “behavioral despair” due to failed attempts to escape, and treatment with antidepressants (vs. the vehicle) prior to exposure to the FS test prolongs the period of escape-directed behavior.^{28,30} For this reason, this test is widely used to assess the activity of antidepressant drugs in pre-clinical models. In agreement with results of other behavioral assays, NK1R $-/-$ mice exhibited significantly shorter immobility (200 seconds vs. 250 seconds in wild-type mice, $p < .05$) in the FS model. This finding provides further evidence for the significant role of the SP-NK1R system in regulation of depressive behavior.

Figure 3. Stress-Induced c-Fos Activation^a

^aL. Santarelli, M.D., unpublished data. Induction of c-Fos expression after a 10-minute trial in the elevated plus maze in neurokinin-1 receptor (NK1R) knockout (-/-) mice was reduced in the paraventricular hypothalamic nuclei (PVN) (A-C) and increased in the cortical amygdala (CoAmy) (D-F). Administration of the substance P antagonist RP67580 was also associated with reduced c-Fos induction in the PVN (C) and increased c-Fos expression in the CoAmy (F) in wild-type (+/+), but not in NK1R -/- mice. Error bars indicate standard error of the mean. * $p < .001$; † $p < .01$; ‡ $p < .05$.

Open Field

Like the EPM, the OF test can be used to determine both the overall locomotor activity (measured as the path length traveled in a certain time) and the exploratory behavior (measured as the number of nose pokes).³¹ While the overall locomotor activity levels of wild-type and NK1R -/- mice were comparable, the number of nose pokes was significantly greater in the latter group (0.5 vs. 2.5, $p < .05$). An increase in exploratory activity in response to a novel and anxiogenic environment is consistent with decreased anxiety, as demonstrated in earlier studies.³²⁻³⁴

Stress-Induced c-Fos Activation

To identify neuronal circuits that may be involved in the NK1R-mediated modulation of anxiety-related responses, we analyzed the induction of c-Fos protein expression (a

measure of neuronal activity) 2 hours after 10-minute exposure to the anxiogenic environment of the EPM (L.S., unpublished data). In wild-type mice, c-Fos expression was induced in various brain structures associated with stress response, similar to the findings previously reported in rats.^{35,36} Interestingly, in the paraventricular nucleus (PVN) of the hypothalamus, a pivotal structure in the coordination of the stress response, c-Fos induction after exposure to the EPM was strongly reduced in both NK1R -/- and RP67580-pretreated NK1R +/+ mice (when compared with vehicle-pretreated NK1R +/+ animals; Figure 3 A-C). In contrast, in the cortical nucleus of the amygdala, which is activated during exploratory behavior and is involved in processing of olfactory information,³⁷ c-Fos induction in NK1R -/- and RP67580-pretreated NK1R +/+ mice was more pronounced than in vehicle-pretreated

wild-type animals (Figure 3 D–F). This observation is consistent with increased exploratory behavior of the NK1R $-/-$ mice seen in the OF test (greater number of nose pokes; see Open Field section above). No significant differences in c-Fos induction were detected in other brain structures, including the medial amygdala, periaqueductal gray, and the PVN of the thalamus.

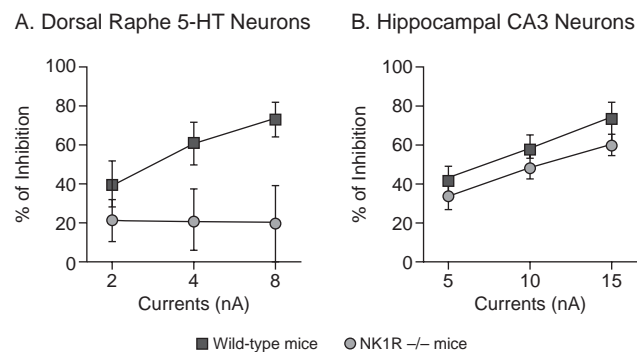
INTERACTION OF SP WITH 5-HT AND NE PATHWAYS

The significant anatomical overlap between the SP-NK1R system and other neurotransmitters (e.g., 5-HT, NE) with well-established roles in affective behaviors suggests the possibility of a functional interaction. To address this issue, the firing rate of 5-HT neurons in the dorsal raphe (DR), a major source of serotonergic projections to forebrain structures, was investigated in wild-type mice (with and without RP67580 pretreatment) and in NK1R $-/-$ mice.²³

The firing rate of DR serotonergic neurons in NK1R $-/-$ and wild-type mice acutely pretreated with RP67580 was significantly higher than in untreated wild-type mice (3 Hz, 4 Hz, and 1.5 Hz, respectively; $p < .01$ for comparison of NK1R $-/-$ and untreated wild-type mice; $p < .001$ for comparison of RP67580-pretreated and untreated wild-type mice),²³ suggesting that 5-HT transmission in DR is negatively regulated by the SP-NK1R pathway. Because the firing of these neurons is known to be inhibited by the presynaptic 5-HT_{1A} autoreceptor, subsequent experiments sought to determine if NK1R inactivation exerts its stimulatory effects on 5-HT neurotransmission by down-regulating this inhibitory autoreceptor.²³ Direct application of a selective 5-HT_{1A} agonist (8-hydroxy-2-[di-n-propylamino] tetralin [8-OH-DPAT]) onto serotonergic neurons in DR inhibited their firing in a dose-dependent manner in wild-type mice, but this effect was markedly reduced in NK1R $-/-$ mice (Figure 4A)²³ and in rats treated with SPA CP-96,345.³⁸ This finding supports the hypothesis that inactivation of the SP-NK1R pathway may increase firing of DR 5-HT neurons by reducing expression of the presynaptic 5-HT_{1A} autoreceptor.²³

