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Neurokinin-1 (NK₁) receptors in cholinergic neurons of the rat ventral pallidum have a predominantly dendritic distribution that is affected by apomorphine when combined with startle-evoking auditory stimulation

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Abstract

Cholinergic neurons of the basal forebrain are implicated in startle reflex inhibition by a prior weak stimulus often referred to as prepulse inhibition (PPI) and used as an index of sensorimotor gating deficits in schizophrenia. Gating deficits can be produced in rodent models by acute systemic administration of apomorphine, a non-selective dopamine D1 and D2 receptor agonist that also affects trafficking of neurokinin-1 (NK₁) receptors induced by startle evoking auditory stimulation (AS) in midbrain neurons. We used electron microscopic immunolabeling of NK₁ receptors and the vesicular acetylcholine transporter (VAChT) to test the hypothesis that the subcellular distributions of these receptors in cholinergic neurons of the rat ventral pallidum are subject to a similar regulation. In vehicle controls, NK₁ immunogold was often seen near cytoplasmic endomembranes in somata and large dendrites, but was more equally distributed in cytoplasmic and plasmalemmal compartments of medium dendrites, and principally located on the plasma membrane of small dendrites. These labeling patterns appeared to be largely independent of whether the NK₁ receptor was co-expressed with VAChT, however only the medium and small VAChT-labeled dendrites showed significant treatment-specific differences in NK₁ immunogold distributions. The NK₁ receptor immunogold particle density on the plasma membrane of medium cholinergic dendrites was significantly enhanced by combined apomorphine and AS, while neither alone affected either the plasmalemmal density or the equality of the plasmalemmal and cytoplasmic distributions of NK₁ receptors in these dendrites. Small cholinergic dendrites showed a significant AS-induced increase in both the plasmalemmal and cytoplasmic density of NK₁ gold particles, and an apomorphine-induced disruption of the preferential plasmalemmal targeting of the NK₁ receptors. These results provide ultrastructural evidence that NK₁ receptors in cholinergic neurons of the ventral pallidum have subcellular locations and plasticity conducive to active involvement in dopamine-dependent sensorimotor processing.

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Keywords

substance P; basal ganglia; limbic system; dopamine; acoustic startle; acetylcholine

Introduction

Cholinergic neurons of the basal forebrain innervate all cortical areas and participate in the gating of information processing and in attention mechanisms (Perry et al., 1999; Sarter and Bruno, 1999; Zaborszky et al., 1999). Specific lesions of basal forebrain cholinergic neurons alter sensory processing capacity (Stoehr et al., 1997; Turchi and Sarter, 1997; 2000) and lead to abnormal cognitive processes common to multiple neuropsychiatric disorders (Sarter and Bruno, 1999). These lesions also impair a physiological inhibitory mechanism of sensorimotor gating called prepulse inhibition (PPI; Ballmaier et al., 2001) in which a weak acoustic, visual or somatic stimulus is able to block the motor response to a closely following intense stimulus (Swerdlow and Koob, 1987). The PPI conditioning prevents over stimulation and thus favours attention and thought cohesion. Patients with schizophrenia (Swerdlow and Koob, 1987; Kumari et al., 2000; Braff et al., 2001) or several other neuropsychiatric disorders show attention deficits and reduced sensorimotor gating ascribed in large part to abnormalities within some portion of the cortico-striato-pallido-thalamic/pallido-tegmental brain circuitry (Kumari et al., 2003a,b).

The basal forebrain cholinergic neurons in the rat are scattered throughout diverse forebrain regions belonging to different functional systems in which the cholinergic neurons at least partially share the circuitry of anatomically linked brain regions (Alheid and Heimer, 1988; Butcher and Semba, 1989; Zaborszky et al., 1999). The ventral pallidum is one of the key components of the cortico-striatopallido-thalamic circuit that has been related to neuropsychiatric disorders (Swerdlow and Koob, 1987; Alheid and Heimer, 1988; Heimer, 2003). Cholinergic neurons located within the ventral pallidum are contacted by striatopallidal inputs (Grove et al., 1986; Zaborszky and Cullinan, 1992), many of which contain substance P, a neuropeptide that is prevalent in the neuropil surrounding the ventral pallidal neurons (Rieck et al., 1995). The cholinergic neurons within the ventral pallidum also express NK₁ receptors (Gergen, 1991; Chen et al., 2001) that have the greatest affinity for substance P (Mantyh, 2002). Thus, NK₁ receptors may be important mediators of substance P evoked excitation in the ventral pallidum (Napier et al., 1995), and specifically affecting the postsynaptic excitability of the cholinergic neurons in this brain region (Nakajima et al., 1991).

The ventral tegmental area (VTA) is a mesencephalic link within the ventral cortico-striato-pallido-tegmental circuit that has parallels with the ventral pallidum in receiving abundant SP-containing projections from the nucleus accumbens portion of the ventral striatum (Lu et al., 1998). The dopaminergic neurons of the VTA, in turn, send projections to the ventral pallidum and ventral striatum, where dopamine potently modulates cortical-subcortical re-entrant circuits serving many diverse functions (Kalivas and Nakamura, 1999). Tonic activation of NK₁ receptors within the VTA is thought to be necessary to maintain the spontaneous activity of dopaminergic neurons (Minabe et al., 1996). Moreover, we have recently shown that the surface expression of NK₁ receptors in VTA dopaminergic dendrites is subject to acute changes following startle-evoking acoustic stimulation (AS). This plasticity was opposed by systemic administration of apomorphine, a non-selective dopamine D1 and D2 receptor agonist (Lessard and Pickel, 2005). Acute systemic apomorphine also is known to significantly disrupt sensorimotor gating, as measured by PPI of the startle response evoked by AS (Mansbach et al., 1988; Davis et al., 1990; Geyer et al., 2001).

In contrast with the VTA dopaminergic neurons, there is no ultrastructural evidence that NK₁ receptors are available at functional plasmalemmal sites or actively modulated by AS or by dopamine in cholinergic neurons of the ventral pallidum. To address these questions, we examined the immunogold labeling for NK₁ receptors in cholinergic neurons identified by immunolocalization of the vesicular acetylcholine transporter (VAChT) in the ventral pallidum of rats receiving startle-evoking AS in presence or absence of apomorphine. The results provide the first evidence that VAChT is expressed in somatodendritic as well as axonal profiles within the ventral pallidum, where the cholinergic dendrites show a size-dependent plasticity of NK₁ receptors following AS that is distinct from that seen in the presence of apomorphine.

