LOCALIZATION AND FUNCTION OF NK₃ SUBTYPE TACHYKININ RECEPTORS OF LAYER V PYRAMIDAL NEURONS OF THE GUINEA-PIG MEDIAL PREFRONTAL CORTEX

M. A. SIMMONS,a,b* C. D. SOBOTKA-BRINERa
AND A. M. MEDDA,b

aDepartment of Neuroscience Biology, AstraZeneca Pharmaceuticals, Wilmington, DE 19850, USA
bDepartment of Integrative Medical Sciences, Northeastern Ohio Universities College of Medicine, 4209 State Route 44, P.O. Box 95, Rootstown, OH 44272-0095, USA

Abstract—The NK₃ subtype of tachykinin receptor has been implicated as a modulator of synaptic transmission in several brain regions, including the cerebral cortex. The localization and expression of NK₃ receptors within the brain vary from species to species. In addition, the pharmacology of NK₃ receptor-specific antagonists shows significant species variability. Among commonly used animal models, the pharmacology of the guinea-pig NK₃ receptor most closely resembles that of the human NK₃ receptor. Here, we provide anatomical localization studies, receptor binding studies, and studies of the electrophysiological effects of NK₃ receptor ligands of guinea-pig cortex using two commercially available ligands, the NK₃ receptor peptide analog senktide, and the quinolinecarboxamide NK₃ receptor antagonist SB-222,200.

Saturation binding studies with membranes isolated from guinea-pig cerebral cortex showed saturable binding consistent with a single high affinity site. Autoradiographic studies revealed dense specific binding in layers II/III and layer V of the cerebral cortex. For electrophysiological studies, brain slices were prepared from prefrontal cortex of 3- to 14-day-old guinea pigs. Whole cell recordings were made from layer V pyramidal neurons. In current clamp mode with a K⁺-containing pipette solution, senktide depolarized the pyramidal neurons and led to repetitive firing of action potentials. In voltage clamp mode with a Cs⁺-containing pipette solution, senktide application produced an inward current and a concentration-dependent enhancement of the amplitude and the duration of activating the receptor. Like adrenergic, opioid, taste and other C-protein-coupled receptors, the deduced amino acid sequence of the tachykinin receptors is consistent with the seven transmembrane domain structure proposed for this family of receptors.

The tachykinins are a family of peptides that primarily act as neurotransmitters or neuromodulators in the CNS and peripheral nervous system. The tachykinin family is defined characteristicly by the conserved carboxy terminal sequence -Phe-Xaa-Gly-Leu-Met-NH₂, where Xaa is a variable amino acid. The endogenous mammalian tachykinins are substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) (Beaujouan et al., 2004).

These endogenous peptides act preferentially at three tachykinin receptors, termed the NK₁, NK₂ and NK₃ subtype tachykinin receptors. The NK₁ receptor exhibits a higher affinity for the binding of SP than for NKA or NKB (Krause et al., 1994). The NK₃ receptor binds NKA with the highest affinity (Buck and Shatz, 1998). For the NK₃ receptor, the order of agonist potency is NKB > NKA > SP (Laurer et al., 1986); however, all three ligands are capable of activating the receptor. Like adrenergic, opioid, taste and other C-protein-coupled receptors, the deduced amino acid sequence of the tachykinin receptors is consistent with the seven transmembrane domain structure proposed for this family of receptors.

The pharmacology of tachykinin receptors has been well characterized in pain, and the roles of tachykinin receptor ligands in lacrimation, salivation, and smooth muscle contraction have been well documented (Regoli et al., 1994). Only recently has evidence emerged regarding their role in psychopharmacology. Interest in the role of NK₃ receptors in the brain has been piqued by the development of the brain permeant, potent, NK₃-selective compounds osanetant (Kamali, 2001) and talnetant (Evangelista, 2005).

Studies of the function and pharmacology of NK₃ receptors have proven to be complicated in several respects. One of the difficulties is that the distribution of NKB expressing neurons and of NK₃ receptors in the brain varies among commonly used animal models (Massi et al., 2000; Langlois et al., 2001) and between some of these models and primates (Nagano et al., 2006). The pharmacology of small molecule antagonists also shows interspecies variation. In general, tachykinin receptor antagonists show selectivity for either rat or mouse or guinea-pig and gerbil.

CORRESPONDENCE TO: M. A. Simmons, Department of Integrative Medical Sciences, Northeastern Ohio Universities College of Medicine, 4209 State Route 44, P.O. Box 95, Rootstown, OH 44272-0095, USA
E-mail address: simmons@neoucom.edu (M. A. Simmons).

