Tachykinin regulation of cholinergic transmission in the limbic/prefrontal territory of the rat dorsal striatum: implication of new neurokinine 1-sensitive receptor binding site and interaction with enkephalin/mu opioid receptor transmission.

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Abbreviation: α-methyl-p-tyrosine, αMPT; βfunaltrexamine, βFNA ; acetylcholine, ACh ; choline acetyl transferase, ChAT ; dopamine, DA ; enkephalin, ENK ; mu opioid receptor, MOR ; neurokinin, NK; NK_1 receptor, NK_1R ; prefrontal, PF ; substance P, SP,

ABSTRACT

The tachykinin neurokinin 1 receptors (NK_1Rs) regulation of acetylcholine release and its interaction with the enkephalin/mu opioid receptors (MORs) transmission was investigated in the limbic/prefrontal territory of the dorsal striatum. Using double immunohistochemistry, we first showed that in this territory, cholinergic interneurons contain tachykinin NK_1Rs and co-express MORs in the last part of the light period (afternoon). In slices of the striatal limbic/prefrontal territory, following suppression of the dopaminergic inhibitory control of acetylcholine release, application of the tachykinin NK_1R antagonist, SSR240600, markedly reduced the NMDA-induced acetylcholine release in the morning but not in the afternoon when the enkephalin/MOR regulation is operational. In the afternoon, the NK_1R antagonist response required the suppression of the enkephalin/MOR inhibitory control of acetylcholine release by βfunaltrexamine. The pharmacological profile of the tachykinin NK_1R regulation tested by application of the receptor agonists ([Pro^9]substance P, neurokinin A, neuropeptide K and substance P(6-11)) and antagonists (SSR240600, GR205171, GR82334 and RP67580) indicated that the subtype of tachykinin NK_1R implicated are the new NK_1-sensitive receptor binding site.

Therefore, in the limbic/prefrontal territory of the dorsal striatum, endogenous tachykinin facilitates acetylcholine release via a tachykinin NK_1R subtype. In the afternoon, the tachykinin/NK_1R and the enkephalin/MOR transmissions interact to control cholinergic transmission.

Key words: Tachykinin NK_1 receptor subtypes, Mu opioid receptor, acetylcholine, tachykinin, striatum

Running title: NK_1/MOR receptors interaction and ACh release

INTRODUCTION

The striatum is a component of multiple cortico-basal ganglia loop circuits that control movement as well as cognitive, motivational and emotional aspects of behavior. In the dorsal striatum, in addition to functional territories defined by their specific cortical afferents (Berendse *et al.* 1992; Deniau and Thierry 1997), two main compartments, the striosomes and the matrix, are distinguished (Graybiel 1990; Desban *et al.* 1993; Gerfen and Wilson 1996). The sensorimotor territory which mainly consists of matrix belongs to the sensorimotor cortico-basal ganglia circuits. The limbic/prefrontal (PF) territory enriched in striosomes belongs to the frontal cortico-basal ganglia circuits. Dysfunction in these latter circuits causes cognitive disorders such as observed in obsessive-compulsive disorder (Bradshaw and Sheppard 2000; Graybiel and Rauch 2000; Carlsson 2001), Parkinson's disease (Dubois and Pillon 1997) and cocaine users (Volkow *et al.* 2004).

Cholinergic interneurones are distributed throughout the striatum and their dense and widespread local axon collateral network is largely restricted to the matrix compartment where it primarily targets the striatal output neurones (Izzo and Bolam 1988; Kawaguchi 1992; Tepper and Bolam 2004; Wang *et al.* 2006). They are tonically active and involved in reward related procedural learning and working memory (Graybiel *et al.* 1994; Aosaki *et al.* 1994; Apicella 2002). Cholinergic interneurones are innervated not only by cortical and thalamic glutamatergic inputs but also by nigral dopaminergic neurones and peptide containing terminals originating from recurrent collaterals of striatal efferent neurones (Bolam and Bennett 1995). Indeed, the neuropeptides, opioids (enkephalins (ENK) and dynorphin) and tachykinins (substance P (SP), neurokinine (NK) A and NKB), are locally released from the recurrent collaterals of the output neurones and participate in the regulation of striatal cholinergic transmission (Lendvai *et al.* 1993; Anderson *et al.* 1994; Aosaki and Kawaguchi 1996; Blanchet *et al.* 1998; Steinberg *et al.* 1998; Kemel *et al.* 2002; Jabourian *et al.* 2004). In

the sensorimotor territory of the dorsal striatum where the regulation of cholinergic transmission by tachykinin has been mainly studied, stimulation of the tachykinin NK_1, NK_2 and NK_3 receptors by endogenously released tachykinins facilitates the NMDA-evoked release of ACh. Due to the potent dopamine (DA)/D2 inhibitory control of acetylcholine (ACh) release, these tachykinin regulations require suppression of dopaminergic transmission (Anderson *et al.* 1994; Steinberg *et al.* 1998; Kemel *et al.* 2002). Consistent with the presence of tachykinin NK_1 receptors (NK_1Rs) in cholinergic interneurons (Gerfen 1991; Kaneko *et al.* 1993), the tachykinin/NK_1 facilitation of ACh release is direct whereas the tachykinin/NK_2 and NK_3 regulations are indirect and require nitric oxide (Steinberg *et al.* 1998; Kemel *et al.* 2002). Three subtypes of tachykinin NK_1 binding sites are present in the brain; classic, septide-sensitive and new NK_1-sensitive (Beaujouan *et al.* 2000). In the striatal sensorimotor territory, the direct tachykinin/NK_1 control of ACh release is mediated by the new NK_1-sensitive subtype of NK_1R binding site (Kemel *et al.* 2003). Because only one type of NK_1R has been characterized by molecular means, this pharmacologically defined new tachykinin receptor binding site which could correspond to a distinct receptor molecule or a conformational state of the NK_1R (Holst *et al.* 2001) was termed new NK_1-sensitive receptor binding site.