Experimental Procedures

Animals

Adult male Sprague-Dawley rats (280-400 g) were purchased 14 days prior to their experimental use (Taconic, Germantown, NY). The animals were housed in pairs in a colony room under a 12 hour light/dark cycle (lights on at 6:00 am and off by 6:00 pm), and maintained on food and water *ad libitum*. Each rat was used in only one experiment. All experiments were conducted in accordance with the NIH regulations of animal care and were approved by the Institutional Animal Care and Use Committee of Weill Medical College of Cornell University. All efforts were made to minimize animal suffering and to reduce the number of animals employed.

Apomorphine Administration and Experimental groups

Rats were injected subcutaneously with 1 mg/kg of apomorphine R-(-)-Apomorphine HCl (ICN Biomedicals Inc, Aurora, OH) prepared in a vehicle solution of isotonic saline containing 0.1 mg/ml of ascorbic acid. These were subdivided into four groups: (1) vehicle without AS (VEH), (2) vehicle and auditory stimulation (VEH+AS), (3) apomorphine alone (APO), and (4) apomorphine plus auditory stimulation (APO+AS).

Startle evaluation apparatus and prepulse inhibition (PPI) testing

All test sessions were performed in a one chamber SR-LAB startle apparatus with digitalized signal output (San Diego Instruments, San Diego, CA). This chamber and testing methods are briefly summarized and were previously described in full detail (Lessard and Pickel, 2005). Startle magnitude was defined as the peak response determined from an average of 250 readings recorded by a microcomputer and interface assembly (San Diego Instruments).

All rats were handled for 10 minutes daily starting two weeks before the AS experiments in order to reduce variation in behavioral results (Swerdlow et al., 2000). Seven days prior to testing, these rats were exposed to a brief startle-matching session. On the day of testing, initial startle-matched rats were placed in the startle chamber and exposed to 65 dB background noise for 5 minutes, followed by 11 PULSE trials of 40 ms 120 dB noise burst, with intervals averaging 15 seconds (range 8 to 23). The peak and average responses from each rat on each of eleven trials were determined. The initial response value was recorded separately, and the remaining ten responses for each rat were averaged together. Data from these sessions were then used to assign rats to two equal groups, sorting the startle reactivity values to give each of the two groups the same mean and variance. The rats were divided into pairs, one of which received apomorphine or saline 5 minutes before being introduced into the startle chamber for testing PPI to the AS. Four different trial types were defined: a PULSE ALONE trial containing only a 40 ms burst of 120 dB noise, three different PREPULSE plus PULSE trial types. The latter consisted of prepulse stimuli preceding the 120 dB pulse stimulus by 100 ms (onset to onset): 3, 6, and 12 dB above the 65 dB background noise with a duration of 20 ms. The difference between the PULSE ALONE and the PREPULSE plus PULSE trials was expressed

as *percent PPI* [$100 \times (\text{Mean Startle Amplitude on PULSE ALONE trials} - \text{Mean Startle Amplitude on PREPULSE plus PULSE trials}) / \text{Mean Startle Amplitude on PULSE ALONE trials}$]. The data showing apomorphine-induced enhancement of amplitude of the startle response and the attenuation of PPI for the animals used in these ultrastructural studies was previously described, along with evidence that the initial low intensity stimulus did not affect NK1 receptor trafficking in the rat midbrain (Lessard and Pickel, 2005).

Immunolabeling

The rats were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.) 60 minutes after the apomorphine or vehicle injection, and perfused through the aortic arch with (1) 5-10 ml of heparin-saline, (2) 50 ml of 3.75% acrolein in 2% paraformaldehyde and (3) 200 ml of 2% paraformaldehyde. The fixative solutions were prepared in 0.1M phosphate buffer (PB), pH 7.4. The brain slices were removed from the cranium, blocked into 2-4 mm coronal slices of tissue and placed in the 2% paraformaldehyde solution for 30 minutes at 4°C. The brains were immersed in PB and sectioned rostrocaudally at a thickness of 40 microns on a vibratome (Leica Microsystems, Bannockburn, IL). Sections from the basal forebrain containing the ventral pallidum at its largest mediolateral extension encompassing the subcommissural and sublenticular ventral pallidum (Paxinos and Watson, 1998) were selected for electron microscopy. Sections were then placed in a 1% sodium borohydride solution for 30 minutes to remove reactive aldehydes. To enhance penetration of the immunoreagents, the sections for electron microscopy were cryoprotected in 25% sucrose and 3.5% glycerol in PB, rapidly frozen in Freon followed by liquid nitrogen, and thawed in room temperature PB. Triton X-100 (0.3%) was used to further enhance penetration in the sections of tissue used for light microscopic immunolabeling.

All of the tissue was processed simultaneously from animals in each treatment group to minimize methodological differences. The prepared vibratome sections were incubated overnight at room temperature and 24 h at 4°C in a solution containing (1) rabbit polyclonal antiserum (Novus Biologicals Inc., Littleton, CO) against a synthetic peptide at the C-terminus of the cloned rat NK₁ receptor conjugated to bovine thyroglobulin and (2) a goat polyclonal antiserum against a C-terminal synthetic peptide sequence corresponding to amino acids 511-530 from the cloned rat VAChT (DiaSorin, Stillwater, MN). Each of these antisera are well characterized, and the NK₁ antiserum also showed a labeling pattern not-distinguishable from that seen with a high quality non-commercial antiserum (Garzón and Pickel, 2000; Pickel et al., 2000; Lessard and Pickel, 2005). The optimal dilutions of the primary antisera were 1: 500 for NK₁ immunogold and 1:1,600 for VAChT-immunoperoxidase labeling.