Abbreviations: ACSF, artificial cerebrospinal fluid; AP5, d-(-)-2-amino-5-phosphonopentanoic acid; Bmax, maximal binding; EPSC, excitatory postsynaptic current; Kd, dissociation constant of the radioligand; Ki, inhibition constant; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydronbenzo[f]quinoxaline-7-sulfonamide; NKA, neurokinin A; NKB, neurokinin B; NK₃, NK₃ subtype tachykinin receptor; RT, room temperature; SP, substance P.
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NK₁ receptors (Chung et al., 1995; Emonds-Alt et al., 2002; Sarau et al., 1997, 2000). The pharmacology of these compounds at the human NK₁ receptor is more accurately replicated in guinea-pig and gerbil models than in rat and mouse (Regoli et al., 1994).

Given the relative receptor distribution, pharmacology and potential utility of the guinea pig as an animal model, we have examined the localization and function of NK₁ receptors in the cerebral cortex of the guinea pig. Here, we provide anatomical localization studies, ligand binding studies, and studies of the physiological effects of NK₁ receptor activation and blockade.

**EXPERIMENTAL PROCEDURES**

**Animals**

Hartley guinea pigs (Charles River Laboratories, Wilmington, MA, USA) were used in these experiments. Animals were housed in a temperature-controlled vivarium with free access to food and water. Animal experiments were approved by the AstraZeneca IACUC and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23). The number of animals used and their suffering were minimized.

**Autoradiography**

Naïve adult male Hartley guinea pigs were killed by decapitation without anesthesia. Brains were removed and frozen in cooled isopentane (−35 °C). Sections (20 μm) were cut in a cryostat at −15 °C, mounted onto gelatin-coated slides, rapidly dried and stored at −80 °C until use. On the day of the experiment, slides were warmed to room temperature (RT, 22–25 °C) and preincubated in 50 mM Tris–HCl, 0.005% polyethylamine, pH 7.4. To determine total binding the sections were incubated in the same buffer with 3 mM MnCl₂, 0.02% BSA, and 1 nM [125I]-MePhe⁷-NKB ligand was used at concentrations of 0.02–0.07 nM. The remainder of the assay was as described above.

Saturation binding data were analyzed by non-linear regression using GraphPad Prism software to yield Kᵦ (dissociation constant of the radioligand) and B_max (maximal binding). For competition binding experiments, inhibition constants (Kᵢ) were calculated using the Cheng-Prusoff equation and XL-Fit software.

**Electrophysiology**

Coronal slices were prepared from the frontal pole of the brain of guinea-pig neonates of either sex at 2–15 days of age, weighing 40–200 g. Animals were decapitated after induction of deep anesthesia with isoflurane. The brain was rapidly removed and immersed in ice cold artificial cerebrospinal fluid (ACSF) gassed with 95% O₂–5% CO₂. The brain was cut in half with a single coronal cut. The front half of the brain was mounted anterior side up in an ice cold gassed bath for slicing. Slices (200 μm) were cut using a Vibratome (Leica Microsystems). The first four slices were discarded. Subsequent slices were stored in a warm (30 °C) holding chamber gassed with 95% O₂–5% CO₂. Slices were allowed to equilibrate for at least 1 h before recording. Individual slices were transferred to a recording chamber which was perfused with gassed ACSF at 2.0 ml/min. Whole cell recordings of single neurons were conducted at RT, 23 °C, using an Axopatch 200C (Hamden, CT, USA) patch clamp amplifier. Electrodes with resistances of 4–8 MΩ were filled with pipette solution. Drugs were applied by bath perfusion.

The composition of solutions used for these experiments is listed below, in mM unless stated otherwise. ACSF: 130 NaCl, 3.5 KCl, 1.5 CaCl₂, 1.5 MgSO₄, 24 NaHCO₃, 1.25 NaH₂PO₄, 10 glucose. K-containing pipette solution: 140 K gluconate, 2 MgSO₄, 0.1 CaCl₂, 0.1 GTP, 2.0 ATP, 10 Hepes, 1 EGTA, pH 7.25, Cs-containing pipette solution: 120 Cs methanesulfonate, 2 MgCl₂, 0.3 GTP, 2.0 ATP, 10 Hepes, pH 7.35.

Excitatory postsynaptic currents (EPSCs) were analyzed by the Minanalysis program. EC₅₀ values were calculated using the logisitic equation: y = E_max *[EC₅₀+x], where x is drug concentration. Of the neurons tested, 90% responded to senktide. Data were obtained from 52 cells in brain slices from 15 different animals. Data are expressed as the mean ± S.E.M.