In the limbic/PF territory of the dorsal striatum, no detailed analysis of the tachykinin regulation of cholinergic transmission similar to that performed in the sensorimotor territory has yet been done. Several observations however point to a territorial specificity in this regulation. Indeed, the limbic/PF territory is enriched in striosomes and a high density of SP fibers and receptors in soma and dendrites has been described in the rim of striosomes (Jakab et al. 1996). This arrangement contrasts with the striatal sensorimotor matrix where, as in the other brain structures, there is no consistent relationship between the amount of SP receptors and the density of SP fibers or cells bodies (Shults *et al.* 1984; Jakab *et al.* 1996; li *et al.*

2000). Moreover, as we recently showed, in the limbic/PF territory of the dorsal striatum, in addition to the DA/D2 inhibitory control of ACh release, ENK locally released by output neurons inhibits ACh release through mu opiod receptors (MORs) located on cholinergic interneurones. Interestingly, the ENK/MOR regulation of ACh release presents a diurnal variation, being only efficient in the last part of the light period (Jabourian *et al.* 2004; 2005). Thus, in the limbic/PF territory, the effectiveness of the direct tachykinin/NK_1 regulation of ACh release might be submitted to a diurnal variation due to its possible interaction with the ENK/MOR transmission.

Therefore, the present study was undertaken to: 1- determine whether, in the limbic/PF territory of rat dorsal striatum, cholinergic interneurones express NK_1Rs as in the sensorimotor territory and if these receptors are co-expressed with MORs 2- analyze the modulation exerted by tachykinins via NK_1Rs on the release of ACh and its interaction with the inhibitory ENK/MOR transmission 3- determine the subtype of receptors involved in the tachykinin NK_1R regulation. Released studies were done in situation of DA depletion required to observe the direct tachykinin regulation of cholinergic transmission.

MATERIALS AND METHODS

Experiments were performed on Sprague-Dawley male rats (225-250g, Charles River, France) treated in accordance with the Guide for Care and Use of Laboratory Animals established by the National Institute of Health and with the European Community Council Directive 86/609 EEC. All efforts were made to minimize animal suffering and only the number of animals necessary to produce reliable data was used. Animals were maintained on a 12-12h light/dark (lights on at 07:00 h) with free access to food and water. They were sacrificed by either decapitation (release experiments) or a lethal dose (120 $mg.Kg^{-1}$, i.p.) of pentobarbital

(Sanofi-Synthelabo, Libourne, France, immunohistochemistry) at 09:00-10:00 h (morning) or 15:00-16:00 h (afternoon).

Ligands and drugs: NMDA, D-serine, hemicholinium-3, βfunaltrexamine (βFNA) and α-methyl-p-tyrosine (αMPT) were obtained from Sigma Aldrich Chimie, L'Isle d'Abeau, France and [^{3}H]-choline from Perkin Elmer life science, Courtaboeuf, France. SSR240600 were kindly given by Sanofi-Synthelabo Recherche, RP67580 by Rhône Poulenc while GR205171, GR82334, [Lys^5,MeLeu^9,Nle^{10}]NKA(4-10) were obtained from Neosystem, Strasbourg, France. NKA, NPK, senktide [Pro^9]SP and SP(6-11), were obtained from Peninsula, Interchim, Montluçon, France.

Localization of the limbic/PF and sensorimotor territories in the rat dorsal striatum: As previously mentioned, the topographical arrangement of the corticostriatal projections defines functional territories in the dorsal striatum (Berendse *et al.* 1992; Deniau and Thierry 1997). The limbic/PF territory lies rostro-medially and the sensorimotor territory laterally. Sagittal slices from these two territories were performed at the following coordinates according to the atlas of Paxinos and Watson (1986): Lateral (L) 1,9 to 2,9 for the limbic/PF slices and L 4 to 6 for the sensorimotor slices.

Immunohistochemistry :

Under deep pentobarbital anesthesia, rats were perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. Brains were removed, post-fixed in the same fixative for 2 hours at 4°C, and cryoprotected in 30% sucrose. Using a freezing microtome, serial sagittal sections were made in the striatum (15 µm thick). Double immunofluorescent were performed: 1- choline acetyl transferase (ChAT)-SP/NK_1R: primary antibodies for ChAT (1:250, mouse monoclonal, Chemicon, Temecula, USA) and SP/NK_1R (1:1000, rabbit polyclonal, Chemicon, Temecula, USA) were visualized using secondary antibodies, TRITC (1:200, goat anti-mouse IgG, red, Southern Biotechnology, Birmingham,

USA)- and Alexa Fluor 488 (1:1000, goat anti-rabbit IgG, green, Molecular Probes, Eugene, USA)-conjugated respectively. 2- MOR-SP/NK_1R: Primary antibodies for MOR (1:500, guinea-pig polyclonal, Chemicon, Temecula, USA) and SP/NK_1R (1:1000, rabbit polyclonal, Chemicon, Temecula, USA) were visualized using secondary antibodies, TRITC (1:100, donkey anti-guinea pig IgG, red, Southern Biotechnology, Birmingham, USA)- and Alexa Fluor 488 (1:1000, goat anti-rabbit IgG, green, Molecular Probes, Eugene, USA)-conjugated respectively. Briefly, after incubating sections in a blocking solution (0.1 M PBS, 5% normal goat serum, 1% bovine serum albumin, Sigma Aldrich Chimie, L'Isle d'Abeau, France) for 2 hours at room temperature, sections were incubated 24 hours at room temperature for the primary antibodies and 2 hours at room temperature for the secondary antibodies. Controls in which primary or secondary antibodies were omitted or replaced with irrelevant antibodies resulted in no detectable staining. Sections were analyzed using a Nikon conventional epifluorescence microscope with Lucia software for neuronal counting. Cell counting was performed by two independent observers who had no information on the animals. Counts were done in the limbic/PF and in the sensorimotor territories of the dorsal striatum in sections taken every 60µm (7 to 9 sections per territory and per striatum); only those cells in which the nucleus was visible were taken into account.