Immediately following the incubation in the primary antisera, the sections of tissue were rinsed and placed for 30 minutes in a 0.1% BSA solution containing biotinylated donkey anti-goat IgG (1:400) followed by 30 minutes in avidin-biotin complex (ABC, Vector Elite Kit, Vector Laboratories, Burlingame, CA) for the detection of the goat VAChT antiserum. The bound peroxidase was identified by reaction of the sections for 6 minutes in a 0.022% solution of 3,3'-diaminobenzidine (Aldrich Chemicals, Milwaukee, WI) in Tris-buffered saline (TS) and 0.01 % hydrogen peroxide. These sections were then rinsed in TS pH 7.6, and placed for 2 hours in a 1:50 dilution of donkey anti-rabbit IgG with bound 0.8 nm colloidal gold (Electron Microscopy Sciences, Fort Washington, PA) for detection of the rabbit NK-1 receptor antiserum. The gold particles were enlarged for microscopic examination by reaction for 7 minutes at room temperature in a silver solution from the IntenS-EM kit (Amersham).

Microscopic Analysis

All immunolabeled sections for light microscopy were mounted on glass slides, dehydrated and coverslipped, then examined on a Nikon microscope (Nikon Inc., Garden City, NY). For

electron microscopy, the sections were postfixated for 1 hour in 2% osmium tetroxide in PB, dehydrated and flat-embedded between two pieces of Aclar plastic. The areas comprising the subcommissural (Fig. 1A) or sublenticular ventral pallidum were cut (excised) from the sections using the anterior commissure and its posterior limb as anatomical landmarks (Paxinos and Watson, 1998). These excised tissues were then glued onto resin blocks and subsequently trimmed into small trapezoids. Ultrathin sections were cut from the outer surface of these trapezoids of tissue using a Leica ultratome (Leica Microsystems, Wein Austria). These sections were collected onto grids, counterstained with Reynold's lead citrate and uranyl acetate, and examined by using a Tecnai 12 Biotwin electron microscope (FEI, Hillsboro, Oregon). Images were obtained by a digital camera attached to the electron microscope (AMT Advantage HrR/HR-B CCD Camera System, Advanced Microscopy Techniques, Danvers, MA). These images were acquired from regions of the tissue exclusively at the resin-tissue interface, where there was optimal dual labeling for both VAChT and NK₁ receptors. Even one immunogold-silver deposit was considered to be evidence for NK₁ immunolabeling in sections in which an internal control (myelin) showed less than 2 particle/100 myelinated axons.

The subcellular distribution of the immunogold labeling of NK₁ receptors within VAChT-labeled dendritic profiles was quantitatively compared in the four experimental groups: (1) vehicle alone (VEH), (2) vehicle plus AS (VEH + AS), (3) apomorphine alone (APO) and (4) apomorphine plus AS. Three vibratome sections were analyzed from each of 12 animals (3 per experimental or treatment group). From these, a minimum of 50 dually labeled dendritic profiles were examined per sample, up to a total number of 1,977 profiles. Dendrites immunolabeled for the NK₁ receptor in each of the four treatment groups (n=224 from 4 animals, 1 per group) were also analyzed in order to compare the receptor distributions in cholinergic *versus* non-cholinergic dendrites. Block numbers were assigned to each section to prevent investigator bias during data gathering steps. Morphological measurements of single and dually labeled dendritic area, perimeter, average diameter and form factor were performed at a magnification of 18,500x using MCID software (MCID; Imaging Research, St. Catherines, Ontario, Canada). Dendrites with an oblong or irregular shape were discarded (form factor <0.500). Gold particles were counted and binned into either plasma membrane or cytoplasmic compartments, the criteria for plasma membrane being restricted to silver intensified gold particles that were touching the membrane, while all others were considered cytoplasmic. Furthermore, we quantitatively compared the immunogold particles identifying NK₁ receptors in dually labeled dendrites in each of the three sizes from all treatment groups measuring the (1) area density or number of gold particles/ dendritic area, (2) the plasmalemmal density or number of gold particles/ dendritic plasma membrane length or perimeter, and (3) the cytoplasmic density or number of gold particles in the cytoplasm not contacting the plasma membrane/ dendritic area.

Statistical Analysis

An analysis of variance (ANOVA) was used to determine whether there were any significant differences in dendritic size between treatment groups. Following this, a cluster analysis of the average diameters of dendrites was performed to statistically sort dendrites by size. These data were then analyzed by a two factor (group × dendritic size) ANOVA to determine the size-dependence of NK₁ receptor immunogold labeling density on the perimeter (number of particles/plasma membrane length in microns) and/or in the cytoplasm (number of particles / area in square microns) of labeled dendrites, as determined from the MCID analysis.

Other analyses were completed using the number of gold particles on the plasma membrane or distributed in the cytoplasm of individual dendrites, thus permitting a direct comparison of the plasmalemmal to cytoplasmic distributions without the confounds of having two different unit measures (microns and square microns). For this, a two factor (gold partitioning × dendritic

size) ANOVA was used for determining changes in labeling patterns dependent upon location within the dendrites. In addition, a two factor (gold partitioning $\times$ group) was used to examine changes in labeling patterns across experimental groups. Plasmalemmal to cytoplasmic partitioning was also examined using Students t-test for individual comparisons. Only probability values (p) less than 0.05 were considered to be statistically significant.

Illustrations

Adobe Photoshop software (Adobe Photoshop, v. 7.0.1., Adobe Systems Inc., Mountain View, CA) was used to enhance the sharpness and contrast of digital images obtained from both light and electron microscopes. The images were assembled into composite figures and lettering was added using PowerPoint (Microsoft Office 2003, Microsoft Corporation, Redmond, WA).

Results

Light microscopic immunoperoxidase labeling for VAChT was seen in dispersed multipolar neurons within the subcommissural ventral pallidum and adjacent structures, whereas a more aggregated cluster of VAChT-labeled neurons was evident in the horizontal diagonal band nucleus (Fig. 1A). Light microscopy also revealed the immunogold-silver labeling for NK₁ receptors within the VAChT-immunoreactive somata and in thick branched processes, presumed to be dendrites of the cholinergic neurons in the ventral pallidum (Fig. 1B,C). Numerous other somata and processes within the adjacent neuropil were immunolabeled exclusively for NK₁ receptors. Thick straight and varicose processes were the principal structures that showed single labeling for VAChT or, more rarely for NK₁ receptors (Fig. 1B,C). Electron microscopy confirmed the presence of immunolabeling for NK₁ receptors and/or VAChT in soma, dendrites and terminals within the ventral pallidum (boxed area in Fig. 1A). Furthermore quantitative analysis of NK₁ receptors immunogold labeling established an AS-induced, size-dependent and apomorphine-sensitive relocation of these receptors in cholinergic dendrites.