**Chemicals**

[125I]-MePhe⁷-NKB, specific activity 2200 Ci/mmol, was purchased from PerkinElmer; NKA and SP were purchased from Sigma (St Louis, MO, USA); senktide, b-(−)-2-amino-5-phosphoentannic acid (AP5) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydronbenzol[if]quinoline-7-sulfonamide (NBQX) from Tocris (Ellisville, MO, USA), and N-MePhe⁷-NKB from Bachem. SB-222,200 and osanetant were synthesized in house. All other chemicals were purchased from Sigma or Fisher Scientific (Pittsburgh, PA, USA). For the electrophysiological experiments, senktide stock solution at a concentration of 5 mM and SB-222,200 at a concentration of 10 mM were prepared in DMSO. At the highest concentration of senktide tested (2 μM), the concentration of DMSO would be 0.04%. To test for effects of this vehicle, 0.05% DMSO was applied to brain slices. No change in current or sEPSCs was observed (n=4 neurons from two different slices).
RESULTS

NK₃ receptor localization

NK₃ receptors were labeled by autoradiography with [³H]-labeled SB-222,200 (1 nM), an NK₃ receptor antagonist (Sarau et al., 2000). The specificity of the binding was determined by incubation with an excess of unlabeled talnetant (10 μM), a structurally related NK₃ antagonist. Except for an intense band of staining that outlined the cerebral ventricles, labeling was confined to the cerebral cortices. Dense specific bands of labeling were observed in layers II/III and in layer V of the cerebral cortex, including medial prefrontal areas (Fig. 1).

NK₃ receptor binding

Both saturation and competition studies have been conducted to characterize the NK₃ receptor binding sites in guinea-pig cerebral cortex. For saturation binding studies, membranes were isolated from guinea-pig frontal cortex. [¹²⁵I]-MePhe⁷-NKB, a selective NK₃ receptor agonist, showed saturable binding consistent with a single high affinity site (Fig. 2). Non-linear regression analysis of five independent experiments yielded a mean $K_d$ of 0.073 nM±0.02 and a $B_{max}$ of 11±2.71 fmol/mg protein.

To further characterize this binding site, $K_i$ values were obtained from competition binding studies with NK₁, NK₂ and NK₃ receptor-selective ligands versus

Fig. 1. Localization of NK₃ receptors in guinea-pig cerebral cortex by autoradiography with [³H]-SB-222,200. (A) Horizontal section at mid level showing pattern of [³H]-SB-222,200 labeling. (B) Sagittal section at the midline showing pattern of [³H]-SB-222,200 labeling. Prominent labeling was observed in the area comprising layers II/III of the cortex as well as in layer V. Binding was also observed lining the third and fourth ventricles. (C, D) Block of [³H]-SB-222,200 labeling in presence of 10 μM talnetant. The scale bar—5 mm in A and refers to all panels.
125I-MePhe7NKB (Table 1). The NK3 receptor-selective ligands MePhe7-NKB, senktide, and SB-222,200 all competed for 125I-MePhe7NKB binding with Kᵢ in the low nM range. The NK1 agonist, SP, and the NK2 agonist, NKA, were less potent. These binding properties are characteristic of the NK3 receptor.

Electrophysiology

Whole cell recordings were made from single neurons in slices of prefrontal cortex from guinea-pig brains. Recordings were made from pyramidal neurons located in layer V of the medial portion of the slice. An example of a biocytin-filled cell and its location in the slice are shown in Fig. 3. To avoid confounding the data with desensitization to agonist or prolonged effects of antagonist, a recording was made from only a single cell in each brain slice exposed to drug.

Effects of senktide on membrane potential.

In current clamp mode with a K⁺/HCl-containing pipette solution, senktide depolarized the pyramidal neurons, as illustrated in Fig. 4. The response of the neuron shown in the figure was typical in that the depolarization in response to senktide was sufficient to lead to repetitive firing of action potentials and that the action potentials persisted for several minutes following washout of senktide. A similar response was observed in four other neurons.

Senktide effects on membrane current.

In voltage clamp mode (V_h = -70 mV) with a Cs⁺-containing pipette solution, senktide produced an inward current and a dramatic increase in EPSCs, as shown in Fig. 5A and B. The amplitude of the inward current produced was dependent on the senktide concentration (Fig. 5C). The concentration response curve for inward current was well fit by the logistic equation with a Hill coefficient of 1. The EC₅₀ from this curve was 836 nM.