Superfusion experimental device: The superfusion was performed as previously described (Kemel *et al.* 1989; Krebs *et al.* 1991). Briefly, brains were rapidly removed and chilled into a 4°C artificial cerebrospinal fluid (ACSF). In each hemisphere, sagittal slices (1.2-1.5 mm) were cut with a vibratome at the appropriate laterality as mentioned above. Slices were then placed into a superfusion chamber containing ACSF maintained at 34°C, saturated with O_2/CO_2 (95/5, v/v), and continuously renewed (750 µl/min). Microsuperfusion devices were vertically placed onto each selected area of the slices using micromanipulators and a dissecting microscope. Oxygenated ACSF was continuously delivered through each

superfusion device. This procedure allows the superfusion of a limited volume of tissue (~ 0.2 mm^3) surrounding the microsuperfusion device. As previously shown, in the limbic territory, the superfused area corresponds to mixed striosome and matrix tissues (~ 60-70 to 30-40% respectively). At the end of each experiment, precise localization of superfused areas was determined using a dissecting microscope and then compared to the localization of the striosomes (Desban *et al.* 1993).

Estimation of [^{3}H]-ACh release: The release of [^{3}H]-ACh synthesized from [^{3}H]-choline was estimated according to previously described procedure (Scatton and Lehmann 1982; Blanchet *et al.* 1997). This procedure is based on the specific transport (through a high affinity uptake system) of [^{3}H]-choline into cholinergic interneurones and [^{3}H]-ACh synthesis from its labeled precursor. Briefly, the labeling period consisted of a 20 min (30 µl/min) delivery of ACSF-enriched in [^{3}H]-choline (81 Ci/mmol, 0.05 µM; Perkin Elmer Life Science, Courtaboeuf, France). Since the NMDA-evoked release of [^{3}H]-ACh only occurs in the absence of magnesium, tissues were then washed for 55 min with a magnesium-free ACSF (60 µl/min) enriched in hemicholinium-3 (10 µM), a specific inhibitor of the high affinity choline uptake process. The release period (30 min) consisted of the constant delivery of the superfusion medium used during the washing period. Superfusates were collected in 5 min serial fractions. Released [^{3}H]-ACh is rapidly hydrolyzed and generates [^{3}H]-choline, whose high affinity transport into cholinergic interneurones is prevented by hemicholinium-3. [^{3}H]-Choline was estimated in 200 µl aliquots of 5 min superfusate fractions. At the end of the superfusion, superfused tissues punched out from slices were dissolved in 200 µl HCl 0.1 N, 0.1% Triton X-100 for the estimation of total radioactivity contained in tissues. [^{3}H]-Choline present in superfusate fractions and in tissues was measured using supermix scintillation fluid and a Perkin Elmer microbeta trilux counter (Perkin Elmer Life Sciences, Boston, MA, USA). The amount of [^{3}H]-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 [^{3}H]-ACh (FR) was estimated during the three fractions preceding the NMDA application and [^{3}H]-ACh released in each successive fraction was then expressed as a percentage of the average spontaneous release of the labeled transmitter.

Pharmacological treatments: The artificial ACSF had the following composition (in mM): NaCl, 126.5; $NaHCO_3$, 27.5; KCl, 2.4; $MgCl_2$, 0.83 ; KH_2PO_4, 0.5; $CaCl_2$, 1.1 ; Na_2SO_4, 0.5 ; glucose, 11.8. When added, αMPT, the tachykinin NK_1R antagonists, SSR240600, GR205171, GR82334 and RP67580 and the selective MOR antagonist, βFNA were applied at the onset of the washing period, up to the end of the superfusion. Finally, NMDA and D-serine were applied for 2 min, 70 min after the beginning of the washing period. When used, the tachykinin receptor agonists, Pro^9]SP, NKA, NPK, SP(6-11), [Lys^5,MeLeu^9,Nle^{10}]NKA(4-10) or senktide were applied 2 min with NMDA. Silicon catheters of peristaltic pumps were often changed to avoid artefacts due to an eventual adsorption of the drugs to these catheters.

Statistical analysis: Statistical analyses were performed using SigmaStat 2.0 SPSS, Chicago, IL, USA. Comparisons were made using a one way ANOVA, followed by the Tukey *post-hoc* test when appropriate. Significance level was set at $P < 0.05$.

RESULTS

Expression of tachykinin NK_1Rs and MOR in cholinergic interneurones of the limbic/PF territory of the dorsal striatum

We first examined if in the limbic/PF of the dorsal striatum as in the sensorimotor territory, cholinergic interneurones express the tachykinin NK_1Rs. In this goal, double immunofluorescent experiments were performed with specific anti-SP/NK_1R and anti-ChAT

antibodies. In the limbic/PF as in the sensorimotor territory, two populations of NK_1R-immunoreactive (-ir) neurones could be distinguished based on their ChAT- immunoreactivity (-IR): those also displaying ChAT-IR and those that did not display ChAT-IR (Fig. 1). In general, the cell body diameter of neurones simply labelled for NK_1R-IR was smaller than that of double labelled ChAT-/NK_1R-ir neurones (Fig. 1). Counting experiments (realized in 3 rats in the morning and 3 rats in the afternoon) indicated that during the whole part of the diurnal cycle, most of the ChAT-ir neurones also express NK_1R-IR. Thus, in the limbic/PF territory, in animals sacrificed in the morning or in the afternoon 97% of the ChAT-ir neurones (n=532 in morning and n=555 in the afternoon experiments) were NK_1R-IR. These double labelled neurones ChAT/NK_1-IR represented about half of the NK_1-ir neurones (50%, n=1070 in the morning; 52%, n=1062 in the afternoon). Similar data were found in the sensorimotor territory, 98% of the ChAT-ir neurones (n=559 in morning and n=549 in the afternoon experiments) were NK_1R-IR and these neurones represented about half of the NK_1-ir neurones (53%, n=1057 in the morning; 57%, n=962 in the afternoon).