Somatic distributions of VAChT and NK₁ receptors

VAChT-immunoperoxidase labeling was most prevalent along endomembranes in the region of Golgi lamellae in somata within the ventral pallidum of rats receiving vehicle injections without AS (Fig. 2A). In VAChT-labeled somata, the NK₁ immunogold particles were also frequently seen near cytoplasmic endomembranes in the region of the Golgi lamellae and sometimes in contact with the cytoplasmic surface of the plasma membrane (Fig. 2A). The immunolabeled surfaces were contacted by glial processes, small axons or axon terminals in the ventral pallidum of rats in each of the four experimental groups: (1) VEH, (2) VEH + AS, (3) APO and (4) APO+AS. Because of the small number of single or dual labeled somata in the ventral pallidum, no between-group comparisons of the NK₁ immunogold distributions were conducted in the somatic profiles.

Dendritic distributions of VAChT and NK₁ receptors

Labeling for VAChT was lightly distributed throughout the cytoplasm and more intensely localized to tubulovesicular organelles in dendrites, some of which expressed NK₁ immunogold labeling. The dual labeled dendrites showed no apparent treatment-specific differences in their size or branching, as indicated by absence of significant differences in the average diameter of NK₁/VAChT-labeled dendrites across the four experimental groups ($F(3, 1338) = 2.12, p > 0.05$). Cluster analysis used the average diameter to statistically sort dendrites into three categories, large (3.13-1.65 microns, $n=223$), medium (1.64-1.03 microns, $n=795$), and small (1.02-0.12 micron, $n=959$), across experimental groups according to size. This analysis revealed significant size-dependent effects of AS on the subcellular location of the NK₁ immunogold within the cholinergic dendrites. In small dendrites, AS resulted in a

significant increase in cytoplasmic ($F(1,1341) = 7.10, p < .05$), plasmalemmal ($F(1,1341) = 3.60, p < .05$) and total ($F(1,1341) = 3.79, p < .05$) NK₁ immunogold labeling as compared to vehicle controls. As compared with the vehicle control group, apomorphine without AS did not significantly affect the subcellular locations of NK₁ receptors in dendrites with ($F(1,1996) = 1.27, p > .05$) or without ($F(1, 224) = 0.65, p > .05$) VAChT immunoreactivity, regardless of their size.

VAChT and NK1 labeling in large dendrites unaffected by AS and/or apomorphine

In large dendrites VAChT immunoreactivity was frequently distributed along membranous intracellular organelles, some of which were located beneath excitatory-type synapses (Fig. 2B). Other vesicular labeling for VAChT was associated with aggregated vesicles in cytoplasmic compartments of these dendrites (Fig. 2C). There were no apparent group differences in VAChT distribution in rats within the VEH (Fig. 2B), VEH + AS (Fig. 2C), APO (not shown) and APO + AS (Fig. 2D) treatment groups.

Many NK₁ immunogold particles in the VAChT-labeled large dendrites were located along endomembranes immediately beneath the surface, but not in contact with the plasma membrane (Fig. 2B-D). The NK₁ receptor labeling on and near the plasma membrane was most evident at sites contacted by unlabeled axon terminals (Fig. 2C), small axons and glia (Fig. 2C,D). There were no significant differences in the plasmalemmal, cytoplasmic, or total densities of NK₁ receptor immunogold particles in the VAChT-labeled large dendrites (Fig. 3A-C). There was no observed difference between the NK₁ immunogold labeling in these dendrites and that seen in large dendrites without VAChT immunoreactivity in which the receptor was mainly associated with cytoplasmic endomembranes beneath axon terminals, the majority of which formed symmetric synapses.

VAChT and NK₁ receptors in medium dendrites in which AS and apomorphine affect plasmalemmal receptor targeting

VAChT-immunoperoxidase labeling in medium dendrites was often faint and diffuse (Fig. 4A-C). In some cases, however, the peroxidase reaction product was more intensely distributed along segments of the plasma membrane (Fig. 4A and 4C) or along subsynaptic endomembranes (Fig. 4B). There was no consistent treatment-specific difference in the subcellular location of VAChT in these dendrites (Fig. 4 B-C).

Immunogold labeling for NK₁ receptors was seen in the medium-sized cholinergic dendrites (Fig. 4A-C). The immunogold labeling in these dendrites appeared to be almost equally prevalent on the plasma membrane and in association with cytoplasmic endomembranes in animals that received either vehicle (Fig. 4A) or apomorphine (not shown). Visual assessment of the distributions of NK₁ receptors in medium VAChT-labeled dendrites suggested more NK₁ receptor immunogold-particles were aligned on the plasma membrane in rats that received APO + AS (Fig. 4C), as compared with any of the other experimental groups. When statistically compared with VEH controls, the APO+AS group of rats showed a significant increase in the plasmalemmal ($F(3,795) = 3.92, p < .05$) and total ($F(3, 795) = 4.07, p < .05$), with no change in cytoplasmic ($F(3, 795) = 2.22, p > .05$) density of NK₁ receptor labeling in medium cholinergic dendrites (Fig. 3).

Medium dendrites without VAChT often showed more cytoplasmic than plasmalemmal gold particles (Fig. 5A), even though there were no quantitative differences in the cytoplasmic and plasmalemmal distributions of NK₁ receptor immunogold labeling in these dendrites. There also were no significant differences in plasmalemmal, cytoplasmic or total gold particle densities among the four experimental groups ($F(3,109)=1.83, p > 0.05$; $F(3,109)=0.33, p > 0.05$; and $F(3,109)=1.08, p > 0.05$, respectively). These dendrites were contacted by numerous

terminals including some that were separately labeled for either VAChT or NK₁ receptors (Fig. 5A).

