

# Neurokinins Activate Local Glutamatergic Inputs to Serotonergic Neurons of the Dorsal Raphe Nucleus

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*It has been proposed that antidepressant effects of neurokinin NK<sub>1</sub> receptor blockade may result from an increase in serotonin (5-HT) transmission. However, the mechanism by which neurokinins influence 5-HT neurons is not known. In this study, local NK<sub>1</sub> and NK<sub>3</sub> receptor-mediated responses in 5-HT neurons of the dorsal raphe nucleus (DRN) were studied using intracellular recording in rat brain slices. Bath application of the NK<sub>1</sub> receptor agonist substance P (SP) or the NK<sub>3</sub> receptor agonists senktide and NKB induced a robust increase in "spontaneous" excitatory postsynaptic currents (EPSCs) in 5-HT neurons. The EPSCs were blocked by the AMPA/kainate glutamate receptor antagonist CNQX and the fast Na<sup>+</sup> channel blocker tetrodotoxin (TTX), indicating that the increase in EPSCs resulted from an increase in impulse flow in local glutamatergic neuronal afferents. The neurokinins agonists had no direct excitatory effects on 5-HT neurons and no NK<sub>1</sub> or NK<sub>3</sub> receptor*

*immunolabeling was found in 5-HT-labeled neurones. However, neurokinins, by increasing excitatory postsynaptic potentials (EPSPs), did increase the spiking of 5-HT neurons. The SP- and NKB-induced EPSCs were preferentially blocked by NK<sub>1</sub> and NK<sub>3</sub> antagonists, and there was minimal cross-desensitization between agonists at the two receptors. We conclude that neurokinins, via distinct NK<sub>1</sub> and NK<sub>3</sub> receptors, could promote 5-HT transmission, at least in part, by exciting a local population of glutamatergic inputs to 5-HT neurons in the DRN. However, these local excitatory effects, viewed within the context of the global effects of neurokinins on 5-HT neurons, reveal important differences between the functional role of NK<sub>1</sub> and NK<sub>3</sub> receptors.*  
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Members of the tachykinin (neurokinin) family of neuropeptides, including substance P (SP), neurokinin A

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(NKA), and neurokinin B (NKB), have been investigated extensively as putative excitatory neurotransmitters/modulators in the central nervous system. The physiological effects of neurokinins are mediated by three distinct receptors, NK<sub>1</sub>, NK<sub>2</sub> and NK<sub>3</sub> (Nakanishi 1991; Regoli et al. 1994; Seabrook et al. 1995). Although all three endogenous neurokinins can interact with multiple neurokinin receptors, SP is a preferential agonist at the NK<sub>1</sub> receptor, whereas NKB is the presumed endogenous agonist at the NK<sub>3</sub> receptor (Horwell 1995; Iversen et al. 1990; Maggi et al. 1993; Stables et al. 1994; Culman and Unger 1995). Neurokinins have been implicated in the pathophysiology of anxiety and depression by variety preclinical and clinical studies (for review see Stout et al. 2001). A recent clinical study

reported an antidepressant effect after chronic treatment with an antagonist of central NK<sub>1</sub> receptors (Kramer et al. 1998). The prospect of neurokinin receptor antagonists as novel therapeutic agents is intriguing, but clearly further study is needed to define the range of clinical applicability of these agents.

Recently, based on studies in NK<sub>1</sub> receptor knockout mice, it has been suggested that the antidepressant action of NK<sub>1</sub> receptor inactivation may result from an increase in central 5-HT neurotransmission through desensitization of 5-HT<sub>1A</sub> autoreceptors (Froger et al. 2001). Current pharmacological treatment of depression frequently employs selective serotonin reuptake inhibitors, which are also known to desensitize 5-HT<sub>1A</sub> autoreceptors (Chaput et al. 1986). Additionally, there are a number of studies showing behavioral and neurochemical interactions between the 5-HT system and various neurokinins. Intracisternal administration of the NK<sub>3</sub> agonist senktide results in a complex behavioral syndrome involving multiple 5-HT receptors and is dependent upon intact stores of endogenous 5-HT (Stoessl et al. 1987; Stoessl et al. 1990). These studies imply that neurokinins may induce 5-HT-mediated behaviors by enhancing the release of endogenous 5-HT. The 5-HT cell bodies located in the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN) give rise to the principal 5-HT projections to the forebrain. There are a number of studies showing that local injections of neurokinins into the raphe nuclei increase 5-HT release and 5-HT mediated behaviors. For example, injection of SP into the MRN induces an increase in 5-HT turnover in the hippocampus (Forchetti et al. 1982). Similarly, local injection of SP into the DRN increases extracellular levels of 5-HT in the ventral hippocampus of rats (Gradin et al. 1992). On a behavioral level, infusion of the selective NK<sub>3</sub> agonist senktide or a SP analog into the MRN induces a 5-HT-dependent increase in locomotor activity (Paris and Lorens 1987; Paris et al. 1989). It should be noted that there are differential effects of local versus intracisternal administration of SP. While the injection of SP directly into the raphe nuclei leads to an increase in 5-HT-mediated behaviors, intracisternal administration of SP fails to elicit 5-HT-mediated behaviors (Stoessl et al. 1987). In contrast, the NK<sub>3</sub> agonists strongly elicit 5-HT-mediated behaviors both after local injection into the raphe and after intracisternal administration. These studies indicate that there are important differences between the local and global effects of activating NK<sub>1</sub> versus NK<sub>3</sub> receptors.

Surprisingly, there have been no electrophysiological studies characterizing the local effects of neurokinin agonists on the activity 5-HT neurons. The purpose of this study was to investigate the effects of not only SP (NK<sub>1</sub> receptor agonist) but also NK<sub>3</sub> receptor agonists upon 5-HT neurons in a DRN brain slice preparation. Both, SP and NKB immunoreactive nerve fibers and ter-

minals have been detected in the DRN (Cuello and Kanazawa 1978; Marksteiner et al. 1992). In situ hybridization studies show that there are NK<sub>1</sub> receptor mRNA-positive neurons in the periaqueductal gray and DRN (Maeno et al. 1993) and NK<sub>3</sub> receptor mRNA-positive neurons in various raphe nuclei, including the DRN (Ding et al. 1996; Mileusnic et al. 1999). However, it has been reported recently that NK<sub>1</sub> receptors in the DRN are located on non-5-HT neurons rather than 5-HT neurons (Froger et al. 2001). Therefore, we not only investigated direct effects on 5-HT neurons but examined possible indirect effects of neurokinins that may be mediated through non-5-HT neurons. In parallel with the electrophysiology, immunocytochemical methods were employed to determine whether NK<sub>3</sub> as well as NK<sub>1</sub> receptors are located upon 5-HT or non-5-HT cells.