In addition to presynaptic expression in the DR, the inhibitory 5-HT_{1A} autoreceptor is also expressed postsynaptically, most notably in the hippocampus. In contrast to the findings reported for DR 5-HT neurons, the application of 8-OH-DPAT at the level of the hippocampus produced a dose-dependent inhibition of the firing of CA3 pyramidal neurons, an effect that was maintained in NK1R $-/-$ mice (Figure 4B).²³ Similar findings were reported in rats after both short- and long-term treatment with SPA CP-96,345.³⁸ In addition, long-term treatment with CP-96,345 was shown to cause tonic activation of postsynaptic 5-HT_{1A} receptors,³⁸ a response also seen with many other antidepressant treatments. Taken together, these findings indicate that the SP-NK1R system primarily regulates the function

Figure 4. Effects of 8-OH-DPAT on 5-HT_{1A} Receptor Function^a



^aReproduced with permission from Santarelli et al.²³ Reduced inhibition of firing of serotonergic neurons in response to administration of 5-HT_{1A} agonist (8-hydroxy-2-[di-n-propylamino] tetralin [8-OH-DPAT]) in neurokinin-1 receptor (NK1R) knockout ($-/-$) mice was observed in dorsal raphe (A), but not in hippocampus (B). Error bars indicate standard error of the mean.

of presynaptic 5-HT_{1A} autoreceptors in DR, although it may also affect the activity of postsynaptic 5-HT_{1A} autoreceptors in the hippocampus.

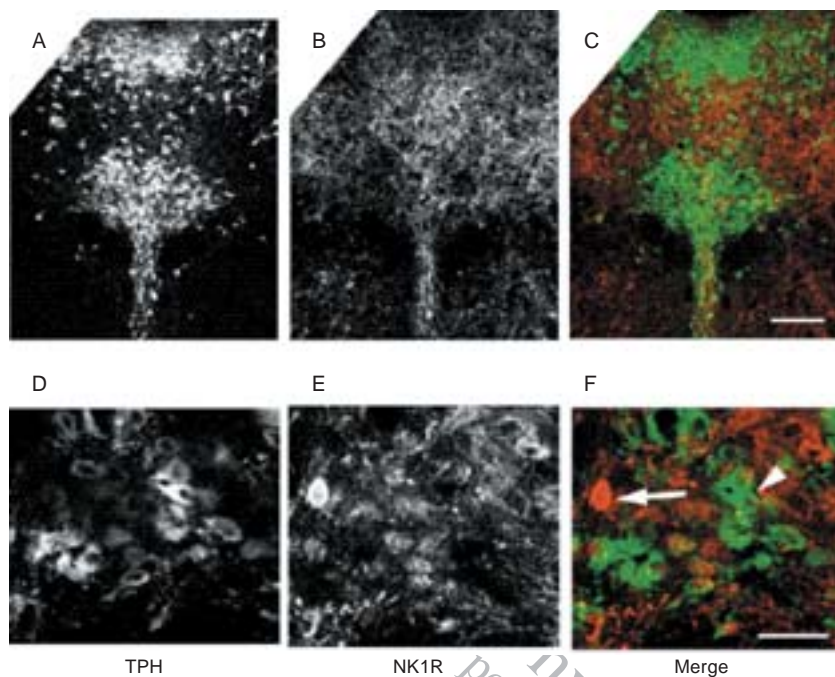
Modulation of presynaptic 5-HT_{1A} autoreceptors in DR by the SP-NK1R system may be direct, involving the presence of NK1R on DR serotonergic neurons. To address this issue, immunocytochemical experiments were performed to compare localization of serotonergic (as identified by the PH8 antitryptophan hydroxylase antibody) and NK1R-expressing (as identified by anti-NK1R antibody) neurons in the DR. Despite the abundance of NK1R immunoreactivity in the DR, only a modest overlap with serotonergic neurons was observed (Figure 5).²³ These findings indicate that the effects of the SP-NK1R pathway on function of serotonergic neurons in DR may be indirect.

This indirect mechanism of SP-NK1R-mediated regulation of DR serotonergic neurons may involve another monoaminergic nucleus, the LC, which was previously shown to stimulate the activity of serotonergic neurons.³⁹ LC contains a large population of NE neurons, and these have been shown to express high levels of NK1R (Figure 6).²³ The localization of NK1R on NE neurons in LC suggests that SPAs may directly regulate function of these neurons. This hypothesis is supported by the finding that SPAs attenuate the inhibition of NE and 5-HT neuronal firing in response to administration of the α_2 -adrenoceptor agonist clonidine.⁴⁰ Therefore, the SP-NK1R pathway appears to exert its effects on the DR 5-HT neurons by directly modulating NE neurotransmission in LC.