Locations of VAChT and NK₁ receptor in small dendrites showing apomorphine-sensitive AS-induced changes in receptor distributions

VAChT-immunoperoxidase reaction product was prominently seen along the plasma membrane (Fig. 4D) and adjacent endomembranes beneath selective axonal contacts on small dendrites (Fig. 4E-F). The afferent terminals were almost all unlabeled and showed non-synaptic junctions or symmetric synapses. These terminals typically contained exclusively small clear vesicles or more rarely a mixture of many small clear and one or more large dense core vesicles (Fig. 4D-E). The affiliation between the VAChT-labeled endomembranes and selective synapses often appeared to be more evident in the VEH + AS group (Fig. 4E) compared with the VEH (Fig. 4D), APO, or APO + AS groups.

In small VAChT-labeled dendrites of vehicle-injected rats, the NK₁ receptor immunogold was sparse, but predominantly located on the plasma membrane (Fig. 4D). Occasionally, the gold particles were seen in the cytoplasm on or near endomembranes in the rats receiving vehicle injections without AS. Following AS, however, the endomembranes as well as plasma membranes of small dendrites appeared to be more frequently associated with NK₁ receptor immunogold, and in some cases the same endomembranes showed both immunogold receptor labeling and peroxidase reaction product for VAChT (Fig. 4E). The apparent increase in plasmalemmal labeling in the VEH + AS group was not evident in rats that received AS in the presence of apomorphine (APO+AS in Fig. 4F).

Quantification confirmed the visual impression that in vehicle-injected rats, AS resulted in a significant increase in plasma membrane ($F(3, 959) = 4.16, p < .05$), cytoplasmic ($F(3, 959) = 5.68, p < .05$), and total ($F(3, 959) = 5.99, p < .05$) density of NK₁ immunogold in small VAChT-labeled dendrites (Fig. 3A-C). In these dendrites, apomorphine alone had no significant effects on the NK₁ immunogold distribution. When combined with AS, however, apomorphine changed the predominantly plasmalemmal distribution seen with AS and vehicle to a predominantly cytoplasmic location of NK₁ immunogold in the small cholinergic dendrites (Fig. 3A). The APO + AS group showed no significant changes in plasmalemmal labeling from vehicle controls, but did show a significant increase above controls in the cytoplasmic (Fig 3B) and total (Fig. 3C) densities of NK₁ receptor immunogold labeling.

A separate quantitative analysis of the subcellular distribution of NK₁ receptor immunogold particles in VAChT-labeled small dendrites revealed that in vehicle-injected controls significantly more ($F(3,1920) = 8.02, p < .05$) gold particles were in contact (plasmalemmal) versus non-contact (cytoplasmic) with the plasma membrane (Fig. 4A). The preferential plasmalemmal to cytoplasmic targeting of NK₁ receptor was not affected by AS in rats receiving vehicle injections (Fig. 4C). After apomorphine and AS, however, there were no longer any significant differences between the numbers of NK₁ immunogold particles associated (or not) with the plasma membrane (Fig. 4C). This may largely reflect the observed increase in cytoplasmic NK₁ immunogold in the small cholinergic dendrites of this treatment group (Fig. 3).

Small dendrites without VAChT preferentially showed plasmalemmal distributions of NK₁ receptor immunogold (Fig. 5A). In contrast with the dual NK₁ receptor and VAChT labeled small dendrites, those without VAChT immunoreactivity showed no significant treatment-specific differences in plasmalemmal ($F(3,106)=1.65, p>0.05$), cytoplasmic ($F(3,106)=0.27, p>0.05$), or total ($F(3,106)=1.22, p>0.05$) NK₁ gold particle densities. These dendrites, as well as other unlabeled small dendrites (Fig. 5B) received input from unlabeled terminals and from terminals containing VAChT-immunoperoxidase reaction product and/or NK₁ immunogold

particles. Usually no more than two gold particles were seen in a single thin section through an axon terminal, but at least one of these particles was commonly located on or near the presynaptic membrane. This presynaptic location was seen both in terminals without VAChT that formed asymmetric synapses (Fig 5A), and in the VAChT-labeled terminals that apposed dendrites (Fig. 5B) or formed symmetric synapses with dendrites.

DISCUSSION

We have shown that in the rat ventral pallidum NK₁ receptors have well defined and highly mobile distributions in cholinergic dendrites, but are also present in non-cholinergic dendrites, some of which receive input from terminals that express NK₁ receptors and/or VAChT. Only the cholinergic dendrites show a significant size-dependent AS-induced change in the local availability of NK₁ receptors that is affected by apomorphine (see Fig. 6, for summary diagram). These observations, together with our localization of VAChT in vesicular endomembranes beneath synapses in dendrites and in axon terminals within the ventral pallidum suggest multiple acetylcholine release sites that are regulated, in part by activation of NK₁ receptors, whose local dendritic availabilities are affected by both sensory input and dopamine. The results have important implications for understanding the role of NK₁ receptors in alerting responses ascribed to activation of cholinergic neurons in the ventral pallidum.

Methodological Considerations

The NK₁ receptor and VAChT antisera used in this study are well characterized and previously used for light and electron microscopic immunolabeling with immunogold or immunoperoxidase labeling methods (Garzón and Pickel, 2000; Lessard and Pickel, 2005). The NK₁ receptor immunogold labeling was often seen as no more than one or two particles in single thin sections through small neuronal profiles, but these particles were usually located on plasma or endomembranes within these profiles. The membrane associations, together with the infrequency of spurious “background” labeling over myelin or other structures not expected to contain NK₁ receptors suggest that these particles are indeed representative of NK₁ immunogold labeling. Similarly, the VAChT-immunoperoxidase labeling was often seen as aggregated deposits over tubulovesicular structures in dendrites or vesicles in axon terminals consistent with identification of a vesicular transporter, and specifically VAChT as seen in cholinergic neurons of other brain regions (Garzón and Pickel, 2000). The low levels and disparate distributions of NK₁ and VAChT immunoreactivities may have resulted in an under estimation of the cholinergic dendrites that express NK₁ receptors as well as the prevalence of synaptic associations between terminals and dendrites that differentially express NK₁ receptors and VAChT. This limitation would be expected, however, to equally affect the detection of NK₁ receptors and VAChT in rats under each set of experimental condition used in our study.

