Facilitation by Endogenous Tachykinins of the NMDA-Evoked Release of Acetylcholine after Acute and Chronic Suppression of Dopaminergic Transmission in the Matrix of the Rat Striatum

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Using a microsuperfusion method *in vitro*, the effects of the NK₁, NK₂, and NK₃ tachykinin receptor antagonists SR140333, SR48968, and SR142801, respectively, on the NMDA-evoked release of [³H]-acetylcholine were investigated after both acute and chronic suppression of dopamine transmission in striosomes and matrix of the rat striatum. NMDA (1 mM) alone or with D-serine (10 μM) in the presence of α-methyl-L-tyrosine (100 μM) markedly enhanced the release of [³H]-acetylcholine through a dopamine-independent inhibitory process. In both conditions, as well as after chronic 6-OHDA-induced denervation of striatal dopaminergic fibers, SR140333, SR48968, or SR142801 (0.1 μM each) reduced the NMDA-evoked release of [³H]-acetylcholine in the matrix but not in striosome-enriched areas. These responses were selectively abolished by coapplication with NMDA of the respective tachykinin agonists, septide, [Lys⁵, MeLeu⁹, Nle¹⁰]NKA(4–10), or senktide. Distinct mechanisms are involved in the effects of the tachykinin antagonists because the inhibitory response of SR140333 was addi-

tive with that of either SR48968 or SR142801. In addition, the SR140333-evoked response remained unchanged, whereas those of SR48968 and SR142801 were abolished in the presence of N^G-monomethyl-L-arginine (nitric oxide synthase inhibitor).

Therefore, in the matrix but not in striosomes, the acute or chronic suppression of dopamine transmission unmasked the facilitatory effects of endogenously released substance P, neurokinin A, and neurokinin B on the NMDA-evoked release of [³H]-acetylcholine. Whereas substance P and neurokinin A are colocalized in same efferent neurons, their responses involve distinct circuits because the substance P response seems to be mediated by NK₁ receptors located on cholinergic interneurons, while those of neurokinin A and neurokinin B are nitric oxide-dependent.

Key words: tachykinin receptor antagonists; NMDA; acetylcholine release; acute and chronic suppression of dopamine transmission; matrix compartment; striatum

The two main compartments of the striatum, the striosomes and the matrix, which can be distinguished by the origin of their afferent and efferent pathways, are connected to limbic and sensorimotor systems, respectively (Graybiel, 1990; Gerfen and Wilson, 1996). The striatal cholinergic interneurons, which are innervated by thalamic and cortical neurons, are tonically active (Aosaki et al., 1995). Their cell bodies are present in higher density in the matrix close to the striosomes, and their neurites innervate both compartments (Graybiel et al., 1986; Blanchet et al., 1997). Thus, these interneurons could contribute to the transfer of information from striosomes to the matrix in which their main targets are the striatal efferent neurons (Kemel et al., 1992; Bernard et al., 1993; Calabresi et al., 2000). Indicating further the role of these cholinergic interneurons in striatal functions, antimuscarinic agents were shown to ameliorate motor abnormalities in Parkinson's disease (Olanow and Koller, 1998) and to reduce neuroleptic-induced catalepsy (Anderson et al., 1995).

Substance P (SP), neurokinin (NK) A, and NKB, the endogenous ligands of NK₁, NK₂, and NK₃ tachykinin receptors, are

colocalized with GABA in striatal efferent GABAergic neurons. SP-containing terminals originating from recurrent collaterals of these neurons make synaptic contacts with cholinergic interneurons (Bolam and Bennett, 1995). In striosomes, the three tachykinins are present in neurons innervating the substantia nigra pars compacta. In the matrix, SP and NKA are located in neurons projecting to the substantia nigra pars reticulata and the entopeduncular nucleus, whereas NKB is found in neurons innervating the external globus pallidus (Besson et al., 1990; Gerfen and Wilson, 1996).

Exogenous agonists of NK₁, NK₂, or NK₃ tachykinin receptors stimulate the release of acetylcholine (ACh) from the rat striatum (Arenas et al., 1991; Petit et al., 1991; Guevara-Guzman et al., 1993; Steinberg et al., 1995). Endogenously released tachykinins facilitate the release of ACh through their action on NK₁ (Anderson et al., 1994) and NK₂ (Steinberg et al., 1998b) receptors. In these studies, endogenous tachykinins are released *in vivo* by stimulation of dopamine (DA) D₁ receptors with either exogenous agonists or neurotensin-induced endogenous release of DA, in the latter situation tachykinin regulations being observed only after the blockade of D₂ receptors (Steinberg et al., 1998b). We have also shown *in vitro* that endogenous SP and NKA are released under potent stimulation of NMDA receptors (Blanchet et al., 2000) and that both tachykinins indirectly inhibit the release of ACh through a DA-dependent process. However, in contrast, in the matrix, under the blockade of DA transmission, SP and

Received Aug. 23, 2001; revised Oct. 30, 2001; accepted Dec. 6, 2001.

This work was supported by Institut National de la Santé et de la Recherche Médicale, Collège de France, and a grant from Sanofi-Synthelabo-Recherche. We are grateful to A. Auclair for her valuable advice in preparing lesioned animals and HPLC estimation of dopamine levels.

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NKA were shown to facilitate the NMDA-evoked release of ACh (Blanchet et al., 1998).