Effects of senktide on EPSCs.

The effect of senktide was to increase both the amplitude and frequency of EPSCs. Histograms of the number of events observed for 5 min periods under control conditions and in the presence of senktide (500 nM) show the increase in EPSC frequency (Fig. 5D). Plots of cumulative EPSC amplitude under control conditions and in the presence of senktide reveal that there were a greater proportion of larger amplitude events in the presence of senktide (Fig. 5E).

Pharmacological analysis of the senktide response

Concentration-response curve for senktide effect on EPSCs. Since senktide increased both the amplitude and frequency of EPSCs the response was quantified by integrating the amplitude of the EPSCs as a function of time to obtain the charge transferred and to fully account for the effect of the drug. This value was measured for the EPSCs that occurred during stable 1 min periods during exposure to various concentrations of drug. These data are plotted as the total area of EPSCs in fC versus senktide concentration (Fig. 6C). Unlike the concentration response curve for inward current, the concentration response curve for EPSC activation did not have an apparent sigmoidal shape and could not be fit by the logistic equation. Based on a peak effect of senktide of 11,000 fC/min, the 50% effect was obtained at a senktide concentration of ~200 nM.

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Table 1. Competition binding to guinea-pig frontal cortex membranes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean Kᵢ±S.E.M. (nM)</th>
<th>n</th>
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<tr>
<td>MePhe-NKB</td>
<td>0.35±0.13</td>
<td>3</td>
</tr>
<tr>
<td>Senktide</td>
<td>6.37±1.3</td>
<td>3</td>
</tr>
<tr>
<td>SB-222200</td>
<td>1.44±0.89</td>
<td>3</td>
</tr>
<tr>
<td>SP</td>
<td>435±86</td>
<td>3</td>
</tr>
<tr>
<td>NKA</td>
<td>1117±215</td>
<td>3</td>
</tr>
</tbody>
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Kᵢ values were obtained from competition binding studies versus 125I-MePhe7NKB. Unlabeled MePhe7-NKB, senktide, and SB-222,200 all competed for binding with Kᵢs in the low nM range, while the NK₁ agonist, SP, and the NK₂ agonist, NKA, were less potent.
Block by NK3 antagonist and glutamate antagonists.
The effects of senktide were blocked by the NK3 antagonist SB-222,200. When added during a senktide response, SB-222,200 attenuated the inward current and EPSC activation produced by senktide (Fig. 6A). When the slice was pre-incubated with SB-222,200 for 15 min, the effect of senktide was almost completely blocked (Fig. 6B). The average effect of SB-222,200 is plotted on the senktide concentration response curve (square on Fig. 6C).

The glutamatergic nature of the EPSCs induced by senktide is demonstrated by block by a 5 min preincubation with the combination of the NMDA receptor antagonist AP5 (50 μM) and the AMPA receptor antagonist NBQX (10 μM) (Fig. 7). These concentrations have been shown to block glutamatergic EPSCs in previous studies (Wuarin and Dudek, 1991; Sheardown, 1993). This result was repeated in five other cells. The inward current in response to senktide was still observed in the presence of AP5 and NBQX. The increase in EPSCs was blocked.

DISCUSSION
In this study we have shown that the NK3 receptor system has a strong excitatory effect on layer V pyramidal neurons of guinea-pig medial prefrontal cortex. Despite the fact that the role of NK3 receptors in modulating cortical function is of considerable interest, the effects of NK3 receptor agonists and antagonists in guinea-pig cortex have not been previously shown.

The pattern of receptor autoradiographic binding of SB-222,200 shown here is consistent with previous studies of NK3 receptor autoradiography with other ligands including the agonist senktide and the antagonist osanetant (SR-142801) (Langlois et al., 2001). Both agonist and antagonist showed the same pattern of distribution with particularly high densities of binding described in the mid-cortical layer and deep cortical layer, corresponding to our layer II/III and layer V binding. A similar pattern of staining was seen with an NK3 receptor antibody (Yip and Chahl, 2001). There appears to be a similar cortical distribution of NK3 receptors in mouse (Duarte et al., 2006), rat (Dam et al., 1990; Langlois et al., 2001; Saffroy et al., 2003; Shughrue et al., 1996; Mileusnic et al., 1999), gerbil (Langlois et al., 2001), monkey (Nagano et al., 2006) and human (Mileusnic et al., 1999; Tooney et al., 2000), although there are species differences in NK3 receptor localization in other brain areas.