We have recently shown that in the limbic/PF territory, in addition to the output neurones of striosomes, MORs are also expressed in 80% of cholinergic interneurones in the late part of the diurnal cycle. These interneurones were mainly observed in the matrix, some at the border of striosomes (Jabourian *et al.* 2005). Considering that most of cholinergic interneurones of this territory identified by their ChAT-IR expressed NK_1R-IR, it was likely that most of these cells co-express NK_1R and MOR. This was confirmed by double immunofluorescent experiments with specific anti-SP/NK_1R and anti-MOR antibodies. Because we previously shown that the MOR positive neurons found in the matrix of the limbic/PF territory are the cholinergic interneurones (Jabourian *et al.* 2005), in the present study it was assumed that the MOR positive neurones corresponded to cholinergic interneurones. All these MOR-ir neurones found in the matrix co-expressed NK_1R-IR (Fig. 1).

The direct control of ACh release by NK1R in the limbic/PF territory shows a diurnal variation

The effect of the NK_1R antagonist, SSR240600, on the NMDA-evoked release of [^{3}H]-ACh was analyzed in morning and afternoon experiments. The tachykinin antagonist (0.1 to 100 nM) was applied in the presence of αMPT (100 µM), 70 min before the 2 min application of 1mM NMDA+10 µM D-serine (NMDA). In this condition, SSR240600 reduced the NMDA-evoked release of [^{3}H]-ACh in the morning but not in the afternoon. Indeed, in the morning, SSR240600 dose dependently (0.1 to 10 nM) decreased the NMDA-evoked release of [^{3}H]-ACh (the amplitude of the SSR240600 effects being similar at 10 nM and 100nM). In contrast, in the afternoon, whatever the concentration used, the SSR240600 failed to modify the NMDA-evoked release of [^{3}H]-ACh (Fig. 2).

The diurnal variation in the effect of NK_1 antagonist results from interaction with ENK/MOR transmission

We have recently shown that in the limbic/PF territory of the dorsal striatum, endogenous ENK inhibits directly the NMDA-evoked release of [^{3}H]-ACh via MORs expressed by cholinergic interneurones. Since this regulation is operational only in the afternoon (Jabourian *et al.* 2004, 2005) the failure of NK_1R antagonist to reduce ACh release in the afternoon could result from interaction of NK_1R regulation with the inhibitory ENK/MOR regulation of ACh release. This interaction was tested by analyzing the direct tachykinin/NK_1R regulation of ACh release in the afternoon, after blockade of both the DA and the ENK/MOR inhibitory control of ACh release. The ENK/MOR regulation was suppressed using the MOR antagonist, βFNA. It has been proposed that βFNA could also act on kappa receptors (Zhu *et al.* 1997). However in the limbic/PF territory, the βFNA-induced modulation of ACh release is totally counteracted by the selective MOR agonist, DAMGO, indicating that these βFNA responses are totally MOR-dependent (Jabourian *et al.* 2004).

Confirming previous observation, βFNA (1 μM) markedly enhanced the NMDA-evoked release of [^{3}H]-ACh observed in the afternoon in the presence of αMPT. In the presence of both αMPT and βFNA, SSR240600 became able to reduce the NMDA-evoked release of [^{3}H]-ACh in the afternoon. This response was concentration-dependent from 0.1 to 10 nM SSR240600 (the amplitude of the responses being similar at 10 and 100 nM) (Fig. 2).

Specificity of the SSR240600-induced reduction of the NMDA-evoked release of ACh

The specific involvement of NK_1Rs in the effect of SSR240600 was tested by analysing the efficacy of potent and selective agonists of NK_1, NK_2 and NK_3 receptors ([Pro^9]SP, [Lys^5,MeLeu^9,Nle^{10}]NKA(4-10) and senktide, respectively) to counteract the inhibitory effect of SSR240600 (10nM) on the NMDA-evoked release of [^{3}H]-ACh (obtained in the morning in the presence of αMPT). Tachykinins agonists were used at the concentration of 1 nM which alone were without effect on the NMDA-evoked release of [^{3}H]-ACh (Fig. 3). When applied 2 min with NMDA, [Pro^9]SP completely reversed the SSR240600-evoked response. In contrast, [Lys^5,MeLeu^9,Nle^{10}]NKA(4-10) and senktide did not modified the inhibitory effect of SSR240600 on the NMDA-evoked release of [^{3}H]-ACh (Fig. 3).

Characterisation of the subtype(s) of NK_1R involved in the SSR240600 reduction of the NMDA-evoked release of ACh: classic versus non classic receptors

On the basis of pharmacological criteria, three different subtypes of tachykinin NK_1 binding sites are demonstrated in the brain; the classic and two non classic, septide-sensitive and new NK_1-sensitive binding sites. While substance P exhibits a high affinity for the three subtypes, NKA, NPK and NPγ recognize with a high affinity only the two non classic septide-sensitive and new NK_1-sensitive sites (Beaujouan *et al.* 2000). Thus, NKA and NPK can be used to distinguish classic from non-classic NK_1Rs and in the present study these substances were used to identify the subtype of NK_1R involved in the SSR240600-evoked response.

Tachykinin agonists were applied 2 min with NMDA and used at concentrations which alone, were without significant effect on the NMDA-evoked release of [^{3}H]-ACh (Table 1, 2). While in the presence of αMPT the selective NK_2 tachykinin receptor antagonist, SR48968 (10 nM) did not modify the NMDA-evoked release of [^{3}H]-Ach, the effect of SSR240600 (10 nM) was completely counteracted by NKA and NPK at 0.1 nM (Table 2). In addition, the effect of NKA was concentration-dependent (Fig 4) and the 50% reversal of the SSR240600 response was observed at very low concentrations (0.01nM) of NKA (Table 3). The potent effect of NKA and NPK indicated that the NK_1R involved in the control of ACh release in the striatal limbic/PF territory was of the septide-sensitive or the new NK_1-sensitive type.