To further clarify the role of the neuromodulators SP, NKA, and NKB on the NMDA-evoked release of ACh in the striatum, we have applied the tachykinin receptor antagonists SR140333 (NK₁), SR48968 (NK₂), and SR142801 (NK₃), *in vitro* in both compartments of the rat striatum. This was achieved after the acute blockade of DA transmission but also 1 month after the 6-hydroxydopamine (6-OHDA)-induced degeneration of the striatal DA innervation, a chronic situation generally used as an experimental model for Parkinson's disease.

MATERIALS AND METHODS

Experiments were performed on Sprague Dawley male rats (200–250 gm; Charles River, Iffa Credo, France) kept for at least 8 d in a controlled environment of light (8:00 A.M., 8:00 P.M.), temperature, and humidity. Animals were killed by decapitation during the light period.

6-hydroxydopamine injections into the fields of Forel. Thirty minutes before operation, animals received an intraperitoneal injection of desipramine (25 mg/kg) to protect the ascending noradrenergic pathways. Rats (150–175 gm) were lesioned under ketamine anesthesia (Imalgene R; Iffamerieux; 150 mg/kg, i.p.) using a David Kopf stereotaxic apparatus (incisor bar 3.4 mm above the interaural line). A microinjection cannula was implanted into the right fields of Forel at the following coordinates: 2.2 mm caudal to bregma, 1.6 mm lateral to the midline, and 8.4 mm under the surface of the skull. 6-OHDA was dissolved in saline containing 0.02% ascorbic acid and injected at a dose of 6 μ g in a volume of 1.5 μ l over 5 min.

One month after the lesion, release experiments were performed as described below, and the efficacy of the lesion was tested by the estimation of dopamine levels in the striatum. In each hemisphere, a sagittal slice (500 μ m) was cut in the central part of the striatum (between slices used for striosomes and matrix release experiments), and a microdisk of tissue (1.2 mm diameter; $\sim$ 43 μ g of protein) was dissected and stored at -20°C in 150 μ l of 0.1N perchloric acid containing 0.05% sodium metabisulfite. After homogenization and centrifugation (20,000 $\times$ g, 15 min), fractions of supernatants (8 μ l) were injected into an HPLC column (80 $\times$ 4.6 mm, 3 μ m particle size; HR-80; ESA Inc., Chelmsford, MA) equilibrated with a mobile phase. Mobile phase (NaH₂PO₄, 75 mM; EDTA, 20 mM; octane sulfonic acid, 2.75 mM; triethylamine, 0.7 mM; acetonitrile 6%; methanol 6%, pH 5.2) was delivered at 0.7 ml/min by an ESA-580 pump. Electrochemical detection was performed with an ESA coulometric detector (Coulchem II 5100A, with a 5014A analytical cell; Eurosep, Cergy, France). The conditioning and detecting electrodes were respectively set at -0.175 and $+0.175$ mV, allowing a good signal-to-noise ratio of the dopamine oxidization current. External standards were used to determine the sensitivity stability (0.3–0.5 pg of dopamine) (Darracq et al. 2001). Only those animals with striatal DA levels decreased by $>92\%$ were kept for further analysis.

Determination of striosome- and matrix-enriched areas on slices of the rat striatum. As previously described (Desban et al., 1993), striosome- and matrix-enriched areas (denominated striosomes and matrix for simplification) were delineated on sagittal brain sections after autoradiographic visualization of [³H]-naloxone binding to μ -opioid receptors, a specific marker of striosomes (Herkenham and Pert, 1981). [³H]-Naloxone binding exhibited a patchy distribution with highly labeled striosomes contrasting with weakly labeled matrix. A prominent striosomes territory was observed in the rostral pole of the striatum, and an extensive unlabeled matrix area was detected on most lateral sagittal sections. Lateral and medial sagittal slices were thus used to superfuse matrix ($4 < L < 5$ according to the atlas of Paxinos and Watson, 1986) and striosomes ($2 < L < 3$) areas, respectively.

Superfusion experimental device. The superfusion was performed as previously described (Krebs et al., 1991). Briefly, brains were rapidly removed and placed into a cool artificial CSF (ACSF). In each hemisphere, two sagittal slices (1.2–1.5 mm) were cut with a vibratome at the appropriate laterality, one for the striosomes ($2 < L < 3$), and the other for the matrix ($4 < L < 5$). Slices were then placed into a superfusion chamber containing ACSF maintained at 34°C , saturated with O₂ and CO₂ (95:5, v/v), and continuously renewed (750 μ l/min) thanks to a peristaltic pump. Microsuperfusion cannulas were vertically placed onto each selected area of the slices using micromanipulators and a dissecting microscope. These microsuperfusion devices consisted of a guide placed

at the surface of the tissue and two inner tubes, one penetrating slightly into the slice (200 μ m) to deliver the superfusion fluid, and the other situated 5 mm above the tissue to collect superfusates. An oxygenated ACSF was continuously delivered through each superfusion device using another peristaltic pump. This procedure allows the superfusion of a limited volume of tissue ($\sim$ 0.5 mm³) surrounding the inner tube of the microsuperfusion device. As previously discussed, the area superfused on medial slices corresponds to a striosome-enriched area slightly contaminated ($\sim$ 25%) by matrix tissue, whereas the area superfused on lateral slices corresponds only to matrix tissue (Krebs et al., 1991; Blanchet et al., 1997).