The NK3 receptor is distinguished by its preference for NKB-like ligands over SP and NKA, which preferentially bind to NK1 and NK2 receptors, respectively. In addition, SB-222,200 is a selective NK3 antagonist. The receptor binding data indicate the presence of an NK3 receptor in guinea-pig cerebral cortical membranes and are consistent with previous studies of NK3 receptor binding properties. Suman-Chauhan et al., 1994, observed a $K_d$ of 0.362 nM for $^{125}$I-MePhe$^7$NKB binding to guinea-pig cortical membranes and are consistent with previous studies of NK3 receptor binding properties. Suman-Chauhan et al., 1994, observed a $K_d$ of 0.362 nM for $^{125}$I-MePhe$^7$NKB binding to guinea-pig cortical membranes. This is higher than the value of 0.073 nM determined here, but within the expected range of error. Both their saturation data and competition data are about two to three times higher than our values for all of the ligands tested. Furthermore, the relative potency profiles for different ligands reported by Suman-Chauhan et al. are the same as reported here.

NK3 receptors have been shown to modulate release of amine neurotransmitters at several sites in guinea-pig...
Injection of senktide into the substantia nigra leads to increased dopamine release in the striatum, injection into the ventral tegmental area increases dopamine release in the nucleus accumbens and injection into the septal area increases hippocampal release of acetylcholine (Marco et al., 1998). The role of NK3 receptors in regulating midbrain dopaminergic neurons is further supported by studies showing a block of activation by osanetant (Gueudet et al., 1999). Our data show that NK3 receptors also modulate synaptic transmission by direct actions in prefrontal cortex.

Previous electrophysiological studies have examined NK3 receptor-mediated effects in rat entorhinal cortex (Stacey et al., 2002) and gerbil cingulate cortex (Rekling, 2004). In the latter study, current clamp recordings from layer V pyramidal neurons revealed a depolarization and increase in excitatory postsynaptic potentials in response to senktide (500 nM). In the former study, voltage clamp recording from layer V pyramidal neurons in medial entorhinal cortex of the rat revealed that senktide (50–500 nM) increased glutamatergic EPSCs. This effect was blocked by osanetant.

The data presented here are consistent with the model presented by Stacey et al. (2002) (see Fig. 8 of their paper). In this model, the effect of activating NK3 receptors is to excite layer V pyramidal neurons. At the cell from which the recording is being made, this is observed as a depolarization and increased spiking in current clamp mode and an inward current under voltage clamp. This leads to increased glutamate release onto neighboring pyramidal neurons via recurrent axon collaterals. Thus, the increase in EPSCs observed after NK3 receptor activation is from neighboring pyramidal neurons that have been depolarized and excited by senktide and are now releasing glutamate onto the cell being monitored.

**Fig. 5.** Effect of senktide on membrane current of a layer V pyramidal neuron. (A) An inward current and a large increase in EPSCs were produced by senktide (500 nM) application, as indicated by the bar. Time calibration = 1 min. (B) At a faster time base, a few small EPSCs are observed in the control condition, prior to senktide application. The response to senktide is shown in the bottom tracing. (C) The amplitude of inward current in response to various concentrations of senktide is shown. *n*=3–5 for each point. Fitting this data to the logistic equation yielded an EC<sub>50</sub> of 836 nM (pEC<sub>50</sub>=6.08±0.31). (D) EPSC amplitude histograms before and after senktide (500 nM) application to a single neuron are shown. EPSC amplitudes were measured during 5 min periods prior to (left panel labeled “Control”) and during application of 500 nM senktide (right panel labeled “Senktide”). Comparison of these plots clearly illustrates a dramatic increase in the frequency of EPSCs in the presence of senktide. (E) Cumulative frequency plots of EPSC amplitude recorded from a single neuron before and after senktide application. In the presence of senktide, larger-amplitude events make up a larger fraction of the EPSCs.
The NK3 receptor system has been shown to have a variety of psychopharmacological actions (Massi et al., 2000). Animal studies have shown NK3 involvement in anxiety, depression and reward systems. Human clinical
studies have been conducted with two NK3 receptor antagonists, osanetant and talnetant. The results have shown that treatment with an NK3 antagonist leads to improvements in the positive symptoms of schizophrenia (Spooren et al., 2005). The site and mechanism of this effect are not fully understood. Given the proposed role of prefrontal cortical areas in psychosis, our data suggest that at least some of the positive actions of NK3 antagonists in schizophrenia could be related to a block of NK3 receptors in the cortex.

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