## MATERIALS AND METHODS

### Brain Slice Preparation

Brain slices were prepared as described previously (Jolas and Aghajanian 1996). Briefly, male albino rats (130–180g, Harlan) were anesthetized with chloral hydrate (400mg/kg, i.p.), in adherence to protocols approved by the Yale University Animal Care and Use Committee. Following decapitation, the brains were removed rapidly and trimmed in ice-cold (~4°C) artificial cerebrospinal fluid (ACSF) in which sucrose (252 mM) was substituted for NaCl (sucrose-ACSF). A block of tissue containing the DRN was dissected and coronal slices (600 µm) were cut in sucrose-ACSF with an oscillating-blade tissue slicer (DSK Microslicer, Ted Pella, Inc., Redding California). A slice containing the DRN was positioned on the stage of a fluid-gas interface chamber with the cerebral aqueduct facing an elevated fluid inlet. Fluid was wicked directly from the elevated inlet onto the slice as previously described (Burlhis and Aghajanian 1987). The standard ACSF (pH~7.35), equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub>, contained: NaCl, 128 mM; KCl, 3 mM; CaCl<sub>2</sub>, 2 mM; MgSO<sub>4</sub>, 2 mM; NaHCO<sub>3</sub>, 24 mM; NaH<sub>2</sub>PO<sub>4</sub>, 1.25 mM; and D-glucose, 10 mM. The slices were incubated at 33.0 ± 0.5°C and perfused at a rate of ~1 ml/min. A 2–3 h recovery period was allowed before collecting data. Drug solutions, in gassed ACSF, were introduced through a stopcock arrangement to the recording chamber with a latency of ~20s.

### Intracellular Recordings

Microelectrodes were pulled from filament-containing 1.5 mm glass tubing using a Brown-Flaming pipette puller (Sutter Instrument Co., Novato, CA) and filled with 2M KCl (30–45 MΩ). Current-clamp and voltage-clamp recordings were made using an AxoClamp 2A

amplifier coupled to a pClamp/Digidata 1200 system (Axon Instruments Foster City, CA). In current-clamp mode, using KCl-containing electrodes, putative serotonergic neurons were recognized by their long spike duration ( $\sim 1$  msec at 50% spike amplitude) and high input resistance. Previously, using a double-labeling method, neurons with these characteristics were shown to be serotonergic (Aghajanian and VanderMaelen 1982). In addition, neurons with these characteristics were shown to respond to applied 5-HT (100  $\mu$ M) with a hyperpolarization  $\geq 5$  mV (Aghajanian and Lakoski 1984).

In voltage clamp, all experiments were performed using the discontinuous single-electrode voltage-clamp mode at sampling frequencies of 5–6 kHz and a loop gain 10 nA/mV (30% duty cycle). The headstage voltage was monitored continuously to ensure that voltage transients decayed fully before voltage was sampled; false clamping was avoided by optimizing the capacitance compensation and selecting sampling frequencies that allowed input voltage to settle to a horizontal baseline. Postsynaptic currents were measured in discontinuous single-electrode voltage clamp mode (10 kHz low pass filter cut-off frequency) and cells clamped near their resting potential ( $-70$  mV). With KCl electrodes, spontaneous inhibitory postsynaptic currents (IPSCs) and excitatory postsynaptic currents (EPSCs) both appear as inward currents because the reversal potential for chloride is shifted from approximately  $-70$  mV to  $-15$  mV. To isolate EPSCs from reverse IPSCs, we first applied neurokinins to determine whether they induced synaptic currents. If this was confirmed, the subsequent experiments were done in presence of bicuculline (10  $\mu$ M) to block IPSCs. Using pClamp 8.0 (Axon Instruments), the effects of drugs on spontaneous and neurokinin-induced EPSCs were sampled by recording 10 one second episodes before and during the peak of action of the drugs.

The firing activity of 5-HT cells in anesthetized rats in vivo is dependent on a tonically active noradrenergic system (Gallager and Aghajanian 1976; Baraban et al. 1978). In brain slices, noradrenergic inputs are severed and 5-HT cells are usually quiescent; activity can be restored with the  $\alpha_1$ -adrenergic agonist phenylephrine (PE) (VanderMaelen and Aghajanian 1983). Thus PE (3  $\mu$ M) was added to the perfusion medium to maintain the firing of 5-HT neuron when testing the effect of neurokinins on the spiking activity of 5-HT neurons.

### Immunocytochemistry

**Animals and Tissue Preparation.** Four rats were deeply anesthetized with sodium pentobarbital (60 mg/kg; i.p.), and then perfused through the heart with 100 ml of normal saline followed by 500 ml of 4% freshly depolymerized paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). The brain stem was removed and 30- $\mu$ m thick transverse serial sections were

cut though the midbrain on a Vibratome and collected in PB. Alternate sections were divided into three sets, each extending from the anterior to posterior aspect of the DRN. The separate sets of the sections (about 10 sections/set) were then double stained for NK<sub>1</sub>+ 5HT, NK<sub>3</sub>+ 5-HT, or NK<sub>1</sub>+NK<sub>3</sub>.

### Double Immunofluorescence Labeling for 5-HT and NK Receptors (NK<sub>1</sub> and NK<sub>3</sub>)

The sections were processed for 5-HT immunofluorescence staining using the TSA (Tyramide Signal Amplification) method, and then for NK<sub>1</sub> or NK<sub>3</sub> staining with routine immunofluorescence staining. This method is optimal for performing double immunofluorescence staining when both primary antibodies are raised in the same species (Shindler and Roth 1996). Briefly, after treatment with blocking buffer, the sections were incubated overnight in rabbit anti-5-HT IgG (160,000; Diasorin, Stillwater, MN), biotinylated donkey anti-rabbit IgG (1:200; Jackson) for 2 h, streptavidin-horseradish peroxidase (1:100) for 1 h, and then FITC-tyramide (1:100) in amplification diluent for 8 min. With the dilution, no signal was detected unless amplified with TSA Kit. After being washed in PBS, the sections were incubated overnight with the second primary antibody, rabbit anti-NK<sub>1</sub> IgG (1:4,000; Advanced Targeting System) or rabbit anti-NK<sub>3</sub> IgG (1  $\mu$ g/ml; Ding et al. 1996), and then with Cy3-conjugated secondary antibody (1:200; Jackson) for 2 h. The sections were washed, mounted onto glass slides, coverslipped, and observed under the confocal microscope for image acquisition and initial processing. Immunohistochemical controls included omitting the primary antibody and replacing the secondary antibody with normal serum from the same species. To control for possible cross-reaction between the first primary antibody and the second secondary antibody, the sections were processed as described above except that the second primary antibody (rabbit anti-NK<sub>1</sub> or NK<sub>3</sub> IgG) was omitted. Thus, the sections incubated with the first primary antibody and revealed with FITC-TSA were incubated with Cy3-conjugated donkey anti-rabbit antibody. The sections were uniformly negative on the red channel.

### Drugs

Tetrodotoxin (TTX) was obtained from Alomone Labs (Jerusalem, Israel); 5-hydroxytryptamine creatinine sulfate (5-HT) was from Sigma, bicuculline methiodide (Bic), substance P, senktide, neurokinin B, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were from Research Biochemical International (Natick, MA); RP 67580 was from Rhone-Poulenc Rorer (Cedex, France) and SB 223412 was from SmithKline Beecham Pharmaceuticals (Harlow, Essex, Italy).