Estimation of [³H]-ACh release. The release of [³H]-ACh synthesized from [³H]-choline was estimated as previously described (Scatton and Lehmann, 1982; Blanchet et al., 1997). This procedure is based on the specific transport (through a high-affinity uptake system) of [³H]-choline into cholinergic interneurons and the synthesis of [³H]-ACh. Briefly, the labeling period consisted of a 20 min (30 μ l/min) delivery of the ACSF-enriched in [³H]-choline (81 Ci/mmol; 0.05 μ M; NEN, Boston, MA). Because the NMDA-evoked release of [³H]-ACh is only observed in the absence of magnesium, the tissue was then washed for 35 min using the magnesium-free ACSF (60 μ l/min) enriched in hemicholinium-3 (10 μ M), a specific inhibitor of the high-affinity choline uptake process. The release period (50 min) consisted of the constant delivery (60 μ l/min) of the superfusion medium used during the washing period. Receptor antagonists, DA or nitric oxide (NO) synthase inhibitors were added throughout the washing and the release periods while NMDA with or without D-serine and tachykinin agonists were applied for a 2 min period 35 min after the beginning of the superfusion (release period). Superfusates were collected in 5 min serial fractions.

Released [³H]-ACh is rapidly hydrolyzed and generates [³H]-choline, whose high-affinity transport into cholinergic interneurons is prevented by hemicholinium-3. [³H]-Choline was estimated in 200 μ l aliquots of 5 min superfusate fractions. At the end of the 50 min superfusion, superfused tissues (striosomes or matrix) were punched out from slices and dissolved in 200 μ l of HCl 0.1N 0.1% Triton for the estimation of total radioactivity. Because variations in the amount of incorporated [³H]-choline were observed from slices of different animals, the amount of [³H]-choline recovered in each successive superfusate fraction was expressed as a percentage of the calculated radioactivity present in the tissue during the time interval corresponding to the collected fraction [fractional release (FR)]. The spontaneous release of [³H]-ACh (FR) was estimated during the two fractions preceding the NMDA application and the NMDA-evoked release of [³H]-ACh in each successive fraction was then expressed as a percentage of the average spontaneous release of the labeled transmitter. The average incorporation of [³H]-choline was $\sim$ 1.5 times higher in matrix-enriched (2230 ± 180 Bq) than in striosome-enriched (1650 ± 20 Bq) areas, but no statistical difference was found for the spontaneous FR of [³H]-ACh release between the striosomes (2.8 ± 0.1) and the matrix (2.7 ± 0.1).

In each experiment, positions of superfused areas were checked and compared with the localization of striosomes as determined from autoradiographic data obtained after [³H]-naloxone binding on sections cut at the same laterality in distinct animals. Variations in these positions were of ± 250 μ m.

Pharmacological treatments. The artificial ACSF had the following composition (in mM): NaCl, 126.5; NaHCO₃, 27.5; KCl, 2.4; MgCl₂, 0.83; KH₂PO₄, 0.5; CaCl₂, 1.1; Na₂SO₄, 0.5; and glucose, 11.8. When added, (–)sulpiride, α -methyl-L-tyrosine (α -MPT), N^G-monomethyl-L-arginine (L-NMMA), the NK₁, NK₂, and NK₃ tachykinin receptor antagonists SR140333, SR48968, and SR142801, respectively, and their antipodes SR140603, SR48965, and SR142806, respectively, were applied at the onset of the washing period, up to the end of the superfusion. Finally, NMDA with or without D-serine was applied for 2 min, 70 min after the beginning of the washing period. When used, the NK₁, NK₂, and NK₃ tachykinin receptor agonists, septide, [Lys⁵,MeLeu⁹,NorLe¹⁰]NKA_{4–10}, and senktide, respectively, were applied 2 min with NMDA. NMDA, D-serine, hemicholinium-3, α -MPT, (–)sulpiride, and L-NMMA were obtained from Sigma (St. Louis, MO); SR140333, SR48968, SR142801, SR140603, SR48965, and SR142806 were kindly given by Sanofi Recherche. Silicon catheters of peristaltic pumps were often changed to avoid artifacts caused by an eventual adsorption of the drugs to these catheters.

Statistical analysis. Differences between treatments were evaluated with the two-tailed Student's *t* test. When multiple comparisons were made, results were analyzed using one-way ANOVA. Individual comparisons between treatments were evaluated with the multiple com-

Table 1. Effects of sulpiride and of α -methyl-*p*-tyrosine on the release of [3 H]-acetylcholine evoked by NMDA with or without D-serine in striatal compartments

	Striosome-enriched areas (%)	Matrix (%)
NMDA 1 mM ^a	345 $\pm$ 24	270 $\pm$ 10
+ Sulpiride 1 μ M	331 $\pm$ 37	277 $\pm$ 19
NMDA 1 mM/D-Ser	189 $\pm$ 6	183 $\pm$ 7
+ α MPT 100 μ M	353 $\pm$ 26*	268 $\pm$ 13*

Experiments were performed on sagittal slices of the rat brain, in either striosome-enriched areas or the matrix of the striatum, as described in Materials and Methods. NMDA (1 mM alone or with 10 μ M D-serine) was applied for 2 min, 70 min after the beginning of the washing period. When used, (–)sulpiride (1 μ M) or α -methyl-*p*-tyrosine (α MPT; 100 μ M) was added to the ACSF from the start of the washing period up to the end of the experiment. In each experiment, the NMDA-evoked release of [3 H]-ACh was estimated in 5 min fractions and expressed as a percentage of the mean spontaneous release determined in the two fractions collected before NMDA application. Results are the means $\pm$ SEM of data obtained in 15–21 experiments. * p < 0.05 when the effect of NMDA + D-Ser in the presence of α MPT was compared with the response induced by NMDA + D-Ser alone in each striatal compartment.