The new NK1-sensitive receptor binding sites are involved in the SSR240600 response

Identification of the non classic NK_1R, septide-sensitive and new NK_1-sensitive, involved in the SSR240600 response was performed using pharmacological criteria. While NKA has a high affinity for the two subtypes, the short C-terminal SP fragment, SP(6-11), has a high affinity for the septide-sensitive and a weak affinity for the new NK_1-sensitive binding sites. Thus, the ratio values of the IC_{50} of SP(6-11) and NKA are higher for the new NK_1-sensitive (21) than for the septide-sensitive binding sites (3) (Table 2). In addition, while the NK_1R antagonists, SSR240600 and GR205171 have a high affinity for the two subtypes, GR82334 and RP67580 have a high affinity for the septide-sensitive binding sites and a weak affinity for the new NK_1-sensitive binding sites (Beaujouan *et al.* 2000).

SP(6-11) applied 2 min with NMDA at concentrations which alone were without significant effect on the NMDA-evoked release of [^{3}H]-ACh (Table 1) counteracted in a concentration-dependent manner the inhibitory effect of SSR240600 on the NMDA-evoked release of [^{3}H]-ACh (Fig. 4). The concentration of SP(6-11) leading to a 50 % reversal of the SSR240600-evoked response was 1.1nM, about 110 times higher than that observed with NKA (Table 3). The ratio of the SP(6-11) and NKA concentrations leading to a 50 % reversal of SSR240600-

evoked response was closed to the ratio value obtained for the new NK_1-sensitive but not for the classic or the septide-sensitive sites in binding studies (Table 3).

An additional finding in favour of the implication of the new NK1-sensitive receptor binding site in the regulation of ACh release was obtained by comparing the effect of the four tachykinin NK_1R antagonists, SSR240600, GR205171, GR82334 and RP67580 (100 µM each), on the NMDA-evoked release of [^{3}H]-ACh. In the presence of αMPT in the morning and of αMPT and βFNA in the afternoon, GR205171 as SSR240600 similarly reduced the NMDA-evoked release of [^{3}H]-ACh whereas RP67580 and GR82334 were without effect (Fig. 5).

DISCUSSION

The present study shows that in the limbic/PF territory of the rat dorsal striatum, cholinergic interneurones contain tachykinin NK_1Rs and co-express MORs in the last part of the light period. In absence of dopaminergic transmission, endogenous tachykinin released under stimulation of glutamatergic NMDA receptors facilitates the release of ACh in the morning but not in the afternoon when the ENK/MOR regulation is operational. In the afternoon, the tachykinin regulation required the suppression of the ENK/MOR inhibitory control of ACh release. Among the three subtypes of tachykinin NK_1 binding sites present in the brain (Beaujouan *at al.* 2000), the pharmacological profile of the tachykinin response indicated that it is the new NK_1-sensitive receptor binding sites which are involved in the control of ACh release.

In the striatum, SP receptors are segregated into cholinergic and somatostatinergic interneurones (Kaneko *et al.* 1993). In agreement with these findings, the present data confirm that in the limbic/PF as in the sensorimotor territory of the dorsal striatum, almost all cholinergic interneurones contain NK_1Rs. These neurones correspond approximately to half

of the NK_1R-positive neurones. In addition, we have recently shown that in the limbic/PF but not in the sensorimotor territory of the dorsal striatum, cholinergic interneurones contain also MORs. Expression of these receptors follows diurnal variation since most (80%) of cholinergic interneurones contain functional MORs only in the afternoon (Jabourian *et al.* 2005). Contrasting with the diurnal variation in the expression of MORs, the percent of cholinergic interneurones expressing NK_1Rs is similar in the morning and the afternoon. Thus, in the limbic/PF territory of the dorsal striatum, cholinergic interneurones contain NK_1Rs in the morning and NK_1Rs and MORs in the afternoon.

NK_1R regulation of cholinergic transmission in the limbic/PF territory of the dorsal striatum, diurnal variation and interaction with ENK/MOR regulation

In line with the potent depolarization of striatal cholinergic interneurones by SP (Aosaki and Kawaguchi 1996) endogenously released tachykinins (Anderson *et al.* 1994, Blanchet *et al.* 1998; Steinberg *et al.* 1998, Kemel *et al.* 2002) enhance the release of ACh in the striatum. In fact, distinct tachykinin NK_1R-mediated regulations of the NMDA-evoked release of ACh have been demonstrated in striatal territories: 1- a prominent indirect DA-dependent inhibitory control obtained in the limbic/PF territory (Blanchet *et al.* 1998), 2- a prominent direct facilitation of ACh release only observed after suppression of DA transmission and operational in the sensorimotor territory (Kemel *et al.* 2002). The present data shows that this latter direct NK_1R regulation is also operational in the limbic/PF territory of the dorsal striatum but display a diurnal variation. Indeed, in the absence of the dopaminergic transmission, the selective tachykinin NK_1R antagonist, SSR240600 (Steinberg *et al.* 2002), markedly reduced the NMDA-evoked release of ACh in the morning but not in the afternoon. Interestingly, we recently described in the limbic/PF striatal territory, another and opposite diurnal regulation of cholinergic transmission exerted by the direct inhibitory ENK/MOR control of ACh release. Contrary to the NK_1R regulation, the ENK/MOR control is

operational in the afternoon but not in the morning (Jabourian *et al.* 2004). This ENK/MOR response is additive with the dopaminergic inhibitory control of ACh release and lead to a strong inhibitory regulation of ACh release. In the afternoon, after blockade of both ENK/MOR and dopaminergic transmissions, the NK_1R antagonist, SSR240600, markedly reduced the NMDA-evoked release of ACh. Thus, in the morning and the afternoon, the tachykinins via NK_1R are able to favour a prominent increase of ACh release. However, the tachykinin/NK_1R response depends on the ENK/MOR control of cholinergic transmission and thus on its diurnal variation. In various CNS structures, including the striatum, the extracellular concentration of ACh displays a circadian rhythm (Day *et al.* 1991; Westerink 1995; De Prado *et al.* 2003). In the limbic/PF territory of the dorsal striatum, the diurnal variation of cholinergic transmission is in relation with its ENK/MOR control (Jabourian *et al.* 2004). Indeed, taking into account the diurnal variation of various component of the ENK system in the striatum (activity of endopeptidase 22.19 (enzyme responsible for the conversion of small ENK-containing peptides into ENK), levels of pre-proENK mRNA, ENK tissue content, and spontaneous as well as evoked release of ENK) (Dauge *et al.* 1996; Ferro *et al.* 1992; Jabourian *et al.* 2005), it appears that the diurnal variation in the ENK/MOR regulation of cholinergic transmission results from synergistic changes in the evoked release of endogenous ENK and in the expression of MORs by cholinergic interneurones (Jabourian *et al.* 2005).