## Analysis and Statistics

EPSCs were acquired by the Clampex module of pClamp 8 and a Digidata 1200 interface. Analysis of PSCs frequency and amplitude was conducted with Mini Analysis software (Synaptosoft Inc, Decatur, GA). This program detects and measures spontaneous synaptic events according to amplitude, rate of rise, duration, and area under the curve (fc). Analysis parameters were as follows: amplitude threshold, 5 pA and area threshold, 75 fc. Statistical comparisons of the extracted amplitude and interspike interval distributions for EPSCs were conducted with the nonparametric Kolmogorov-Smirnov (K-S) test. This test gives a measure of the relative dispersion between two distributions (Goodman 1954). Cumulative probability distributions were considered significantly different with  $p < .01$  (K-S test). Descriptive statistics are given as mean  $\pm$  SEM. Mean amplitude, frequency, and other data were analyzed by 2-tailed paired Student's *t*-test.

## RESULTS

Of 285 neurons recorded in the DRN, 180 met criteria for classification as being serotonergic and these were used for the experiments with the neurokinins. These cells had the following characteristics: long spike duration ( $1.02 \pm 0.02$  msec at half amplitude), an hyperpolarizing response ( $\geq 5$  mV) to applied 5-HT ( $100 \mu\text{M}$ ), an average resting potential of  $-63.2 \pm 1.6$  mV, mean action potential amplitude of  $91.8 \pm 1.3$  mV, and high input resistance ( $226.1 \pm 3.1 \text{ M}\Omega$ ).

### Substance P and Senktide Have No Direct Effect on 5-HT Neurons of the DRN

The effects of both SP and senktide on 5-HT neurons in the DRN were tested under voltage clamp. SP and senktide frequently produced an increase in spontaneous postsynaptic currents (PSCs) in 5-HT neurons, which could be blocked by the AMPA/kainate antagonist CNQX (see below). However, after giving CNQX to remove the contaminating influence of superimposed PSCs, senktide and SP had no direct effects on any of the 5-HT cells tested ( $n = 20$ ). Because washout of CNQX is prolonged, the data from these cells were not included in the results from subsequent experiments on the induction of synaptic currents by neurokinins. Figure 1, panel A, shows a recording from a typical 5-HT neuron, which had a typical outward current and increased conductance ( $\sim 20\%$ ) in response to  $100 \mu\text{M}$  5-HT. When senktide was bath applied ( $1 \mu\text{M}$ , 2 min) prior to the administration of CNQX, it induced a large increase in spontaneous PSCs (arrow, Figure 1) making it difficult to assess postsynaptic changes in isolation. Follow-

ing CNQX, neither senktide nor SP produced observable changes in holding current or membrane conductance.

### Immunolabeling Shows Lack of Co-localization of 5-HT and NK Receptors in the DRN

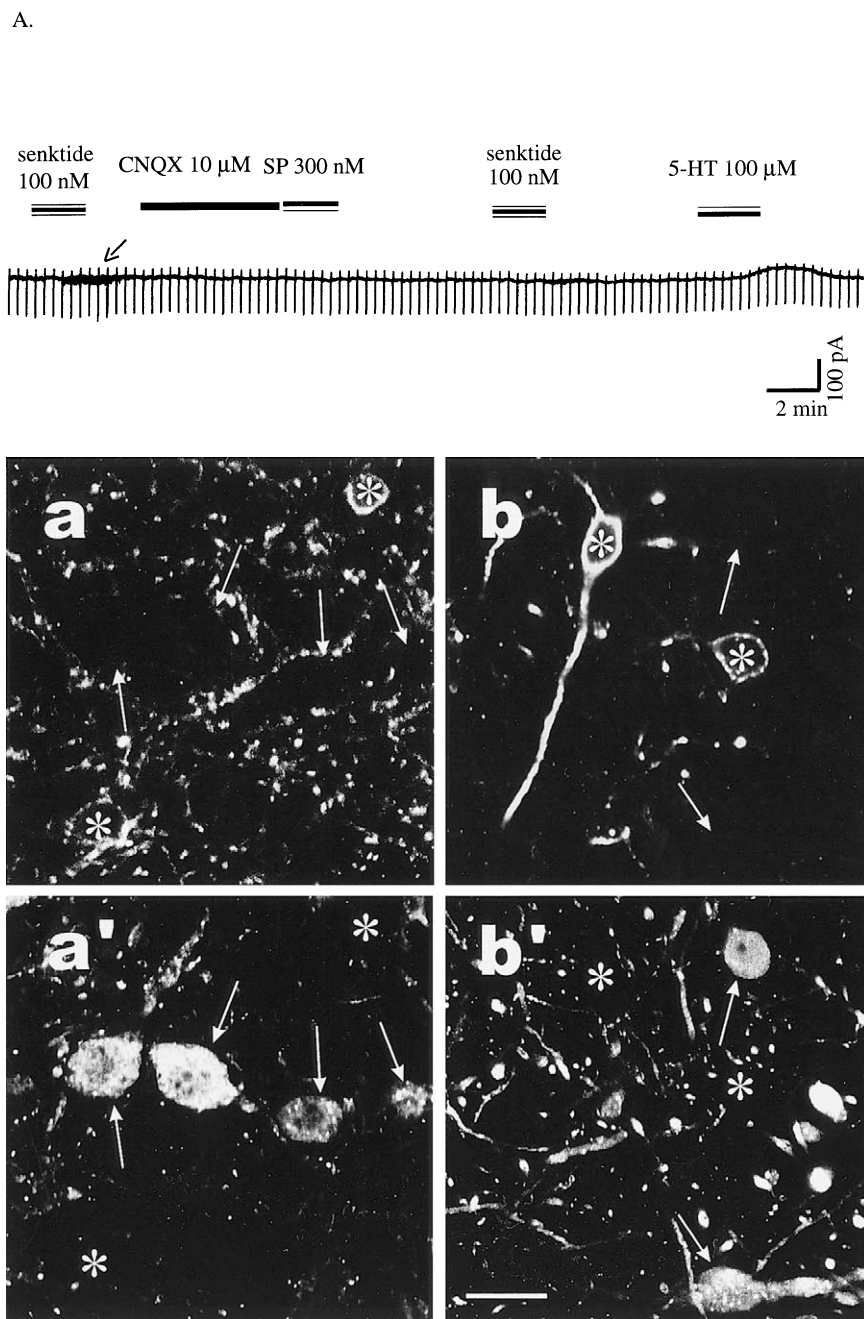
Sets of sections extending from the anterior to posterior DRN were examined after treatment by each of the double staining combinations ( $n = 4$  rats). The distribution of 5-HT-containing in the DRN was consistent with previous studies (Dahlström and Fuxe 1964; Ungerstedt 1971). Although 5-HT-positive and  $\text{NK}_1$  and  $\text{NK}_3$  receptor-expressing neurons were intermingled in the DRN, no neurons were doubly immunostained for 5-HT and neurokinin receptors (5-HT and  $\text{NK}_1$  or 5-HT and  $\text{NK}_3$ ) (Figure 1, panel b). These observations are consistent with the electrophysiological results which failed to show any direct postsynaptic effects of  $\text{NK}_1$  or  $\text{NK}_3$  agonists upon 5-HT neurons. In addition, no  $\text{NK}_1/\text{NK}_3$  doubly-labeled neurons were found in the DRN.