^aData from Blanchet et al. (2000).

parisons Tukey test or Dunnett's test. The level of significance was set at p < 0.05.

RESULTS

Reduction by SR140333, SR48968, or SR142801 of the NMDA-evoked release of [3 H]-ACh in the matrix under the acute suppression of the dopamine inhibitory regulation

We have previously shown in both striatal compartments that in absence of magnesium, the stimulation of NMDA receptors increases the release of DA (Krebs et al., 1991) and that endogenously released DA exerts a marked inhibitory effect on the release of ACh (Blanchet et al., 1997). As illustrated in Table 1, the release of [3 H]-ACh evoked by potent stimulation of NMDA receptors (2 min application of 1 mM NMDA and 10 μ M D-serine, in the absence of magnesium) was markedly enhanced in striosomes as well as in the matrix when DA synthesis was acutely inhibited by the coapplication of 100 μ M α -MPT. In contrast, this DA inhibitory regulation was not observed when NMDA receptors were stimulated with NMDA in the absence of D-serine, probably because of a low level of DA release. Hence, responses induced by NMDA alone were identical to those evoked by NMDA + D-serine in the presence of α -MPT. In addition, NMDA-evoked responses were not affected by sulpiride (1 μ M), an antagonist of D2 receptors.

Thus, to further explore the regulatory role of endogenous tachykinins on the NMDA-evoked release of [3 H]-ACh in absence of the DA inhibitory control, NMDA receptors were stimulated by either 1 mM NMDA (NMDA) or 1 mM NMDA + 10 μ M D-serine in the presence of 100 μ M α -MPT (NMDA + D-serine + α -MPT). In both situations, when used at 0.1 μ M but not at 10 nM, the selective NK1 (SR140333), NK2 (SR48968), and NK3 (SR142801) tachykinin receptor antagonists decreased the evoked release of [3 H]-ACh in the matrix but were without effect in striosomes. In the matrix, responses induced by each tachykinin antagonist were of similar amplitude whatever the paradigm used for the stimulation of NMDA receptors, either NMDA alone or NMDA + D-serine + α -MPT (Fig. 1). As a control, the inactive isomers of SR140333, SR48968, and SR142801

Figure 1. Effects of SR140333, SR48968, or SR142801 on the NMDA-evoked release of [3 H]-ACh in absence of the dopamine inhibitory control of cholinergic interneurons in striatal compartments. Superfusion experiments and expression of data were performed as described in Materials and Methods and in Table 1 legend. NMDA (1 mM alone or with 10 μ M D-serine) was applied for 2 min, 70 min after the beginning of the washing period. When used, α -MPT (100 μ M), SR140333, SR48968, or SR142801 (10 nM or 0.1 μ M for each) were added to the ACSF from the start of the washing period up to the end of the experiment. Results are the means $\pm$ SEM of data obtained in 8–25 experiments. * p < 0.05 when NMDA responses (1 mM alone or with D-serine and α -MPT) obtained in the presence of NK₁ (SR140333), NK₂ (SR48968), or NK₃ (SR142801) receptor antagonists were compared with the respective effects of NMDA treatments in the absence of the tachykinin antagonists in the matrix.

(SR140603, SR48965, and SR142806, respectively) were applied to the slice at the active concentration (0.1 μ M) and did not reduce the evoked release of [3 H]-ACh in the matrix (data not shown). Thus, the increase in ACh release brought about by NMDA stimulation is reduced by blockade of NK1, NK2, and NK3 tachykinin receptors when DA transmission is acutely disrupted in the matrix but not in the striosomes of the striatum.

Reduction by SR140333, SR48968, or SR142801 of the NMDA-evoked release of [3 H]-ACh in the matrix after chronic 6-OHDA-induced dopamine denervation of the striatum

Because 6-OHDA-induced degeneration of dopaminergic nigrostriatal neurons is generally used as an experimental model for Parkinson's disease, the involvement of endogenous tachykinins in the regulation of the evoked release of ACh was investigated in this model. The effects of SR140333 (NK1), SR48968 (NK2), and

Table 2. Effects of SR140333, SR48968, or SR142801 on the NMDA-evoked release of [³H]-ACh in matrix and striosomes after chronic 6-OH-DA-induced dopaminergic denervation of the striatum

	Matrix		Striosomes
	Unlesioned (%)	6-OHDA-lesioned (%)	6-OHDA-lesioned (%)
NMDA/D-serine	204 ± 11	279 ± 29	290 ± 7
+SR140333 0.1 μM	179 ± 13	180 ± 10*	297 ± 18
+SR48968 0.1 μM	279 ± 8*	201 ± 3*	281 ± 17
+SR142801 0.1 μM	267 ± 30*	171 ± 22*	281 ± 16

One month after the 6-OH-DA injection into the fields of Forel, superfusion experiments and expression of data were performed as described in Materials and Methods and in Table 1 legend. NMDA 1 mM + 10 μM D-serine was applied for 2 min, 70 min after the beginning of the washing period. When used, SR140333, SR48968, or SR142801 (0.1 μM for each) was added to the ACSF from the start of the washing period up to the end of the experiment. Results are the means ± SEM of data obtained in four to six experiments. **p* < 0.05 when NMDA responses obtained in the presence of NK₁ (SR140333), NK₂ (SR48968), or NK₃ (SR142801) receptor antagonists were compared with the respective effect of NMDA in the absence of the tachykinin antagonists in the matrix.

