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***In situ* Hybridization Histochemical and Immunohistochemical Evidence that Striatal Projection Neurons Co-containing Substance P and Enkephalin are Overrepresented in the Striosomal Compartment of Striatum in Rats**

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Abstract

In a prior study, we showed that the few striatal projection neurons that contain both substance P (SP) and enkephalin (ENK) in rats may preferentially project to the substantia nigra pars compacta. Since striatal neurons that project to the pars compacta are thought to preferentially reside in the striosomal compartment, we investigated if striatal neurons that contain both SP and ENK are preferentially localized to the patch compartment. We used *in situ* hybridization histochemistry to double-label sections for SP and ENK to identify SP/ENK co-containing neurons, and immunolabeling of adjacent sections for the mu opiate receptor (MOR) to define the striosomal compartment. We found that 32.3% of neurons containing both SP and ENK were localized to the striosomal compartment, which itself only made up 12.8% of the striatum. Our results further showed that the density of neurons co-containing SP and ENK was three-fold higher in striosomes than in the matrix compartment. These results are consistent with the notion that SP/ENK colocalizing neurons preferentially project to pars compacta, and these and our prior results additionally raise the possibility that neurons of this type in the striatal matrix may also project to the pars compacta.

Keywords

Striatum; Substance P; Enkephalin; Projection Neuron; Colocalization

Introduction

Models of mammalian basal ganglia organization have dichotomized striatal projection neurons into two neurochemically and functionally distinct types: neurons containing substance P (SP) that project to the internal pallidal segment (GPi), the substantia nigra pars reticulata (SNr), and/or the substantia nigra pars compacta (SNc), and neurons containing enkephalin (ENK) that project to the external pallidal segment (GPe) [1,4,6,8,11,13,16,23, 26]. The view that striatal projection neurons consist of two major neurochemically distinct types was based on immunohistochemical and ISHH (in situ hybridization histochemistry) double-label studies that reported low incidence of SP colocalization with ENK in individual

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striatal perikarya [2,3,5,14,18,19] and immunolabeling studies that reported low incidence of SP colocalization with ENK in fibers and terminals in striatal targets [2,16,21,24].

Some recent findings, however, raised the possibility that the dichotomy of striatal projection neurons into two types (an SP+ type and an ENK+ type) might be overly simplified, and that a third neurochemically distinct type that co-contains SP and ENK might be common [7,12,14,27,31,33,35]. In a recent study [36], we used single-cell RT-PCR (scRT-PCR), ISHH, and immunolabeling to address this issue, and found that SP/ENK colocalizing striatal neurons, represent a small fraction of striatal projection neurons in rats (4% in adults and 10% in juveniles). Our results suggested that these neurons may have the SNc as their main project target, based on the presence of SP/ENK colocalizing terminals in SNc but not other striatal target areas. Since striato-SNc neurons reportedly reside in striosomes, which comprise about 10–15% of striatal neurons [15,16,17], one would expect SP/ENK colocalizing neurons to be preponderantly found in the striosomes, if they in fact preferentially target SNc. In the present study, we used ISHH double-labeling for SP and ENK in combination with MOR immunolabeling of adjacent sections to define striosomes to examine this issue. We found that SP/ENK neurons are overrepresented in striosomes and may thus be a subset of striato-SNc neurons.