### Substance P and Senktide Induce an Increase in Spontaneous EPSCs in 5-HT Cells

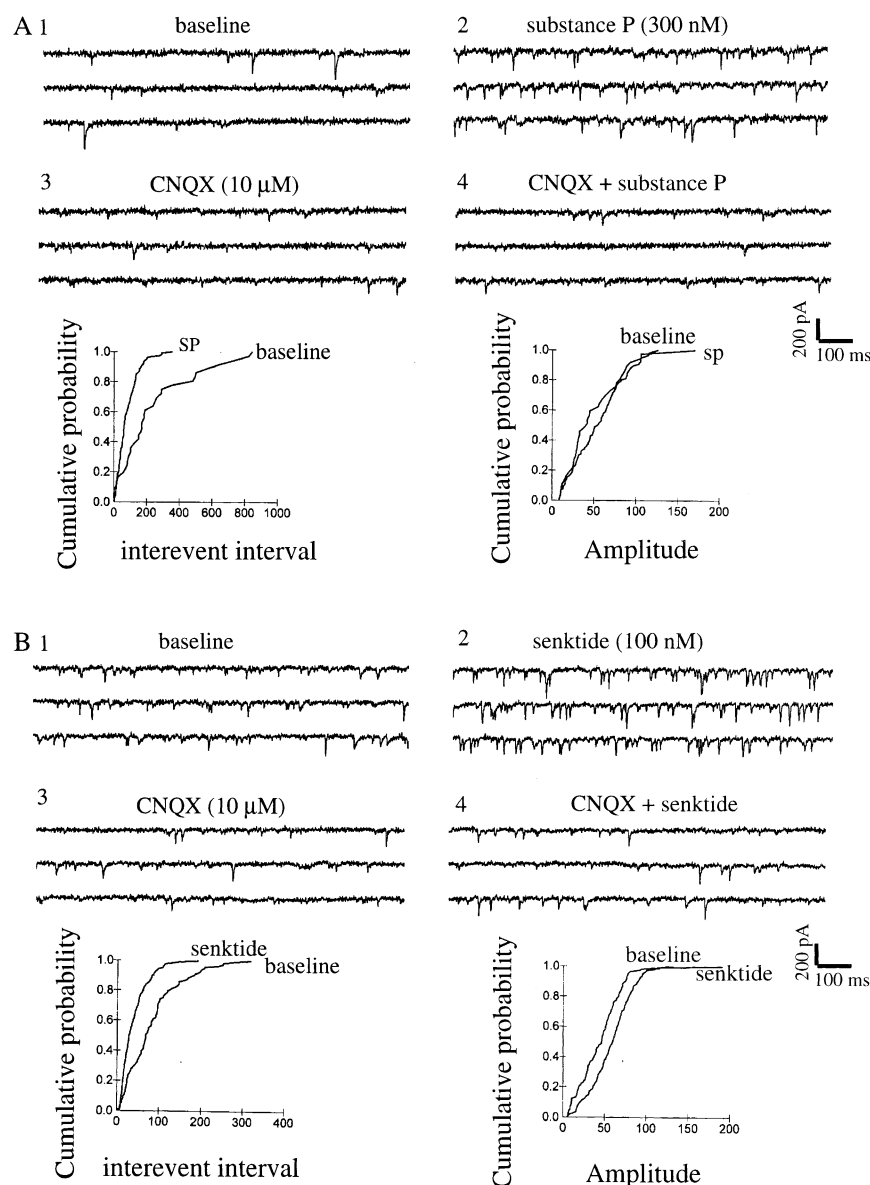
Although SP and senktide had no direct effect on 5-HT neurons both were capable of producing an increase in "spontaneous" (non-electrically evoked) PSCs (Figure 1, panel A, and Figure 2). Table 1 shows that a high proportion of 5-HT neurons exhibited PSC responses to one or both of the agonists. In the great majority of responsive cells (91/104), SP or senktide primarily induced an increase in EPSCs as indicated by their sensitivity to CNQX ( $10 \mu\text{M}$ ) (Figure 2). However, in 10% (10/104) of the responsive neurons, the SP- or senktide-induced PSCs consisted of a mixture of CNQX sensitive and CNQX-insensitive PSCs. Furthermore, in three of the cells tested with SP (3/104), the PSCs were completely insensitive to CNQX but were blocked completely by application the  $\text{GABA}_A$  receptor antagonist bicuculline ( $10 \mu\text{M}$ ), indicating that they were  $\text{GABA}_A$ -mediated IPSCs. It should be noted that EPSCs and IPSCs induced by SP or senktide also could be distinguished on the basis of time constant ( $\tau$ ) of decay from the peak response (not shown).

### Substance P and NKB-induced EPSCs Are Concentration-dependent and TTX Sensitive

As shown in Table 2, under basal conditions spontaneous EPSC frequency and amplitude were  $9.4 \pm 0.08 \text{ Hz}$  and  $61.1 \pm 0.04 \text{ pA}$ , respectively. SP, senktide, or NKB significantly increased mean EPSC frequency in all responding cells (K-S test,  $p < .0001$ ,  $n = 86/86$ ) and amplitude in most responding cells (K-S test  $p < .01$ ,  $n = 51/86$ ). Increases in EPSCs could be detected 1 min af-



**Figure 1.** Lack of direct interactions between neurokinins and 5-HT cells: electrophysiological and histochemical evidence. A. Intracellular recording illustrating the lack of direct effect of a NK<sub>1</sub> agonist SP and a selective NK<sub>3</sub> agonist senktide on 5-HT neurons of the DRN. This intracellular voltage clamp recording from a 5-HT neuron shows a typical outward current and increased conductance in response to 100 μM 5-HT (right part of trace). Initially, when the NK<sub>3</sub> agonist senktide was bath applied (1 μM, 2 min), it appeared to produce no significant changes in holding current; however, because of an increase in PSCs as indicated by a thickening of the baseline trace (arrow), it was difficult to judge the exact membrane potential. After CNQX, when the PSCs were blocked, SP and senktide clearly could be seen to have no effect either on membrane potential or  $R_{in}$  ( $n = 20$ ).  $R_{in}$  was assessed by hyperpolarizing pulses applied through the recording electrode to the 5-HT neuron. (duration 400 ms, at 20 s intervals). B. Confocal micrographs of double immunofluorescent staining for either NK<sub>1</sub>/5-HT (a/a') or NK<sub>3</sub>/5-HT (b, b') in the DRN of the rat. In both cases, no doubly labeled neurons are seen in the DRN. The absence of neurokinin receptors on 5-HT cells is consistent with the lack of direct electrophysiological effects illustrated in panel A. Arrows point to 5-HT-positive neurons. NK<sub>1</sub> and NK<sub>3</sub>-expressing neurons are indicated by asterisks. Scale bar = 40 μm. Double staining for NK<sub>1</sub>/NK<sub>3</sub> was also performed (not shown).