SR142801 (NK₃) tachykinin antagonists on the NMDA-evoked release of [³H]-ACh were examined in both striatal compartments after chronic 6-OH-DA-induced DA denervation of the striatum. In this chronic condition, as observed under the acute suppression of the striatal DA transmission, the NMDA (1 mM) + D-serine (10 μM)-evoked release of [³H]-ACh was markedly enhanced in both the matrix (+179 ± 2% vs +104 ± 11% of the spontaneous release in unlesioned matrix) and the striosomes (+190 ± 7% vs +89 ± 6% of the spontaneous release in unlesioned striosomes). In the unlesioned matrix, the NMDA + D-serine-evoked release of [³H]-ACh was enhanced in the presence of either SR48968 (0.1 μM) or SR142801 (0.1 μM) but not in the presence of SR140333 (0.1 μM). In contrast, in the DA-lesioned matrix, the NMDA + D-serine-evoked release of ACh was significantly reduced in the presence of each of the three antagonists. The three tachykinin antagonists were without effect in DA-lesioned striosomes (Table 2). Thus, as observed in the acute preparation, blockade of NK₁, NK₂, and NK₃ receptors lead to a reduction of ACh release resulting from NMDA stimulation in the matrix, but not the striosomes of the striatum from rats chronically deprived of DA.

Reversal by selective tachykinin agonists of the reduction of the NMDA-evoked release of [³H]-ACh induced by the NK₁, NK₂, and NK₃ tachykinin receptor antagonists in the matrix

To confirm that the responses induced in the matrix by the tachykinin receptor antagonists resulted indeed from the selective blockade of NK₁, NK₂, or NK₃ tachykinin receptors, attempts were made to counteract the effects of these antagonists by the coapplication with NMDA + D-serine of the respective selective receptor agonists; septide (NK₁), [Lys⁵,MeLeu⁹,NLe¹⁰] NKA(4–10) (NK₂), or senktide (NK₃). Although each agonist alone (0.1 μM each) was without effect on the NMDA + D-serine + α-MPT-evoked release of [³H]-ACh, septide, [Lys⁵,MeLeu⁹,NLe¹⁰] NKA(4–10), and senktide completely counteracted the reduction of the evoked release of [³H]-ACh induced by SR140333, SR48968, and SR142801, respectively (Fig. 2). In addition, each tachykinin receptor agonist was effective solely against the corresponding antagonist i.e., septide was without significant effect on

Figure 2. Selective suppression by NK₁, NK₂, and NK₃ tachykinin agonists of the reduction of the NMDA-evoked release of [³H]-ACh induced by corresponding tachykinin antagonists in the matrix. NMDA treatment was achieved under complete suppression of DA transmission with α-MPT in the matrix. NMDA (1 mM + 10 μM D-serine) alone or with septide, [Lys⁵, MeLeu⁹, NLe¹⁰] NKA(4–10) ([X]NKA(4–10)), or senktide was applied for 2 min, 70 min after the beginning of the washing period. α-MPT (100 μM) and, when used, SR140333, SR48968, or SR142801 were added to the ACSF from the start of the washing period up to the end of the experiment. Results are the means ± SEM of data obtained in 7–15 experiments. **p* < 0.05 when the combined effects of NMDA and septide, [Lys⁵, MeLeu⁹, NLe¹⁰]NKA(4–10), or senktide in the presence of SR140333, SR48968, or SR142801, respectively, were compared with respective control responses obtained in the absence of the tachykinin agonists.

responses evoked by the NK₂ (SR48968) and NK₃ (SR142801) antagonists, [Lys⁵,MeLeu⁹,NLe¹⁰]NKA(4–10) did not modify responses induced by the NK₁ (SR140333) and NK₃ (SR142801) antagonists and, finally, senktide was without effect on responses induced by either the NK₁ (SR140333) or the NK₂ (SR48968) antagonists (Fig. 2). These results demonstrate that the reductions by tachykinin receptor antagonists in ACh release evoked by NMDA stimulation are brought about by specific action of these antagonists at NK₁, NK₂, and NK₃ receptors.