Subcellular localization of VAChT and NK₁ receptors in large dendrites

The present localization of VAChT to tubulovesicular organelles in dendrites, and particularly those located beneath synaptic inputs in the ventral pallidum is consistent with the VAChT localization seen in other groups of cholinergic neurons (Garzón and Pickel, 2000). A similar location also exists for the vesicular monoamine transporter-2 (VMAT2) in dopaminergic dendrites of the substantia nigra, where there is known dendritic release of dopamine (Geffen et al., 1976; Cuello, 1982; Nirenberg et al., 1996). In contrast with dopamine, there is limited evidence for somatodendritic acetylcholine release in brain, although depolarization does elicit this type of acetylcholine release in parasympathetic ganglia (Johnson and Pilar, 1980). The tubulovesicular storage and release of acetylcholine from dendrites at synapses could act through presynaptic muscarinic cholinergic receptors to decrease the release of glutamate, gamma-aminobutyric acid (GABA), or other neurotransmitters (Marchi et al., 1989; Raiteri et al., 1990a; b). This suggests that cholinergic drugs targeting muscarinic receptors in the ventral

pallidum are potentially useful in treating certain forms of psychosis thought to largely involve brainstem groups of cholinergic neurons (Yeomans, 1995). It is also conceivable that the dendritic release of acetylcholine in the ventral pallidum could activate presynaptic nicotinic receptors resulting in increased release of these and other neurotransmitters including dopamine (Wonnacott et al., 1996; Dajas-Bailador et al., 2000; Biton et al., 2007).

The subcellular localization of NK₁ immunogold in most VAChT-labeled somata and large dendrites in the ventral pallidum confirms and extends an earlier light microscopic study (Chen et al., 2001) showing high levels of NK₁ receptor expression in cholinergic basal forebrain neurons. We also show that in larger cholinergic as well as non-cholinergic dendrites expressing NK₁ receptors, the receptor labeling is principally localized to endomembranes. These endomembranes may be involved in intracellular trafficking of the receptors to the plasma membrane (Grady et al., 1995; Quartara and Maggi, 1997).

AS and apomorphine effects on subcellular localization of NK₁ receptors in medium and small dendrites

In contrast to larger dendrites, the smaller dendrites showed more extensive NK₁ labeling on non-synaptic portions of the plasma membrane or beneath symmetric (inhibitory-type) or asymmetric (excitatory-type) synapses. This ultrastructural localization is in keeping with a recent description of NK₁ receptors in pallidal neurons from the monkey internal and external globus pallidus (Levesque et al., 2006). The presence of NK₁ receptors at postsynaptic sites near inhibitory terminals on dendrites of the ventral pallidum follows the reported prevalence of GABAergic terminals on dendrites in this region (Chang et al., 1995). The location is consistent with the known input to this region from striatal projection neurons that co-express GABA and substance P (Marksteiner et al., 1996; Mounir and Parent, 2002), and these terminals form symmetric, inhibitory-type synapses with both cholinergic and non-cholinergic neurons of the basal forebrain (Bolam et al., 1986).

The location of dendritic NK₁ receptors within and near asymmetric, excitatory-type synapses on small cholinergic or non-cholinergic dendrites is more unexpected, although a few substance P-containing terminals in the ventral pallidum do form asymmetric axodendritic synapses (Bolam et al., 1986). The VAChT-labeled terminals may arise not only from local neurons, but also from the mesopontine tegmentum (Semba et al., 1988), where a subset of cholinergic neurons contain substance P (Vincent et al., 1983; Vincent et al., 1986). Postsynaptic NK₁ receptors located at excitatory-type synapses in the cholinergic dendrites are strategically positioned to mediate substance P-induced postsynaptic excitation, which is comparable to glutamate, except for a slower onset and longer activation (Napier et al., 1995).

The similarities in NK₁ receptor distributions in dendrites of both cholinergic and non-cholinergic neurons in the ventral pallidum support the notion that cholinergic and non-cholinergic neurons in this region are exposed to common downstream and upstream information (Grove et al., 1986; Haber, 1986). Despite these similarities, however, only the cholinergic neurons showed significant AS-induced changes in NK₁ receptor distribution, suggesting that the inputs to cholinergic and non-cholinergic neurons substantially differ in the sensory-evoked release of tachykinins.

The AS and apomorphine induced changes in NK₁ receptor distribution in the cholinergic neurons of the ventral pallidum have both similarities and differences with that seen in non-dopaminergic, putative GABAergic neurons of the VTA (Lessard and Pickel, 2005). The observed significant increase above controls in the plasmalemmal density of NK₁ receptors in the cholinergic dendrites of the ventral pallidum after AS is comparable to that seen in the non-dopaminergic dendrites of the VTA. In the ventral pallidum, however, the AS-induced increase in plasmalemmal, as well as cytoplasmic and total NK₁ immunogold occurred exclusively in

the small cholinergic dendrites. This spatial restriction may reflect in part a preferential distal dendritic targeting of AS-activated tachykinin containing striatopallidal projection neurons (Grove et al., 1986; Zaborszky et al., 1986). Cholinergic neurons in the ventral pallidum are substantially less entwined by striatal axons than those in the globus pallidus (Fox et al., 1974; Haber and Nauta, 1983; Hazrati and Parent, 1992), and in fact, the largely GABAergic striatal inputs to the ventral pallidum seem to preferentially terminate on small diameter dendrites (Ingham et al., 1985).

Presynaptic distributions of NK₁ receptors and VAChT

The NK₁ receptor labeling was often associated with the pre-synaptic membrane of axon terminals, the majority of which were devoid of VAChT and formed asymmetric, excitatory-type synapses. This morphology is consistent with that of glutamatergic terminals in the ventral pallidum, which originate from diverse cortical and subcortical structures (Groenewegen et al., 1993). Cortical, thalamic and amygdalar afferents to the ventral pallidum, in fact, express NK₁ receptors (Mantyh et al., 1989; Nakaya et al., 1994; Yip and Chahl, 2001). The presynaptic location of NK₁ receptors is ideally suited for mediation of tachykinin-induced facilitation of glutamatergic transmission from these regions such as the amygdala (Mitrovic and Napier, 1998; Turner et al., 2001). In addition to glutamatergic projections, serotonergic axon terminals derived from the dorsal raphe nucleus (Gasbarri et al., 1999) could also account for asymmetric contacts, since some of the dorsal raphe serotonergic neurons also express NK₁ receptors (Lacoste et al., 2006).