**Figure 2.** Induction of EPSCs by SP and senktide in 5-HT cells. A. (1–4) In voltage-clamp mode (holding potential =  $-65$  mV), SP (300 nM) induced an increase in frequency of PSCs in a 5-HT cell; this effect was blocked by CNQX (10  $\mu$ M) indicating they were glutamatergic excitatory PSCs (EPSCs). Below are plots of normalized cumulative distributions of EPSC amplitude and frequency for this cell before and during the application of SP (data taken from ten 1-s episodes for each condition). K-S tests showed a highly significant increase in frequency ( $p < .01$ ), but in this cell no significant change was found in amplitude. B. (1–4) In another cell senktide (100 nM) induced an increase in frequency of PSCs; this effect was blocked by CNQX (10  $\mu$ M). Below are plots of normalized cumulative distributions of EPSC amplitude and frequency for this cell before and during the application of senktide. K-S tests showed a significant increase in both for amplitude ( $p < .05$ ) and frequency ( $p < .001$ ).

ter switching to the agonist-containing solution, approaching a maximum within the next 2–3 min and then slowly returning to the basal levels over 5–10 min. Figure 3 shows that the increases were concentration-dependent for SP ( $EC_{50}$  357 nM,  $n = 5$ ) and NKB ( $EC_{50}$  295 nM,  $n = 5$ ). The increases in EPSCs induced by SP,

NKB, or senktide were blocked completely by administration of the fast sodium channel blocker TTX (2  $\mu$ M, 8 min,  $p < .01$   $n = 5$ , Figure 4), indicating that the effects of SP or NKB were due to release of impulse-flow dependent glutamate, and not due to an increase in spontaneous quantal release (i.e. miniature EPSCs).

**Table 1.** Proportion of DRN neurons in which PSCs were increased\* by one, both, or neither of the neurokinins

	Number of Cells Responding (%)
SP and Senktide	72/150 (48%)
Senktide only	24/150 (16%)
SP only	8/150 (5%)
Neither SP nor Senktide	46/150 (31%)

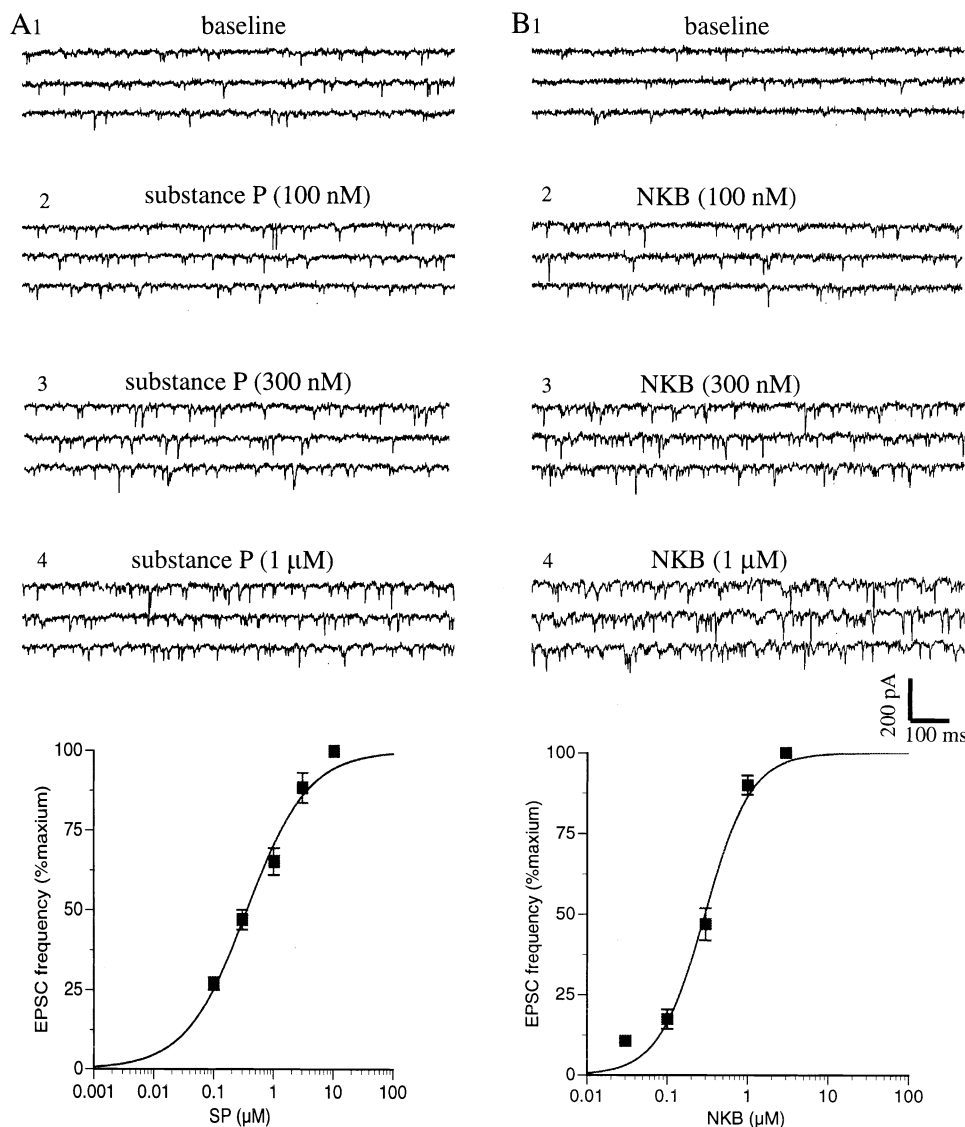
\*  $\geq 20\%$

**Table 2.** Amplitude, Frequency and Decay Time ( $\tau$ ) of Spontaneous and Neurokinin-induced EPSCs

	Frequency (Hz)	Amplitude (pA)	Decay Time ( $\tau$ )	n
Baseline	$9.4 \pm 0.08$	$61.1 \pm 0.04$	$1.05 \pm 0.03$	
SP	$23.3 \pm 0.02^{**}$	$69.2 \pm 0.08^*$	$0.67 \pm 0.05$	32
Senktide	$30.3 \pm 0.01^{**}$	$78.7 \pm 0.03^{**}$	$0.69 \pm 0.06$	34
NKB	$25.4 \pm 0.03^{**}$	$71.9 \pm 0.2^*$	$0.70 \pm 0.06$	25

\*\*  $p < .01$ .

\*  $p < .05$  (paired  $t$ -test).



**Figure 3.** SP- and NKB-induced EPSCs are concentration-dependent. A. (1–4) In a 5-HT cell, EPSCs induced by SP were concentration-dependent. Below is normalized concentration-response curve for a group of five randomly selected DRN cells where the SP-induced increase in EPSC frequency at each concentration (0.1, 0.3, 1, 3, 10  $\mu$ M) were expressed as the percent of the 10  $\mu$ M SP response for each cell. The  $EC_{50}$  of SP was 357 nM and a near maximally-effective concentration was 3  $\mu$ M. B. (1–4) In another 5-HT cell, EPSCs induced by NKB were also concentration-dependent. Below is normalized concentration-response curve for a group of five randomly selected DRN cells where the NKB-induced increase in EPSC frequency at each concentration (0.1, 0.3, 1, 3, 10  $\mu$ M) were expressed as the percent of the 3  $\mu$ M NKB response for each cell. The  $EC_{50}$  of NKB was 295 nM and a near maximally-effective concentration was 1  $\mu$ M.