Figure 3. Effects of the combined application of tachykinin antagonists on the NMDA-evoked release of [³H]-ACh in the matrix. NMDA treatment was achieved under complete suppression of DA transmission with α-MPT. NMDA (1 mM + 10 μM D-serine) was applied for 2 min, 70 min after the beginning of the washing period. α-MPT (100 μM) and, when used, SR140333, SR48968, or SR142801 alone or in combination were added to the ACSF from the start of the washing period up to the end of the experiment. Results are the means ± SEM of data obtained in 11–16 experiments. **p* < 0.05 when the effects of the NMDA treatment in the presence of combined application of SR140333 and either SR48968 or SR142801 were compared with the response of the NMDA treatment performed in the presence of SR140333 alone; □*p* < 0.05 when the response of the NMDA treatment in the presence of combined application of SR140333 and SR48968 was compared with the response of the NMDA treatment in the presence of SR48968 alone; §*p* < 0.05 when the response of the NMDA treatment in the presence of the combined application of SR140333 and SR142801 was compared with the response of NMDA treatment in the presence of SR142801 alone.

Effects of combined applications of tachykinin receptor antagonists on the dopamine-independent NMDA-evoked release of [³H]-ACh in the matrix

The excitatory effects of endogenously released SP, NKA, and NKB on ACh release could be either direct or indirect depending on the localization of NK₁, NK₂, and NK₃ receptors. Additional experiments were thus performed in the combined presence of tachykinin receptor antagonists (SR140333 with SR48968, SR140333 with SR142801, and SR48968 with SR142801; 0.1 μM each) to determine whether endogenously released tachykinins act through distinct (additive responses) or common (no additive responses) processes.

As shown in Figure 3, the reduction of the NMDA + D-serine + α-MPT-evoked release of [³H]-ACh was much greater in the combined presence of SR140333 and SR48968 than in the presence of either the NK₁ or the NK₂ antagonist alone. In contrast, the effects of SR48968 and SR142801 were not additive because

the reduction evoked by the coapplication of the two antagonists was identical to that induced by either the NK₂ or the NK₃ receptor antagonist alone. More complex effects were observed in the combined presence of SR140333 (NK₁) and SR142801 (NK₃) receptor antagonists. Indeed, in half of the experiments, the reduction of the NMDA-evoked release of [³H]-ACh induced by both antagonists was much more prominent than that observed with each antagonist alone while this was not the case in other experiments (Fig. 3). Altogether these results suggest that the facilitatory effect of endogenously released SP is mediated by a mechanism that is distinct from that evoked by either NKA or NKB, whereas a common process could be involved in the facilitatory response induced by NKA and NKB.

Effects of N^G-monomethyl-L-arginine on the tachykinin receptor antagonist-induced inhibition of the dopamine-independent release of [³H]-ACh evoked by NMDA in the matrix

It has been shown *in vitro* that NO enhances membrane excitability of cholinergic interneurons through a direct postsynaptic action and that this effect, which is not secondary to the release of endogenous neurotransmitters, persists in the presence of dopamine or SP receptor antagonists (Centonze et al., 2001). In addition, it has also been reported *in vivo* that NO is involved in the facilitation of ACh release induced by the stimulation of NK₂ tachykinin receptors (Steinberg et al., 1998b). Therefore, attempts were made to determine whether this is also the case in our *in vitro* conditions and whether differences or not can be observed under stimulation of NK₁, NK₂, or NK₃ receptors. L-NMMA, a potent inhibitor of NO synthase (NOS) activity was used for this purpose. Experiments performed with L-NMMA in the absence of DA transmission indicated that, in the matrix, at a concentration of 1 μM, L-NMMA reduced by 31% the NMDA + D-serine + α-MPT-evoked release of [³H]-ACh, whereas no effect was observed at 0.1 μM (L-NMMA 1 μM: +118 ± 5%; L-NMMA 0.1 μM: +183 ± 8% vs control: +171 ± 6% of the spontaneous release).

As shown in Figure 4, whatever the concentration of L-NMMA (0.1 μM or 1 μM), the NK₁ receptor antagonist (SR140333, 0.1 μM) still primarily inhibited the NMDA + D-serine + α-MPT-evoked release of [³H]-ACh. In contrast, in the presence of 1 μM L-NMMA, SR48968 (NK₂) and SR142801 (NK₃) no longer reduced the NMDA + D-serine + α-MPT-evoked release of [³H]-ACh. Moreover, the suppression of the inhibitory effects of these tachykinin antagonists was already either partial (SR48968) or total (SR142801) when L-NMMA was used at a smaller concentration (0.1 μM). These results suggest that the modulation of ACh release mediated by NK₂ and NK₃ receptors is dependent on NO.

DISCUSSION

The main findings of this study are that the marked increase of NMDA-evoked release of ACh observed after suppression of DA transmission is reduced by blockade of tachykinin receptors with specific antagonists in the matrix but not in the striosomes of the rat striatum. These data were obtained both in the acute absence of the DA inhibitory regulation [NMDA alone (NMDA) or with D-serine in the presence of α-MPT (NMDA + D-serine + α-MPT)] and after the chronic suppression of DA transmission (6-OHDA-induced degeneration of dopaminergic nigrostriatal neurons).