Materials and Methods

Four 4-month old rats were used for the present study. Note that we use primate terminology for the pallidal segments in rat, as per Paxinos and Watson [25], because of the accepted homology of the nonprimate globus pallidus to the primate GPe and of the nonprimate entopeduncular nucleus to the primate GPi. Double-label ISHH was performed on 20µm thick fresh frozen cryostat sections from the four Sprague-Dawley rats. The sections were collected onto precleaned Superfrost®/Plus microscope slides as they were sectioned, dried on a slide warmer and stored at –80°C until used for ISHH. To process the tissue for ISHH, the slides were removed from the –80°C, quickly thawed and dried by a hair dryer. After fixation with 2% paraformaldehyde in saline sodium citrate (2× SSC) for 5 minutes, the sections were acetylated with 0.25% acetic anhydride/0.1M triethanolamine hydrochloride (pH 8.0) for 10 minutes, dehydrated through a graded ethanol series, and air-dried. Digoxigenin-UTP labeled and ³⁵S-UTP labeled cRNA probes (i.e. riboprobes) for preproenkephalin (PPE) and preprotachykinin (PPT), respectively, were transcribed from plasmids with PPE cDNA or PPT cDNA inserts (935bp and 475bp in size, respectively), generously provided by W.S Young of NIMH [30]. For double-labeling, ³⁵S -UTP-labeled PPT riboprobe and digoxigenin-UTP-labeled PPE riboprobe were mixed and applied to individual section. In our prior study [36], we found this combination to be more effective than digoxigenin-PPT and ³⁵S-PPE. For the double-label hybridization, the sections were incubated with digoxigenin (DIG)-labeled and/or ³⁵S -labeled (1×10⁷ dpm/ml) probe(s) in hybridization buffer containing 50% formamide, 4× SSC, 1× Denhardt's solution, 200µg/ml denatured salmon sperm DNA, 250µg/ml yeast tRNA, 10% dextran sulfate, and 20mM DTT at 58°C overnight. After hybridization, the slices were washed at 55°C in 4× SSC, 50% formamide with 2× SSC, 2× SSC, treated with RNase A (20µg/ml) for 30 min at 37°C, and washed in 0.5× SSC at 55°C. Sections were then dehydrated through a graded ethanol series, and air-dried. Digoxigenin labeling was detected using anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (AP), as visualized with nitroblue tetrazolium (NBT) histochemistry (Roche, Indianapolis, IN). Autoradiographic (ARG) labeling was detected by coating slides with Ilford-K5D emulsion (Polysciences, Warrington, PA), keeping them in the dark for 28 days at 4°C, developing them in Kodak D-19 developer (Kodak, Rochester, NY), and fixing them in Kodak fixer. Detection of the radioactive signal was carried out first, followed by digoxigenin detection. Sections were coverslipped with a 1% gelatin-based aqueous solution when both labeling steps were complete.

The distribution and abundance of SP/ENK colocalizing neurons were determined by using Neurolucida-assisted reconstructions of labeled perikarya. For these reconstructions, the contours of the striatum for individual double-labeled sections were outlined at low power using a Neurolucida system (MicroBright Field Inc, Vermont) connected to a Nikon Optiphot-2 microscope with a motorized stage, and SP/ENK double-labeled neurons were subsequently plotted as they were encountered during a systematic examination of the striatum using a 20× objective. Neurons with clear DIG signal for ENK overlain with at least 3× above background ARG signal for SP were considered double-labeled, as in our prior study [35]. The reconstructions of striatum with double-labeled perikarya were viewed and printed using NeuroExplorer (MicroBright Field Inc). Canvas software was used to render one of these reconstructions as an illustration for the present paper.

Sections adjacent to those used for the ISHH were immunolabeled for MOR, to identify the striatal patch compartment. The sections used for MOR immunolabeling were air-dried, and fixed by 5 minute immersion in 4% paraformaldehyde, 5 minutes in Bouin's solution and 5 minutes in 80% ethanol. Following rinsing and drying, peroxidase-antiperoxidase single-labeling was carried out on-the-slide, using a commercially available guinea pig polyclonal anti-mu opiate receptor (MOR) antibody (1:1000, Neuromics, Minneapolis, MN), whose specificity has been established previously [9,34]. Our on-the-slide immunolabeling methods have also been described previously [9,10,28]. The distribution of striosomes in the adjacent section was mapped using an overhead projector at the same magnification as the print for each Neurolucida reconstruction. The Neurolucida mapping of SP/ENK neurons and the mapping of striosomes were then aligned for each pair of adjacent sections, and the abundance of SP/ENK cells in striosome and matrix thereby was determined for each ISHH mapping. Three-four section pairs were typically bilaterally mapped for each rat analyzed. The relative areal extent of striosome and matrix for each MOR-immunolabeled section was determined by digitizing the mapping with a scanner and using NIH Image to measure matrix and striatal area. The regional density of SP/ENK neurons in striosomes and matrix was thereby calculated. A student's t-test was used to assess the statistical significance of the difference between striosomes and matrix in the density of SP/ENK neurons, with a probability of $p < 0.05$ considered significant.