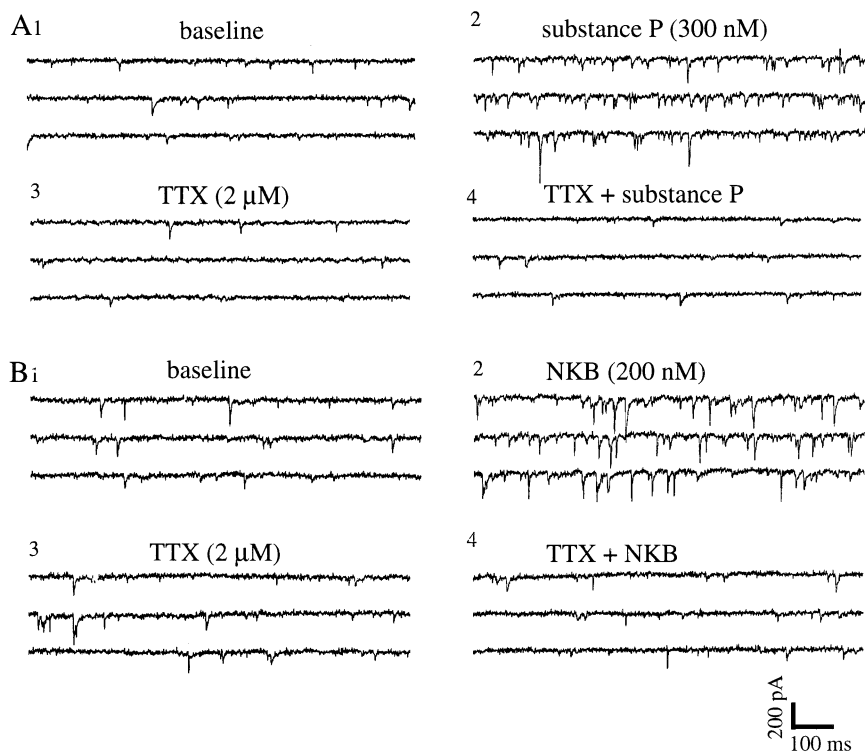
### Pharmacological Characterization of Neurokinin-Induced EPSCs

To determine whether the effects of neurokinin SP or NKB were mediated by selective activation of  $NK_1$  or  $NK_3$  neurokinin receptors, the ability of preferential non-peptide antagonists to block these effects was studied. The selective  $NK_1$  receptor antagonist RP 67580 (Fong et al. 1992; Garret et al. 1991) (10  $\mu$ M, 25 min) reduced the SP-induced EPSCs by 52%, but had no effect on the enhancement of EPSCs by NKB. Higher concentrations (up to 30  $\mu$ M) inhibited the SP effect on EPSCs by 96%, while the NKB effect on EPSCs was inhibited by 52%. In contrast, the selective  $NK_3$  receptor antagonist, SB 223412 (Sarau et al. 1997) (10  $\mu$ M, 25 min) reduced NKB-induced EPSC by 37%, but had no effect on the enhancement of EPSCs by SP. Higher concentrations (up to 30  $\mu$ M) reduced the NKB effect on EPSCs

by 56%, but also inhibited SP effect on EPSCs by 15%. Thus, while the antagonists showed preferential blocking activation, they were not absolutely selective.

### Minimal Cross-desensitization Between Senktide and SP

It has been shown that desensitization with a selective agonist can discriminate between multiple neurokinin receptors (Laufer et al. 1988). In view of the incomplete selectivity of the above antagonists in blocking SP and NKB in the DRN, we turned to a cross-desensitization approach to explore further the independence of  $NK_1$  and  $NK_3$  effects in the DRN. We employed the  $NK_3$ -selective agonist, senktide, which selectively and potently activates  $NK_3$  receptors in rat brain, but is virtually devoid of activity at  $NK_1$  receptors (Laufer et al. 1986a,b;



**Figure 4.** SP- and NKB-induced EPSCs in 5-HT cells are suppressed by bath applied TTX (2  $\mu$ M). A. (1–4) In a 5-HT cell, EPSCs induced by SP (300 nM, 2 min) were blocked totally by bath applied TTX (5 min). B. (1–4) In another 5-HT cell EPSCs induced by NKB (200 nM, 2 min) were blocked totally by bath applied TTX (5 min).

Wormser et al. 1986). In the example shown in Figure 5, panel A, the initial application of SP (1  $\mu$ M) induced a strong increase in EPSC frequency in a 5-HT neuron. After washout of SP, senktide (100 nM) also was found to induce a large initial increase in EPSC frequency. However, after continued senktide application (20 min), EPSCs were reduced to  $\sim 30\%$  of the original response. A subsequent application of SP induced a robust increase of EPSCs indicating that there is little cross-desensitization between senktide and SP. Figure 5, panel B, summarizes data for five cells. Note that the response to SP following senktide did not exceed the original response to SP despite the elevated baseline.

#### Neurokinin-induced excitatory postsynaptic potentials (EPSPs) Can Increase the Spiking of 5-HT Neurons

Five 5-HT neurons that displayed strong EPSPs in response to both SP and senktide were selected for these experiments. PE (3  $\mu$ M) was added to the perfusate, to induce a slow, tonic level of baseline firing (see Methods). As shown in Figure 6, panel A, a slow depolarizing ramp usually preceded the triggering of spikes in the presence of PE. Under these conditions, the firing rate of all five cells was increased by 300 nM SP ( $191 \pm 27\%$ ) or 100 nM senktide ( $279 \pm 34\%$ ). In the presence of senktide (Figure 6, panel B) or SP (not shown), a population of spikes emerged that were triggered by EPSPs. In presence of PE alone, only 16% of spikes were triggered by EPSPs ( $n = 5$ ); following SP ( $n = 4$ ) or senk-