Endogenously released SP and NKA exert two opposite effects on the NMDA + D-serine-evoked release of ACh: a DA-dependent inhibition occurring in both striatal compartments,

Figure 4. Effects of N^G -monomethyl-L-arginine on the inhibition of the NMDA-evoked release of [3 H]-ACh induced by tachykinin receptor antagonists in the matrix. NMDA treatment was achieved under complete suppression of DA transmission with α -MPT. NMDA (1 mM + 10 μ M D-serine) was applied for 2 min, 70 min after the beginning of the washing period. α -MPT (100 μ M), N^G -monomethyl-L-arginine (L-NMMA), and when used, SR140333, SR48968, or SR142801 were added to the ACSF from the start of the washing period up to the end of the experiment. Results are the means $\pm$ SEM of data obtained in 13–26 experiments. * p < 0.05 when responses of the NMDA treatment in the presence of the combined application of L-NMMA (0.1 μ M) and either SR140333 or SR48968 were compared with the response of the NMDA treatment in the presence of L-NMMA (0.1 μ M) alone; ✕ p < 0.05 when the response of the NMDA treatment in the presence of the combined application of L-NMMA (1 μ M) and SR140333 was compared with the response induced by the NMDA treatment in the presence of L-NMMA (1 μ M) alone.

whereas a DA-independent stimulation is observed only in the matrix (Blanchet et al., 1998). The present results confirm and extend these findings by several observations. (1) The reduction of NMDA responses induced in the matrix by the NK_1 (SR140333) and NK_2 (SR48968) antagonists occurred under complete suppression of DA transmission (NMDA + D-serine + α -MPT) as well as with moderate release of DA (NMDA). (2) NKB is also involved in the regulation of the release of ACh in the matrix since the NK_3 tachykinin receptor antagonist (SR142801) reduced the evoked release of ACh under the two acute conditions of reduced DA transmission. (3) The reductions of the evoked release of ACh induced by SR140333, SR48968, and SR142801 were of similar amplitude in the presence or absence of D-serine, suggesting that the amount of tachykinins released under NMDA alone is already sufficient to maximally facilitate the release of ACh. (4) The effects of SR140333,

SR48968, and SR142801 were specific because the corresponding inactive isomers were devoid of activity, and the inhibitory response of each of these antagonists could be selectively reversed by the corresponding NK_1 , NK_2 , and NK_3 receptor agonists, septide, [Lys⁵,MeLeu⁹,NLe¹⁰] NKA(4–10), and senktide, respectively. (5) Finally, the inhibitory control of the NMDA-evoked release of ACh induced by SR140333, SR48968, or SR142801 observed under the acute suppression of DA transmission, was found after chronic dopaminergic denervation, further demonstrating the physiological relevance of this regulation.

In our conditions *in vitro*, as under low neostigmine *in vivo* (DeBoer and Abercrombie, 1996), a prevalent D_2 -mediated inhibition rather than an opposing D_1 -mediated stimulation of ACh release was found. Indeed, under potent stimulation of NMDA receptors, which markedly enhances the endogenous release of DA, the D_1 receptor-mediated stimulation of ACh release could only be observed in the matrix in the presence of a D_2 receptor antagonist (Blanchet et al., 1997). Similarly, inhibitory responses of tachykinin receptor antagonists, SR140333, SR48968, and SR142801 on the evoked release of ACh were only found under low dopaminergic transmission in the matrix but not in the striosomes. Moreover, the facilitation of ACh release induced by neurotensin, which involves the action of endogenously released DA on D_1 receptors, and which is suppressed by a NK_2 antagonist, was only observed after D_2 receptor blockade, i.e., in the absence of the DA inhibitory regulation (Steinberg et al., 1998b). However, the indirect facilitation of ACh release induced by the D_1 receptor agonist (SKF38393), which involves NK_1 (Anderson et al., 1994) and NK_2 (Steinberg et al., 1998b) tachykinin receptors, did not require the presence of a D_2 receptor inhibitor. This could result from the reduction of DA release triggers by local circuits occurring under the stimulation of D_1 receptors (Acquas and Di Chiara, 1999). Finally, when DA transmission is reduced or D_2 -mediated inhibition is blocked, endogenously released SP, NKA, and NKB facilitate the release of ACh.

The diffusible messenger arachidonic acid facilitates indirectly the NMDA-evoked release of ACh in the striatum (Blanchet et al., 1999). This is also the case for NO that is formed in NOS-somatostatin-containing interneurons under NMDA receptor stimulation (Marin et al., 1992). Indeed, the competitive NOS inhibitor, L-NMMA, which decreases the NMDA-evoked release of NO (Luo et al., 1993), reduced the NMDA-evoked release of ACh in the matrix. There is already evidence *in vitro* that NO depolarizes cholinergic interneurons through a direct postsynaptic action which is independent of SP regulation (Centonze et al., 2001). In addition, *in vivo*, NO is involved in the NK_2 but not the NK_1 receptor-mediated facilitation of ACh release because the NKA- but not the septide-evoked release of ACh was abolished after NO synthesis inhibition (Steinberg et al., 1998b). In support of these observations, our results obtained in the matrix in the absence of DA transmission, indicate that the reduction of the NMDA-evoked release of ACh evoked by the NK_2 antagonist, SR48968, is completely suppressed by L-NMMA while the reducing effect of the NK_1 antagonist, SR140333, can still be observed (Fig. 5). Confirming the involvement of distinct mechanisms in the facilitatory effects of endogenous SP and NKA, the coapplication of SR140333 and SR48968 reduced to a much larger extent the NMDA + D-serine + α -MPT-evoked release of ACh than the application of SR140333 or SR48968 alone. It cannot be excluded that SP and NKA, which share common precursors (Krause et al., 1987), are released, at least partly, from different nerve terminals because of differential processing of these precursors and address-