SUMMARY

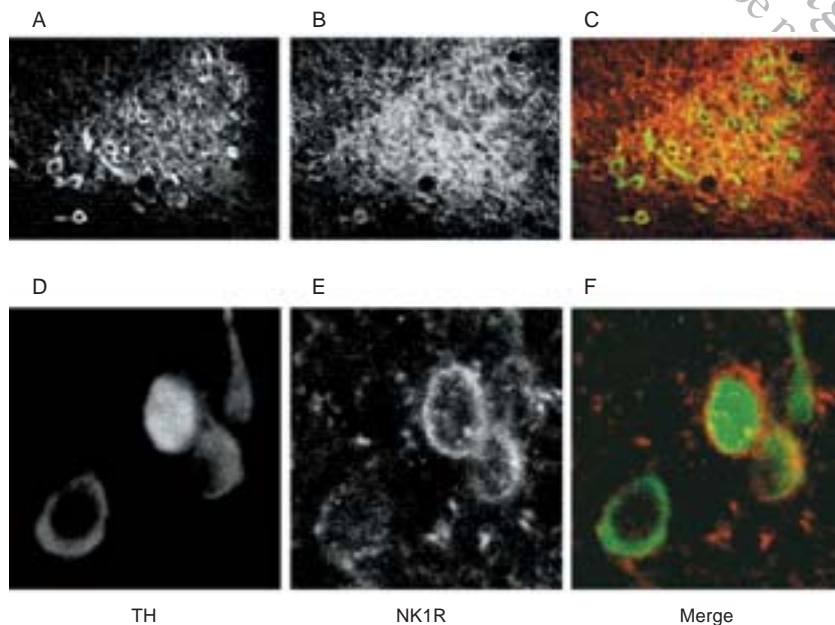
Increasing evidence supports an important role for the SP-NK1R pathway in the control of affective behavior. In

Figure 5. Serotonergic Neurons in Dorsal Raphe (DR) Do Not Express Neurokinin-1 Receptor (NK1R)^a



^aReproduced with permission from Santarelli et al.²³ Immunofluorescent double-labeling of serotonergic neurons (using monoclonal antibody PH8 directed against tryptophan hydroxylase [TPH], in green) and NK1R-expressing neurons (using anti-NK1R antibody, in red) in DR. Arrowhead indicates a neuron stained with the PH8 antibody, while arrow shows a nearby NK1R-positive neuron. Scale bar: 200 μm (A–C) and 50 μm (D–F).

Figure 6. Noradrenergic Neurons in the Locus Ceruleus Also Express Neurokinin-1 Receptor (NK1R)^a



^aReproduced with permission from Santarelli et al.²³ Immunofluorescent double-labeling of tyrosine hydroxylase (TH), which indicates noradrenergic neurons (in green) and NK1R-expressing neurons with anti-NK1R antibody (in red) in the locus ceruleus. Scale bar: 100 μm (A–C) and 10 μm (D–F).

the clinical setting, antagonism of this pathway has resulted in both antidepressant and anxiolytic effects.¹⁰ Animal studies demonstrate that genetic or pharmacologic inactivation of NK1R leads to an attenuation of anxiety-like behavior in several different model systems.^{10,21,23} While it has been proposed that the antidepressant activity of SPA may be independent of serotonergic and noradrenergic pathways,¹⁰ more recent findings suggest that the effect of these agents may involve complex interactions with these key neurotransmitter systems.^{23,40} Inhibition of NK1R has been shown to attenuate the inhibition of the firing activity of NE cells in the LC in response to α_2 -adrenoceptor agonist, and this effect may contribute to enhanced 5-HT activity in DR. Increased firing of DR serotonergic neurons in response to SPA administration is permitted by the decreased inhibitory function of presynaptic 5-HT_{1A} autoreceptors.

Interestingly, the effects of SPAs mimic some of the key changes associated with long-term administration of antidepressant drugs, such as an increase in serotonergic activity and a slow, gradual desensitization of presynaptic 5-HT_{1A} autoreceptors.^{41,42} However, the behavioral and physiologic effects of SPAs occur acutely, suggesting that these agents may trigger delayed adaptive changes in monoaminergic system to account for the slow onset of clinical actions. Nevertheless, these drugs have unique and novel mechanism of actions in depression/anxiety symptoms. It is also possible that, in some regions of the brain, the SP-NK1R system may act independently of other neurotransmitter pathways. Additional studies involving region-specific inactivation of NK1R will provide a better insight into the role of the SP-NK1R pathway in modulation of affective behavior.

Drug names: buspirone (BuSpar and others), chlordiazepoxide (Librium and others), clonidine (Catapres and others), diazepam (Valium)

and others), fluoxetine (Prozac and others), imipramine (Tofranil and others), paroxetine (Paxil), phenelzine (Nardil), venlafaxine (Effexor).

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