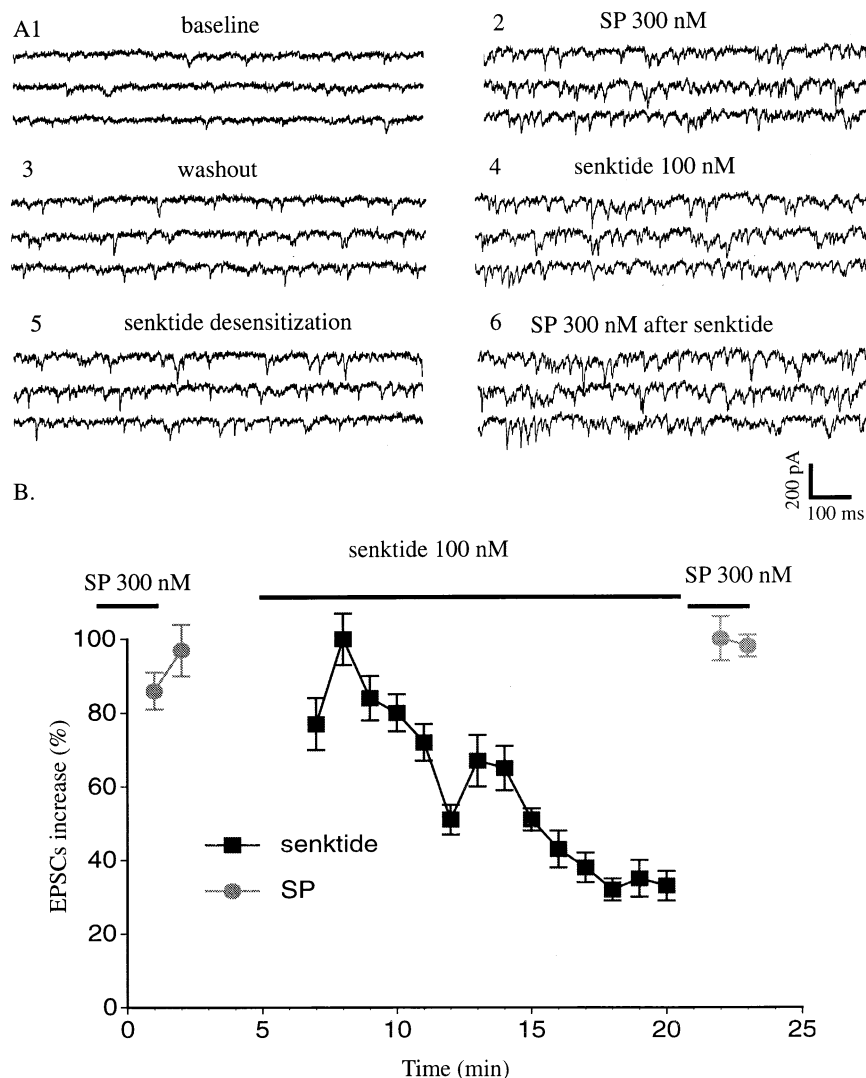
tide ( $n = 5$ ) the proportion of spikes triggered by EPSPs was increased to 59% and 64%, respectively. Figure 6, panel C, shows that the firing induced by PE was not reduced by CNQX ( $n = 5$ ). In contrast, the increase induced by SP or senktide was blocked by CNQX, as would be expected if it was being driven by EPSPs (Figure 6, panel D,  $n = 5$ ).

## DISCUSSION

### Neurokinins Excite 5-HT Neurons in the DRN Indirectly by Activating a Local Population of Glutamatergic Neurons

There are a number of studies indicating that neurokinins may induce 5-HT-mediated behaviors by enhancing the release of endogenous 5-HT (Reisine et al. 1982; Reubi et al. 1978; Solti and Bartfai 1987; Stoessl et al. 1987, 1990). It has been proposed that neurokinins may increase 5-HT release by activating 5-HT cell firing in the raphe nuclei. This proposal is based on the fact that local injections of SP into the MRN (Forchetti et al. 1982) or DRN (Gradin et al. 1992) increase 5-HT turnover or 5-HT release in terminal regions of the hippocampus. However, the mechanism by which neurokinins might alter the activity of 5-HT neurons in the DRN (or other raphe nuclei) was not delineated by those studies. The present results show that NK<sub>1</sub> receptor agonist SP and the NK<sub>3</sub> receptor agonists senktide and NKB do not have direct excitatory effects on 5-HT neurons in the



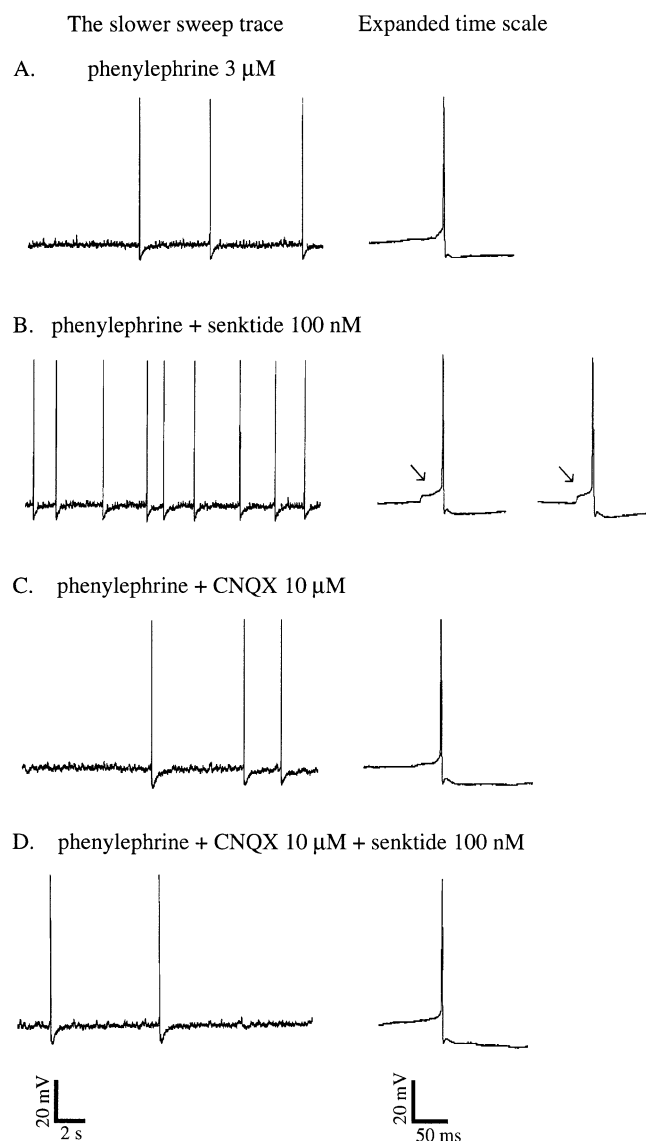


**Figure 5.** The induction of desensitization to the NK<sub>3</sub> agonist senktide does not desensitize the response to the NK<sub>1</sub> agonist SP. **A.** Intracellular recording from a cell under the following conditions: (1) baseline, (2) initial SP application (3) washout, (4) the initial period of senktide, (5) after continued senktide application (20 min), (6) SP application after senktide. **B.** Summarized data for five tested cells. Events were counted at 1 min intervals. Each point represents the mean of 5 neurons ( $\pm$  SEM). Ten consecutive 1-sec episodes were used to determine EPSC frequencies. Note that in preliminary experiments it had been determined that recovery of 5-HT neurons from desensitization to senktide did not begin until after 20 min or more of washout. Therefore, because the second SP application was given immediately after cessation of the senktide perfusion, the latter was not included in the SP solution.