Figure 5. Schematic representation of the tachykinergic regulation of the NMDA-evoked release of ACh in the presence and absence of dopaminergic transmission in the striosomes and the matrix of the rat striatum. Cholinergic interneurons (ACh) whose cell bodies are mainly located in the matrix innervate both striatal compartments. *Top*, In the presence of dopaminergic transmission, endogenously released substance P (SP) and neurokinin A (NKA) in striosomes and NKA in the matrix indirectly inhibit the evoked release of ACh through a dopamine (DA)-dependent process. More precisely, endogenously released tachykinins facilitate the release of DA, which in turn inhibits the release of ACh by acting on D2 receptors located on cholinergic interneurons (Blanchet et al., 1998). *Bottom*, After acute or chronic suppression of dopaminergic transmission, endogenously released SP, NKA, and NKB facilitate the evoked release of ACh in the matrix but not in the striosomes. The SP response is nitric oxide (NO)-independent and mediated by NK₁ receptors located on cholinergic interneurons. NKA and NKB responses are NO-dependent and mediated through NK₂ and NK₃ receptors. The activation of these receptors facilitates the release of NO, which in turn directly stimulates the activity of cholinergic interneurons.

ing of these peptides. More probably, the additivity of the SP and NKA responses results from their respective action on NK₁ and NK₂ receptors distributed on distinct cells. Because NK₁ receptors are densely located on cholinergic interneurons (Gerfen, 1991; Kaneko et al., 1993; Jakab et al., 1996), and because SP induces potent depolarization of these interneurons (Aosaki and Kawaguchi, 1996) stimulation of these NK₁ receptors by endogenously released SP may greatly contribute to the DA-independent facilitation of the NMDA-evoked release of ACh (Fig. 5). However, the additional intervention of presynaptic NK₁ receptors present on afferent striatal fibers (Jakab and Goldman-Rakic, 1996) cannot be excluded. There is now direct evidence for the presence of NK₂ receptors in some brain structures (Steinberg et al., 1998a), but these receptors have not yet been identified in the striatum. Because of the main contribution of NO in the NKA response, the NK₂ receptors involved in this regulation could be located on NOS–somatostatin-containing interneurons.

The DA-independent facilitation of the NMDA-evoked release of ACh by endogenously released NKB is also mediated by NO. Indeed, as with the NK₂ antagonist, SR48968, the reduction of the NMDA response induced by the NK₃ antagonist, SR142801 disappeared under the L-NMMA treatment. In addition, the nonadditivity of the effects of SR48968 and SR142801 further indicates that NKA and NKB act through a common process (Fig. 5). Moreover, the identification of NK₃ receptor mRNAs in NOS–somatostatin-containing interneurons (Preston et al., 2000) provides complementary evidence for the involvement of NO in the NKB response. However, surprisingly, additive inhibitory responses of SR140333 and SR142801 were only observed in half

of the experiments. Because SP and NKB are localized on distinct efferent neurons, and because NKB-positive cells mainly located in the lateral part of the striatum are arranged in clusters (Marksteiner et al., 1992), these peptides could be partly released in different matrix areas. Although the additive responses likely occurred when the superfusion was precisely located in a matrix area possessing both SP- and NKB-containing nerve terminals, this could explain the nonadditive effects of SR140333 and SR142801 in half of the experiments and provide further evidence for the functional heterogeneity of the matrix (Malach and Graybiel, 1988; Kemel et al., 1992).

In conclusion, using the selective NK₁, NK₂, and NK₃ tachykinin receptor antagonists, SR140333, SR48968, and SR142801, respectively (Emonds-Alt et al., 1992, 1993, 1995), our study indicates that endogenous tachykinins, SP, NKA, and NKB are released from collaterals of striatal efferent neurons after stimulation of NMDA receptors. In the matrix, SP, NKA, and NKB facilitate the NMDA-evoked release of ACh and these effects only occur and/or are unmasked after either acute or chronic suppression of dopaminergic transmission. Although the SP-induced facilitation of ACh seems to be a direct process involving NK₁ receptors located on cholinergic interneurons, the NKA- and NKB-induced facilitations are mediated by NO. Therefore, in the matrix, tachykinins present in efferent GABAergic neurons from both the direct (NKA and SP) and indirect (NKB) pathways of the basal ganglia can contribute to the DA-independent facilitatory regulation of the evoked release of ACh.

Several modifications of neuropeptide levels have been observed in the striatum after degeneration of dopaminergic neurons (Agid and Javoy-Agid, 1985; Gerfen et al., 1990; Graybiel, 1990; De Ceballos and Lopez-Lozano, 1999). These changes in peptide synthesis and/or neurotransmission could be directly or indirectly responsible for some of the functional dysregulations associated with striatal dopamine deficiency and activation of the cholinergic interneurons. According to our findings, NK₁, NK₂, and NK₃ tachykinin receptor antagonists alone or combinations of the NK₁ antagonist with either the NK₂ or NK₃ antagonists that induce more potent reduction of cholinergic transmission could be appropriately used as indirect cholinergic antagonists in the treatment of Parkinson's disease. Such a therapeutic strategy could ameliorate the mental state of Parkinson's patients because there is evidence that these tachykinin receptor antagonists, NK₁ antagonists particularly, exert an antidepressant action (Rupniak and Kramer, 1999).

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