Other terminals containing NK₁ receptor labeling were apposed to dendrites and/or formed symmetric junctions comparable to those of the cholinergic terminals, and indeed some of these terminals contained both NK₁ and VAChT immunoreactivities. This suggest that pre-synaptic NK₁ receptors play a role in tachykinin modulation of acetylcholine release in the ventral pallidum as has been shown previously in the hippocampal formation, where the released acetylcholine is actively involved in memory promotion (Feuerstein et al., 1996). Other inhibitory-type terminals expressing NK₁ receptors in the ventral pallidum may be dopaminergic, since NK₁ receptors are present in midbrain dopaminergic neurons (Lessard and Pickel, 2005) that send collateral axons to the ventral pallidum along with their direct projections to the nucleus accumbens (Prensa and Parent, 2001). In the striatum, the activation of presynaptic NK₁ receptors can produce suppression of synaptic transmission by enhancing extracellular dopamine and adenosine, an effect resembling those of cocaine and suggesting that the tachykinins are endogenous psychogenic peptides (Kombian et al., 2003a;b).

Interpretation of the AS and apomorphine-induced plasticity in the ventral pallidum

The cellular mechanism accounting for the AS-induced increase in NK₁ receptor density on the plasma membrane as well as in the cytoplasm of small, but not medium cholinergic dendrites, which instead show an increased receptor density exclusively on their plasma membrane is not known. One possibility is that the excitation produced by AS induces the translation of NK₁ mRNA beneath inputs to small dendrites resulting in the generation of new NK₁ receptors as has been shown to rapidly occur in other experimental systems (see Martin and Zukin, 2006; Poon et al., 2006). This novel form of NK₁ receptor plasticity could occur alone, or together with concomitant changes in NK₁ receptor trafficking affected by the tachykinin release (Grady et al., 1995; Quartara and Maggi, 1997; Jones et al., 2006). Alternatively, AS may specifically affect the efficacy of NK₁ receptor plasmalemmal trafficking in smaller cholinergic dendrites.

The increased availability of NK₁ receptors within and near the postsynaptic membrane in medium and small cholinergic dendrites in the ventral pallidum of vehicle-injected rats subject to AS could markedly facilitate tachykinin activation of these cholinergic neurons. In cultured

cholinergic neurons of the basal forebrain, substance P produces postsynaptic depolarization by inhibiting an inward rectifier K⁺ channel mediated by protein kinase C (Stanfield et al., 1985; Nakajima et al., 1991; Takano et al., 1995). Furthermore, the local infusion of substance P in the cholinergic neurons of the basal forebrain elevates the extracellular levels of acetylcholine in the rat frontal cortex (Souza Silva et al., 2000). Therefore, an AS-evoked release of substance P and/or increased availability of plasmalemmal NK₁ receptors in distal cholinergic dendrites of the ventral pallidum may increase cortical acetylcholine release resulting in activation of neural circuits implicated in attention and processing of behaviorally relevant stimuli (Hasenohrl et al., 2000).

Apomorphine opposed the AS-induced enhancement of NK₁ receptors on the plasma membranes of small cholinergic dendrites, while concurrently increasing the cytoplasmic density and disrupting the preferential plasmalemmal compared with cytoplasmic targeting of NK₁ receptors in these dendrites. Radioimmunoassay studies in the basal forebrain show a dose-dependent decrease in extracellular levels of substance P after acute administration of apomorphine that is most effective at the low (1mg/kg) doses (Janicki et al., 1981). Thus, the acute systemic administration of a low dose of apomorphine in our study also may have resulted in a pronounced suppression of the AS-evoked release of substance P from striatopallidal terminals. The diminished release of substance P in animals receiving apomorphine could significantly affect the AS-induced NK₁ receptor plasmalemmal trafficking as well as the disruption of the plasmalemmal to cytoplasmic compartmentation of these receptors in small cholinergic dendrites. These actions may contribute to apomorphine-induced disruption of prepulse inhibition of AS-induced startle responses affecting sensorimotor gating in rodent models and in schizophrenic patients in which there are known deficits in D2 receptors that are most highly implicated in the gating abnormalities (Mansbach et al., 1988; Davis et al., 1990; Geyer et al., 2001; Kumari et al., 2006).

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List of Abbreviations

ABC, avidin-biotin complex
 ac, anterior commissure
 Ach-te, cholinergic terminal
 ANOVA, analysis of variance
 APO, apomorphine
 AS, auditory stimulation
 BNSTv, ventral bed nucleus of the stria terminalis.
 BSA, bovine serum albumin
 CYT, Cytoplasmic
 de, dendrite
 d-de, dual dendrite
 d-so, dual soma
 em, endomembrane
 f, filaments
 GABA, gamma-aminobutyric acid
 Go, Golgi
 HDB, horizontal diagonal band nucleus
 PB, phosphate buffer
 PM, plasmalemmal
 PPI, prepulse inhibition
 TS, Tris-buffered saline

T-ve, tubulovesicles
 U-de, unlabeled dendrite
 Ut, unlabeled terminal
 VEH, vehicle
 VAChT, vesicular acetylcholine transporter
 VP, ventral pallidum
 VTA, ventral tegmental area

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Fig. 1. Photomicrographs showing immunoperoxidase labeling for VAcT and immunogold-silver labeling for the NK₁ receptor in rat ventral pallidum. **A**, The region analyzed for electron microscopy is shown by the trapezoid in the subcommissural ventral pallidum (VP) just below the anterior commissure (ac) and above the large cholinergic cell group in the horizontal diagonal band nucleus (HDB). These cells and others within the ventral pallidum show brown peroxidase labeling for VAcT (arrows), which is also seen in punctate processes within the ventral bed nucleus of the stria terminalis (BNSTv). **B,C**, Higher magnification image of the dually labeled somata (d-so) and dendrites (d-de) from the region of the trapezoid shows both the brown immunoperoxidase VAcT and immunogold silver (black granules) NK₁ labeling.

Within the neuropil numerous varicose processes show either immunoperoxidase-labeling (brown) for VAChT (arrow heads) or immunogold-silver labeling (arrow) for NK₁ receptors. B is a single photomicrograph, and C is a photomontage of two images taken at different focal depths to enable better visualization of the elongated processes within the depths of the tissue. Scale bars = 500 microns in A, 50 microns in B and C.