DRN. Instead, the neurokinins were found to have an indirect excitatory effect on 5-HT neurons by inducing an increase in EPSCs in 5-HT neurons. The EPSCs were blocked by the AMPA/kainate glutamate receptor antagonist CNQX and were prevented by the fast Na<sup>+</sup> channel blocker TTX, indicating an increase in impulse flow in local glutamatergic neuronal afferents. Previously, it has been shown that 5-HT neurons receive projections from local glutamatergic neurons present in the DRN slice preparation (Jolas and Aghajanian 1997). Consistent with the recent report of Froger and colleagues (Froger et al. 2001), no neurons were doubly immunostained for 5-HT and for NK<sub>1</sub> receptors despite the intermingling of 5-HT-positive and NK<sub>1</sub>-positive neurons within the DRN. The present results additionally show that there was no co-localization of 5-HT and NK<sub>3</sub> receptors or NK<sub>1</sub> and NK<sub>3</sub> receptors. Taken together, the present results provide converging histochemical and electrophysiological evidence that neurokinins have a local excitatory influence upon 5-HT

neurons, but that this effect is through an indirect rather than direct mechanism.

The present study provides several lines of evidence that SP and NKB are preferential agonists, respectively, for NK<sub>1</sub> and NK<sub>3</sub> receptors in the DRN. First, some neurons can be excited by SP but not by senktide, while other neurons are sensitive to senktide but not to SP. Second, low concentrations of the non-peptide NK<sub>1</sub> receptor antagonist RP 67580 (10  $\mu$ M) reduced the SP-induced EPSCs but was inactive against NKB-induced EPSCs. Conversely, low concentrations of selective NK<sub>3</sub> receptor antagonist SB 223412 (10  $\mu$ M) reduced NKB-induced but not SP-induced EPSCs. Third, minimal cross-desensitization occurred between SP and senktide. In addition, we found that the NK<sub>3</sub> agonists generally were more efficacious in inducing EPSCs as well as increasing 5-HT firing rate (Table 1). In addition to this agonist selectivity, immunohistochemical double labeling showed that NK<sub>1</sub> and NK<sub>3</sub> receptors were not co-localized. This opens up the possibility that NK<sub>1</sub> and NK<sub>3</sub>



**Figure 6.** Senktide-induced EPSPs increase the spiking of 5-HT neurons. A. Intracellular recording from a 5-HT DRN neuron in which tonic firing was maintained by phenylephrine (3  $\mu$ M). Note, in the expanded time scale, that the spikes were triggered off slow depolarizing ramps (inset at right). B. Increase in action potential frequency induced by 100 nM senktide. Note that in the presence of senktide, there was an emergence of spikes that were triggering by EPSPs rather than from depolarizing ramps (inset at right). C. CNQX did not decrease phenylephrine dependent spiking. D. The senktide-induced increase in spike frequency was blocked in the presence of CNQX.

receptive glutamatergic neurons could operate within independent circuits, as discussed below.

### Differences between the Local and Global Effects of Activating NK<sub>1</sub> and NK<sub>3</sub> Receptors

As mentioned at the beginning of this article, *in vivo* studies show that there are differential effects of local

versus intracisternal administration of SP and other NK<sub>1</sub> agonists. While the injection of SP directly into the raphe nuclei leads to an increase 5-HT release and 5-HT-mediated behaviors, intracisternal administration of SP fails to elicit 5-HT-mediated behaviors (Gradin et al. 1992; Stoessl et al. 1987). In another *in vivo* study, it was found that the systemic administration of a SP (NK<sub>1</sub>) antagonist produced an increase rather than a decrease in 5-HT cell firing (Santarelli et al. 2001), again indicating that SP does not have a global activating effect on the 5-HT system. The latter authors suggest that while our *in vitro* studies show a local activating effect of SP on 5-HT cell firing in brain slices, in intact preparations this is likely to be opposed by effects of SP elsewhere in the CNS. In contrast to NK<sub>1</sub> agonists, the local and global effects of NK<sub>3</sub> agonists are not different with respect to activating the 5-HT system. Behavioral studies show that NK<sub>3</sub> agonists strongly elicit 5-HT mediated behaviors both after local injection into the raphe and after intracisternal administration. For example, the NK<sub>3</sub> receptor agonist senktide strongly elicits 5-HT-mediated behaviors following *both* intracisternal (Stoessl et al. 1988) and local injections into the raphe nuclei (Paris et al. 1989). The latter results are consistent with our *in vitro* findings that the local application of NK<sub>3</sub> albeit indirectly, activate 5-HT neurons in the DRN. Taken together, the *in vivo* and *in vitro* studies indicate that there are important differences between the overall influence of NK<sub>1</sub> and NK<sub>3</sub> receptors upon the 5-HT as well as other systems in the brain.

### Behavioral and Clinical Implications

In a number of animal behavioral models, contrasting effects of NK<sub>1</sub> and NK<sub>3</sub> antagonists or agonists have been reported. Blockade of NK<sub>1</sub> receptors has an anxiolytic-like effect in rats (File 1997) and mice (Teixeira et al. 1996; Santarelli et al. 2001), while stimulation of NK<sub>1</sub> receptors by SP has an anxiogenic profile (Aguilar and Brandao 1996). The opposite behavioral profile has been observed after stimulation of NK<sub>3</sub> receptors. For example, treatment of mice with the NK<sub>3</sub> agonist NKB or senktide has an anxiolytic-like effect but the reverse effect is seen with an NK<sub>3</sub> antagonist (Ribeiro et al. 1999). Thus, although SP and senktide are similar in activating 5-HT neurons, they differ in their overall effect in preclinical models of anxiety. As discussed above, these differences may result from actions of neurokinins in regions of the central nervous system outside of the DRN. These differences may be explained by the differing expression and distribution of NK<sub>1</sub> and NK<sub>3</sub> receptors in many regions of the brain (Lucas et al. 1992; Maeno et al. 1993; Ding et al. 1996). In addition, the present study shows that even within a single region such as the DRN, NK<sub>1</sub> and NK<sub>3</sub> receptors do not necessarily co-localize on the same subpopulation of neurons.

Given the contrasts between NK<sub>1</sub> and NK<sub>3</sub> receptors in animal model systems, it is likely that such differences will emerge in clinical situations. The NK<sub>1</sub> receptor antagonist MK-869 has been reported to have therapeutic efficacy of in depressed patients (Kramer et al. 1998). As mentioned earlier, based on recent studies in NK<sub>1</sub> knockout mice, it has been suggested the antidepressant effect of blocking NK<sub>1</sub>-receptors may result from desensitization/downregulation of 5-HT<sub>1A</sub> autoreceptors (Froger et al. 2001). At this time, no information is available as to the effects of NK<sub>3</sub> receptor antagonists or agonists in depression or other clinical disorders. NK<sub>3</sub> receptor mRNA expression and receptor immunoreactivity have been found in human brain tissue (Buell et al. 1992; Mileusnic et al. 1999), providing a potential substrate for any therapeutic action of drugs targeting NK<sub>3</sub> receptors. In view of the strong activating effects of NK<sub>3</sub> agonists upon the 5-HT system, both locally and globally, further exploration into the role of 5-HT in clinical aspects of NK<sub>3</sub> receptor function would be warranted.

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