The functional interaction observed in the afternoon between the two main families of striatal peptides, tachykinin and opioid, in the regulation of ACh release might implicate NK_1Rs and MORs co-express in cholinergic interneurones or MORs present at a presynaptic level. Indeed, in striosomes, in addition to their somatodendritic localization, MORs are present at presynaptic level within axon terminals (Wang *et al.* 1996). Thus, MORs could inhibit the release of endogenous tachykinins from recurrent collaterals of efferent neurones. However,

either the expression of MORs in striosomes or the indirect DA-dependent inhibitory control of ACh release involving MORs of efferent neurones are not diurnal dependent (Jabourian *et al.* 2004; 2007). In contrast, the direct ENK/MOR transmission is operational to inhibit ACh release only in the afternoon (Jabourian *et al.* 2004; 2005). Altogether, these data strongly suggest that interaction between ENK/MORs and tachykinin/NK_1Rs transmissions involved MORs and NK_1Rs co-expressed by cholinergic interneurones.

NK_1R regulation of cholinergic transmission in the limbic/PF territory of the dorsal striatum involves new NK1-sensitive receptor binding sites

The inhibitory response of SSR240600 is specifically mediated by NK_1Rs because this effect was reversed by a selective agonist of tachykinin NK_1R, [Pro^9]SP, but not by a selective tachykinin agonists of either NK_2 or NK_3 receptors. In addition to the classic tachykinin NK_1 binding sites two other subtypes, septide-sensitive and new NK_1-sensitive, have also been demonstrated in the CNS (Beaujouan *et al.* 2000; 2004). In contrast to classic NK_1 sites, these NK_1 subsites were found to have a high affinity for NKA, NPK and NPγ. These two non-classic NK_1 subsites are distinguished by their pharmacological properties. Indeed, the tachykinin NK_1 agonist, SP(6-11), and antagonists, GR82334, RP67580 and CP96345, have a higher affinity for the septide-sensitive than for the new NK_1-sensitive binding sites (Beaujouan *et al.* 2000). In the limbic/PF territory of the dorsal striatum, [Pro^9]SP, NKA and NPK at low concentration (0.1nM) completely counteracted the SSR240600 response suggesting that the direct tachykinin facilitation of ACh release is mediated by the non-classic, tachykinin NK_1 septide-sensitive or new NK1-sensitive receptor binding sites. In addition, the much higher concentration of SP(6-11) (~1.1 nM) than of NKA (~0.01 nM) required to induce a 50% reversal of the SSR240600 effect clearly underlines the lower activity of SP(6-11) compared to NKA in this response. Moreover, the ratio of these SP(6-11) and NKA values was consistent with the IC_{50} ratio of SP(6-11) and NKA obtained for the

new NK_1-sensitive sites but far from those obtained for both classic and septide-sensitive sites. Further stressing the involvement of the new NK1-sensitive receptor binding site in the control of ACh release, solely the selective tachykinin NK_1R antagonists, SSR240600 and GR205171, markedly reduced the release of ACh while RP67580 and GR82334 even at a 100 nM did not modified the release of ACh. Altogether, these data strongly suggest that the facilitatory regulation by endogenous tachykinins of the NMDA-evoked release of ACh is mediated by tachykinin new NK1-sensitive receptor binding sites in the limbic/PF territory of the dorsal striatum. There is evidence for the presence of the NK_1R mRNA and protein in cholinergic interneurones (Gerfen 1991; Kaneko *et al.* 1993; Jakab and Goldman-Rakic 1996) but no direct molecular evidence for the existence of NK_1R subtypes is yet available. Because only one type of NK_1R has been characterized by molecular means, the new NK1-sensitive receptor binding site could correspond to a distinct receptor molecule or a conformer of the classic NK_1R as already proposed for the septide-sensitive receptor (Glowinski 1995; Maggi and Schwartz 1997; Sagan *et al.* 1999; Holst *et al.* 2001).

In conclusion, the present data and those previously obtained in the striatal sensorimotor territory (Kemel *et al.* 2003) clearly show that the tachykinin NK_1Rs present in cholinergic interneurones of the striatum are the tachykinin new NK1-sensitive receptor binding sites. Therefore, endogenously released SP, NKA and NPK are the ligands of these receptor binding sites. Interestingly, in the limbic/PF territory of the dorsal striatum, cholinergic interneurones co-express tachykinin new NK_1-sensitive receptor binding sites and MORs in the afternoon. Thus, tachykinins (SP, NKA and NPK) and opioid (ENK) peptides which are contained in distinct output neurones of the striatum interact to control the release of ACh.

As previously mentioned, the limbic/PF territory of the dorsal striatum belongs to the fronto-cortico-basal ganglia circuits. Dysfunctions in these circuits cause cognitive disorders

(Bradshaw and Sheppard 2000; Graybiel and Rauch 2000; Dubois and Pillon 1997; Volkow *et al.* 2004) which are associated with alterations in multiple neurotransmission systems as DA, ACh, serotonin and peptides (Gillman and Sandyk 1986; Mangold *et al.* 2000; Carlsson 2001; Nieoullon 2002; Lundberg *et al.* 2004). Because these striatal neurotransmissions systems participate in information processing in the fronto-basal ganglia circuits, alteration in the balance between these neurotransmissions might contribute to cognitive impairments observed in fronto-striatal disorders. A better understanding of the interactions between striatal neurotransmissions could provide new therapeutic strategies in these diseases. According to ours findings, tachykinin participate to the strong hypercholinergie resulting from the suppression of inhibitory controls of ACh release i.e. dopaminergic in the morning and both dopaminergic and ENK/MOR in the afternoon. Thus, by reducing this excess of ACh release, tachykinin antagonists having a high affinity for the new NK_1-sensitive receptor binding sites such as SSR240600 and GR205171 could be appropriately used as indirect anticholinergic compounds.