Results

We performed ISHH double-labeling for SP and ENK, using digoxigenin-labeled riboprobes for ENK mRNA and ^{35}S -labeled riboprobes for SP mRNA (Fig. 1). Our ISHH studies revealed a patchy distribution of the SP/ENK neurons in striatum, and clusters of SP/ENK neurons frequently overlapped striosomes (Fig. 1). We counted double-labeled neurons in striatal sections double-labeled by ISHH (DIG for ENK and ARG for SP). Comparison of the SP/ENK Neurolucida mappings to the adjacent MOR-immunolabeled sections confirmed that apparent SP/ENK clusters commonly coincided with striosomes (Figs. 1, 2). Quantification of SP/ENK neuron frequency in the two compartments revealed that 32.3% of the SP/ENK neurons were in the striosomal compartment, while striosomes made up only 12.8% of the areal extent of the striatum. The regional density of SP/ENK neurons was thus more than three-fold higher in striosomes than in matrix: 7.23 ± 1.45 neurons/ mm^2 in striosomes versus 2.02 ± 0.24 neurons/ mm^2 in matrix. This difference was significant ($p = 0.026$) by a student's t-test.

Discussion

Our results show that while SP/ENK neurons are not an abundant striatal cell type in rats (10% in juveniles, 4% in adults), they appear to represent many among the striosomal neurons that target the substantia nigra pars compacta. Double-label ISHH is affected by several problems that potentially affect interpretation. First, combining DIG and ARG labeling diminishes the

DIG labeling. Secondly, with heavy silver grain ARG labeling over a cell, the underlying DIG labeling of the cell can be obscured, making it more difficult to determine if an ARG-labeled cell is also DIG-labeled. To a large extent, high-power viewing (which we employed) can overcome this difficulty, but reduced DIG labeling when combined with ARG could reduce the frequency of double-labeled cell detected. Notwithstanding these possible problems, the frequencies of SP/ENK co-localizing cells detected by double-label ISHH by us matches that which we have detected by single-cell RT-PCR [36]. Thus, we believe our SP/ENK neuron detection reliably reflects their abundance in rat striatum.

Previously, we observed SP/ENK colocalization in fibers and terminals in the pars compacta and the ventral tegmental area in young rats [35]. Since striato-SNc neurons reportedly reside in striosomes, which comprise about 10–15% of striatal neurons [15,16,17], one would therefore expect some or all SP/ENK striatal neurons to reside in striosomes. We did find in the present study that 32.3% of SP/ENK neurons reside in striosomes. Given their frequency in striatum overall and the magnitude of the striosomal compartment, this observation indicates that SP/ENK neurons make up about 10% of the striosomal neurons in adult rats, and thus presumably at least about 10% of striato-SNc neurons. The presence of SP/ENK neurons in the matrix, however, raises the possibility that some extrastriosomal neurons also project to the pars compacta and ventral tegmental area. The SP/ENK neurons projecting to SNc appear to be only one of several neurochemically distinct types projecting to SNc. Other types include neurons co-containing ENK and neurotensin [32], and neurons co-containing SP and dynorphin [2,29,36]. How these various types may differ in their functional properties is unknown, but is likely that the perikarya of both ENK/neurotensin and SP/dynorphin striato-SNc neurons make up the remaining projection neurons found in striosomes. Although SP/ENK striatal neurons may be infrequent in rodents, this does not rule out the possibility they are more common in other species, for example primates, in which considerable overlap of SP+ and ENK+ fibers occurs in the ventral tegmental area, SNc and medial SNr [27]. Finally, delineation of striatal projection neurons into neurochemically distinct types does not assume each projects to only one target. For example, direct pathway striatal neurons appear to have a major target (GPI or SNr), and collateral targets as well (GPe, GPI and/or SNr) [4,11,20,22,23,27].

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1. Schematic drawing of a transverse section through the striatum of a 4 month old rat that had been double-labeled by ISHH for SP (PPT) by autoradiographic ISHH and for ENK (PPE) by DIG-ISHH, overlain with the distribution of MOR+ striosomes as visualized by immunolabeling of the adjacent section. Perikarya labeled by ISHH for both SP and ENK are shown as dots, while the striosomes are shown as irregular enclosed contours. Abbreviations: AC – anterior commissure; CC – corpus callosum; V – ventricle.

2.

Images of striatum in a 4 month old rat in adjacent coronal sections that had been either immunolabeled for MOR (A, B, D) or double-labeled for SP (PPT) and ENK (PPE), using ARG to visualize the SP and DIG to visualize the ENK (C, E, F). The MOR images show successively higher power views of the region of two double-labeled neurons (arrows) shown in C, E, F, with the box in A showing the field enlarged in B, and the box in B showing the field enlarged in D. C and D are at the same magnification and show the alignment of the two double-labeled neurons with the striosomal compartment. The images E and F show two focal planes of view, one focusing on the ARG silver-grain labeling (E), and one focusing on the DIG labeling (F). E and F are at the same magnification.