Fig. 2.

Electron micrographs showing immunogold labeling for NK₁ receptors in VAcHT-immunoperoxidase labeled soma (A) and large dendrites (B-D) in the ventral pallidum of rats in VEH, VEH+AS or APO+AS experimental groups. **A**, Cytoplasmic (straight arrows) and plasmalemmal (white chevrons) NK₁ immunogold particles are seen in the cytoplasm outside the nucleus in a neuronal soma that also contains VAcHT-immunoperoxidase labeling. The VAcHT labeling is particularly notable near membranes of the Golgi (Go) lamellae. **B**, **C**, and **D**, NK₁ immunogold is localized within the cytoplasm (straight arrows) near endomembranes (em) or in contact with the plasma membrane (white chevrons) in large dendrites containing VAcHT peroxidase labeling. In **B**, the peroxidase reaction product (block arrow) is most evident near tubulovesicles (T-ve) in the dendritic cytoplasm beneath an asymmetric (curved arrow) excitatory-type synapse formed by an unlabeled terminal (Ut-1), while virtually absent in the region beneath a second unlabeled terminal (Ut-2). In **C**, the dually labeled dendrite is also contacted by unlabeled axon terminals (Ut-1 and Ut-2), while another small dendrite shows exclusively plasmalemmal (chevrons) NK₁ immunogold (NK₁-de) that is restricted to the plasma membrane in contact with unlabeled terminal-3 (Ut-3), but not -4 (Ut-4). In **D**, the NK₁-immunogold-labeled dendrite shows a more diffuse distribution of VAcHT immunoreactivity throughout the cytoplasm. This dendrite also shows endomembranes and one coated vesicle, the latter of which is located on portions of the membrane in contact with unlabeled small axons (a). Appositional contacts also are seen with unlabeled terminals (Ut-1 and Ut-2) and with glia (asterisk). There are no apparent treatment-specific differences in the subcellular localization of NK₁ receptors in these dendrites. Scale bar = 2 microns in **A**; 0.5 microns in **B-D**.

Fig. 3.

Bar graphs showing treatment-dependent differences in densities of NK₁ immunogold particles in VachT-labeled dendrites of rat ventral pallidum. Cluster analysis of NK₁ receptor labeling was used to divide these dendrites into size groups defined by cross-sectional diameter in microns as large (n=190, 1.55-2.9), medium (n=552, 0.99-1.54) and small (n=600, 0.12-0.98). Asterisks indicate significant size and treatment-specific differences in (A) plasmalemmal, (B) cytoplasmic, and (C) total (plasmalemmal and cytoplasmic) NK₁ gold particle density in the VachT-labeled dendrites. Results in the apomorphine (APO) group did not significantly differ from VEH (data not shown).

Fig. 4.

NK₁ receptor immunogold distribution in medium (A-C) and small (D-F) VAcHT-immunoperoxidase labeled dendrites in the ventral pallidum of rats in three treatment groups. The medium dendrites show an almost equal number of NK₁ immunogold particles on the plasma membrane (white chevrons) and within the cytoplasm (straight arrows) in the rats receiving vehicle either without (A) or with AS (B). In rats receiving AS and apomorphine, (APO+AS) in C, most NK₁ gold labeling is located near the plasma membrane (chevrons), and more rarely associated with cytoplasmic endomembranes (em) or multivesicular bodies (mvp). The peroxidase labeling for VAcHT (block arrows) in the medium dendrites is seen on or near the plasmalemmal surface in all treatment groups. Small dendrites, however, showed the greatest prevalence of NK₁ gold labeling on the plasma membrane (chevrons) in the VEH + AS group compared with VEH (D), while the APO+AS group (F) appears to have less plasmalemmal and more cytoplasmic (small arrow) immunogold labeling. Endomembranes (em) are also immunolabeled in the section of E and F, but not in D. Both medium and small dendrites are contacted by varying numbers of largely unlabeled axon terminals (Ut-1-3) and/or glia (*), some of which contain bundles of filaments (f). The faint VAcHT-immunoperoxidase labeling in C is best seen by comparison with the adjacent dendrite that contains exclusively NK₁ immunogold without immunoperoxidase. Scale bar = 0.5 throughout and shown only in F.

Fig. 5.

Pre- and post-synaptic immunogold labeling for the NK₁ receptor in the ventral pallidum of rats in the APO + AS treatment group. **A**, The NK₁-immunogold is seen on the presynaptic membrane (arrowhead) of an axon terminal (NK₁-te) in continuity with a pre-terminal axon also containing NK₁-immunogold (arrow) and endomembranes (em). This terminal forms an asymmetric synapse (curved arrow) with a dendrite (NK₁-de1) that also shows cytoplasmic immunogold labeling for the NK₁ receptor and endomembranes. This medium dendrite is also recipient to inputs from multiple unlabeled terminals (Ut1-7) and from one VAcHT-immunoperoxidase labeled terminal (Ach-te). The only other NK₁-immuno gold labeling is seen on the plasma membrane (chevron) and within the cytoplasm (small arrow) of a small dendrite (NK₁-de2). Immunoperoxidase VAcHT labeling is seen in a small axon (Ach-a). **B**, Immunoperoxidase labeling for VAcHT is co-localized with NK₁-immunogold in a dually labeled axon terminal (d-te) in contact with a small unlabeled dendrite (U-de). The immunogold particles within this terminal are located on the plasma membrane (arrowhead) and within the cytoplasm. Single peroxidase labeling for VAcHT is also seen in a nearby axon terminal (Ach-te) and a small dendrite (Ach-de). Myelinated axon = ma. Scale bars = 0.5 microns.

Fig. 6.

A schematic diagram showing the size- and treatment-dependent distribution of NK₁ receptor in dendrites of a cholinergic neuron in the ventral pallidum. The neuron is artificially divided by a dotted line so as to compare experimental groups. In small dendrites, the VEH+AS group had greater densities of plasmalemmal (black circles) and cytoplasmic (white circles) NK₁ receptor compared to the VEH group. In medium dendrites, the APO+AS group had a greater density of plasmalemmal NK₁ receptor than the VEH group. No differences between experimental groups were observed in the large dendrites.