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Table 1: Absence of effect of tachykinin agonists on the NMDA-evoked release of [^{3}H]-ACh after suppression of DA transmission in the limbic/PF territory

NMDA/D-Ser, αMPT	334±8
+ NKA 1 pM	348±26
+ NKA10 pM	324±19
+ NKA 100 pM	333±19
+ NKA 1nM	327±19
+ SP(6-11) 0.1 nM	335±18
+ SP(6-11) 1 nM	351±15
+ SP(6-11) 10 nM	352±24

NMDA treatment was achieved under suppression of DA transmission with αMPT in morning experiments. NMDA (1 mM+10 µM D-serine) alone or with NKA (1 pM to 1 nM) or SP(6-11) (0.1 nM to 10 nM) was applied for 2 min, 70 min after the beginning of the washing period. αMPT (100µM) was added to the ACSF from the start of the washing period up to the end of the experiment. Results, expressed in percentage of spontaneous release of [^{3}H]-ACh, are the means ± S.E.M. of data obtained in 6 to 20 experiments.

Table 2: Counteracting effect of NKA and NPK on the effect of SSR240600 on the NMDA-evoked release of [^{3}H]-ACh in the limbic/PF territory

	NMDA/D-Ser αMPT	+ Neurokinin A	+ neuropeptide K
NMDA/D-Ser, αMPT	334±8 %	333±19 %	353±20 %
+ SSR240600	237±8 %	333±7 % *	343±14 % *
+ SR48968	367±32 %		

NMDA treatment was achieved under suppression of DA transmission with αMPT. NMDA (1 mM+10 µM D-serine) alone or with NKA or NPK (0.1 nM each) was applied for 2 min, 70 min after the beginning of the washing period. αMPT (100 µM) and when used SSR240600 or SR48968 (10 nM each) were added to the ACSF from the start of the washing period up to the end of the experiment. Results, expressed in percentage of spontaneous release of [^{3}H]-ACh, are the means ± S.E.M. of data obtained in 6 to 20 experiments. Comparison of the effect of NMDA+ NKA or NKP in the presence SSR240600 versus the effect of NMDA alone in the presence of SSR240600, one-way ANOVA: $F_{(5,78)}=15.51$, $P<0.001$; Tukey Test for multiple comparison *$P<0.05$

Table 3: Comparison of the affinity of NKA and SP(6-11) for the tachykinin NK_1 classic, septide-sensitive and new NK_1-sensitive binding sites with the concentration of NKA and SP(6-11) leading to 50% reversal of the response of SSR240600 in the sensorimotor and the limbic/PF territories of the striatum

	NKA (nM) (1)	SP(6-11) (nM) (2)	R=2/1
Affinity for NK_1 sites	*IC_{50}*	*IC_{50}*	
Classic	250	540	2
Septide-sensitive	2.2	5.7	3
New NK_1-sensitive	5.4	116	21
Activity	*50% reversal*	*50% reversal*	
Sensorimotor, SSR240600	0.13	6.8	52
Limbic/PF, SSR240600	0.01	1.1	110

The affinities of NKA and SP(6-11) (IC_{50}) for the tachykinin NK_1 classic, septide-sensitive and new NK_1-sensitive binding sites correspond to values already published, (Beaujouan et al., 2000). The concentration of NKA and SP(6-11) leading to 50% reversal of the SSR240600 response were from Kemel et al. 2003 in the sensorimotor and calculated from experiments shown in figure 4 in the limbic/PF territory of the dorsal striatum.

Figure 1: Tachykinin NK_1R-IR is present in cholinergic interneurones and in MOR-ir neurones of the limbic/PF territory of the dorsal striatum.

Sagittal sections were stained for ChAT-IR (red) and tachykinin NK_1R-IR (green) in the sensorimotor and in limbic/PF territories of the dorsal striatum and for MOR-IR (red) and tachykinin NK_1R-IR (green) in the limbic/PF territory. Digital images were obtained as described in materials and methods. In the limbic/PF as in the sensorimotor territory, NK_1R-IR (green) is found in ChAT-ir neurones (red), co-localization is shown in yellow in the overlay; some neurones being only NK_1R-IR. In the limbic/PF territory, NK_1R-IR is found in all MOR-ir neurones. Scale bar: 20 µm

Figure 2: Effect of SSR240600 on the NMDA-evoked release of [^{3}H]-ACh after suppression of DA and/or ENK/MOR transmissions in the limbic/PF territory of the dorsal striatum

Superfusion experiments and expression of data were performed as described in Materials and Methods. NMDA (1mM with 10µM D-serine) was applied for 2 min, 70 min after the beginning of the washing period. αMPT (100µM) and when used SSR240600 (0.1nM to 100nM) and βFNA (1µM) were 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 ± S.E.M. of data obtained in 6 to 20 experiments. Comparison of the effect of NMDA in the presence of combined application of αMPT and SSR240600 versus the effect of NMDA in the presence of αMPT alone in the morning, one-way ANOVA: $F_{(4,67)}=26.98$, $P<0.001$; Tukey Test for multiple comparison *$P<0.05$. Comparison of the effect of NMDA in the presence of combined application of αMPT, βFNA and SSR240600 versus the effect of

NMDA in the presence of αMPT and βFNA in the afternoon, one-way ANOVA: $F_{(4,39)}$=16.19, P<0.001; Tukey Test for multiple comparison *P<0.05.

Figure 3: Suppression by NK_1, but not by NK_2 and NK_3 tachykinin agonists of the SSR240600 reduction of the NMDA-evoked release of [^{3}H]-ACh

NMDA treatment was achieved in the presence of αMPT in morning experiments. NMDA (1mM+10μM D-serine) alone or with [Pro^9]SP, [Lys^5,MeLeu^9,Nle^{10}] NKA(4-10) ([X]NKA(4-10)) or senktide was applied for 2 min, 70 min after the beginning of the washing period. αMPT (100 μM) and when used SSR240600 were added to the ACSF from the start of the washing period up to the end of the experiment. Results are the means ± S.E.M. of data obtained in 6 to 20 experiments. Comparison of the effect of NMDA + [Pro^9]SP in the presence of SSR240600 versus the effect of NMDA in the presence of SSR240600 alone, one-way ANOVA: $F_{(7,95)}$=13.72, P<0.001; Tukey Test for multiple comparison *P<0.05.

Figure 4: Counteracting effects of NKA and SP(6-11) on the SSR240600 reduction of the NMDA-evoked release of [^{3}H]-ACh.

NMDA treatment was achieved in the presence of αMPT in the morning. NMDA (1mM+10μM D-serine) alone or with either NKA (1pM to 1nM) or SP(6-11) (0.1 to 10 nM) was applied for 2 min, 70 min after the beginning of the washing period. αMPT (100μM) and SSR240600 (10nM) were added to the ACSF from the start of the washing period up to the end of the experiment. Results correspond to the effect of NMDA alone or with either NKA or SP(6-11) minus the corresponding effect of NMDA alone or with either NKA or SP(6-11) in the presence of SSR240600. Data used for these calculations are the means ± S.E.M. (S.E.M. <8%) of results obtained in 6 to 15 experiments.

Figure 5: Effects of SSR240600, GR205171, RP67580 and GR82334 on the NMDA-evoked release of [^{3}H]-ACh after suppression of DA and/or ENK/MOR transmissions

NMDA treatment was achieved in the presence of αMPT and/or βFNA in the limbic/PF territory of the dorsal striatum. NMDA (1 mM with 10 µM D-serine) was applied for 2 min, 70 min after the beginning of the washing period. αMPT (100 µM) and when used βFNA (1 µM), SSR240600, GR205171, RP67580 and GR82334 (100 nM each) were added to the ACSF from the start of the washing period up to the end of the experiment. Results are the means ± S.E.M. of data obtained in 5 to 20 experiments. Comparison of the effect of NMDA in the presence of combined application of αMPT and SSR240600, GR205171, RP67580 or GR82334 versus the effect of NMDA in the presence of αMPT alone in the morning, one-way ANOVA: $F_{(4,63)}$=15.04, P<0.001; Tukey Test for multiple comparison *P<0.05. Comparison of the effect of NMDA in the presence of combined application of αMPT, βFNA and SSR240600, GR205171, RP67580 or GR82334 versus the effect of NMDA in the presence of αMPT and βFNA in the afternoon, one-way ANOVA: $F_{(4,40)}$=18.90, P<0.001; Tukey Test for multiple comparison *P<0.05.

Figure 2

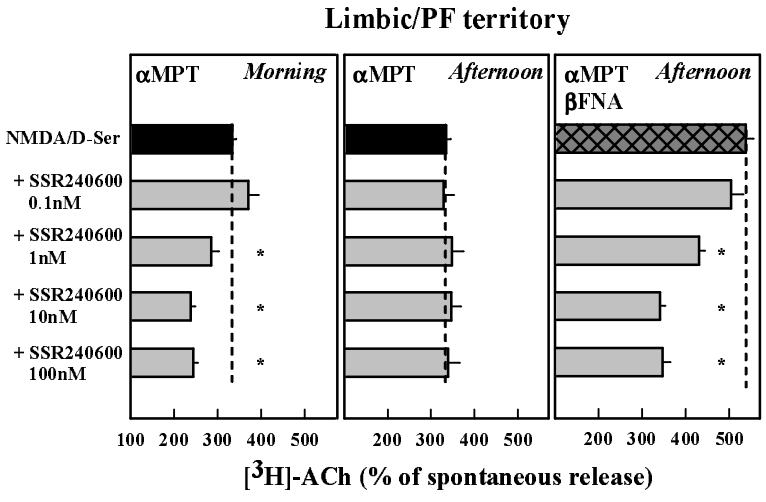

Figure 3

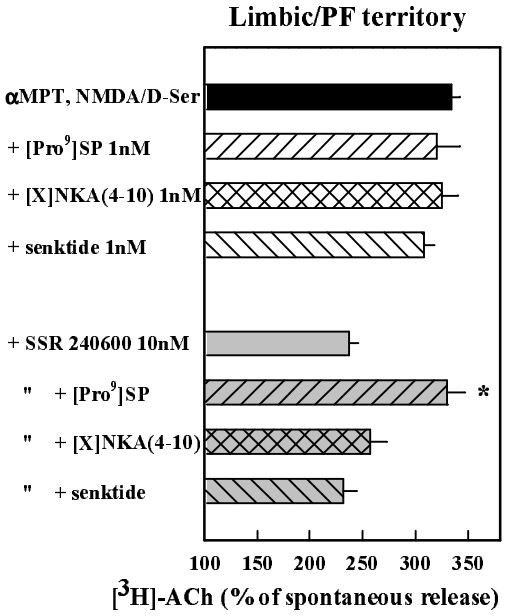

Figure 4

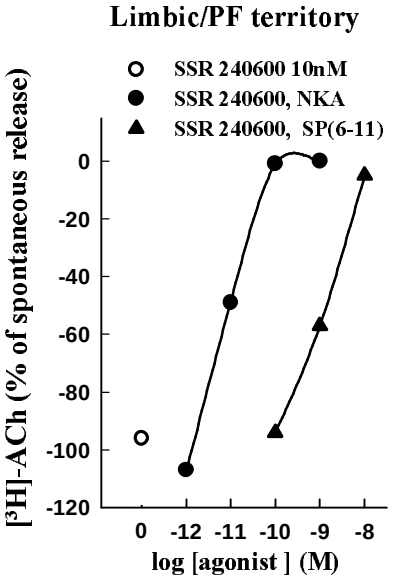

Figure 5