Metabotropic Glutamate Receptor 4-Immunopositive Terminals of Medium-Sized Spiny Neurons Selectively Form Synapses With Cholinergic Interneurons in the Rat Neostriatum

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ABSTRACT
Metabotropic glutamate receptor 4 (mGluR4) is localized mainly to presynaptic membranes in the brain. Rat neostriatum has been reported to contain two types of mGluR4-immunoreactive axon varicosities: small, weakly immunoreactive varicosities that were distributed randomly (type 1) and large, intensely immunoreactive ones that were often aligned linearly (type 2). In the present study, most type 1 terminals formed asymmetric synapses on dendritic spines, whereas type 2 terminals made symmetric synapses on dendritic shafts, showing immunoreactivity for GABAergic markers. After depletion of neostriatal neurons, type 2 but not type 1 varicosities were largely decreased in the damaged region. When medium-sized spiny neurons (MSNs) were labeled with Sindbis virus expressing membrane-targeted green fluorescent protein, mGluR4 immunoreactivity was observed on some varicosities of their axon collaterals in immunofluorescence and immunoelectron microscopies. Furthermore, type 2 varicosities were often positive for substance P but mostly negative for striatal interneuron markers and preproenkephalin. Thus, striatonigral/striato-entopeduncular MSNs are likely to be the largest source of type 2 mGluR4-immunopositive axon terminals in the neostriatum. Next, in the double-immunofluorescence study, almost all choline acetyltransferase (ChAT)-immunopositive and 41% of NK1 receptor-positive dendrites were heavily associated with type 2 mGluR4-immunopositive varicosities. Neuronal nitric oxide synthase (nNOS)-positive dendrites, in contrast, seemed associated with only a few type 2 varicosities. Conversely, almost all type 2 varicosities were closely apposed to NK1 receptor-positive dendrites that were known to be derived from cholinergic and nNOS-producing interneurons. These findings indicate that the mGluR4-positive terminals of MSN axon collaterals selectively form synapses with neostriatal cholinergic interneurons. J. Comp. Neurol. 500:908–922, 2007. © 2006 Wiley-Liss, Inc.

Indexing terms: choline acetyltransferase; projection neurons; target neuron-specific localization; basal ganglia; confocal laser scanning microscopy; immunoelectron microscopy

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L-Glutamate is the principal excitatory neurotransmitter in the mammalian brain. The physiological effects of glutamate are mediated by ligand-gated cation channels and G protein-coupled receptors, referred to as ionotropic and metabotropic glutamate receptors (mGLURs), respectively. Eight mGLUR genes have so far been identified and subdivided into three subgroups on the basis of their amino acid sequence homology, pharmacological profiles, and intracellular signaling pathways (for review see Nakamichi, 1994; Pin and Duvoisin, 1995; Nakamishi et al., 1998). Among the three groups, group III mGLURs, including mGLUR4, mGLUR6, mGLUR7, and mGLUR8, are located predominantly on presynaptic membranes of axon terminals, with the exception of mGLUR6, and their activation results in the inhibition of adenylyl cyclase through Gi protein (for review see Conn and Pin, 1997; Cartmell and Schoepp, 2000). The reduction of cAMP caused by group III mGLUR activation is known to depress synaptic transmission by suppressing the neurotransmitter release from nerve terminals (Baskys and Malenka, 1991; Trembley and Westbrook, 1992; Pisani et al., 1997; Mitchell and Silver, 2000; Wittmann et al., 2001; Valenti et al., 2003, 2005).

The neostriatum is the primary input nucleus of the cerebral basal ganglia and abundantly receives excitatory glutamatergic afferents from the cerebral cortex and thalamic nuclei (for review see Gerfen and Wilson, 1996). Moderate mGLUR4 and intense mGLUR7a immunoreactivities have been observed in the rat neostriatum. Most mGLUR7a-immunonegative terminals formed asymmetric synapses on dendritic spines and less frequently on dendritic shafts of neostriatal neurons (Kosinski et al., 1999). Because intense expression of mGLUR7 mRNA was reported in the cerebral cortex and thalamic nuclei (Ohishi et al., 1995), and because mGLUR7a immunoreactivity in the striatum was reduced by cortical ablation (Kosinski et al., 1999), mGLUR7a is considered to be located principally on excitatory axonal terminals of corticostriatal and thalamostriatal afferents. On the other hand, although mGLUR4 immunoreactivity was initially reported to be very weak in the neostriatum (Bradley et al., 1999), Corti et al. (2002) recently demonstrated that rat neostriatum showed marked immunoreactivity for mGLUR4 and that mGLUR4 immunoreactivity was located mainly on presynaptic profiles that formed symmetric, presumably inhibitory synapses. The latter finding suggests that mGLUR4 in the neostriatum was not restricted on the glutamatergic afferent terminals, although a considerable amount of mGLUR4 mRNA was expressed in the cerebral cortex and thalamic nuclei (Testa et al., 1994; Ohishi et al., 1995). Since mGLUR4 mRNA was also expressed by a large number of striatal neurons (Testa et al., 1994; Ohishi et al., 1995), and since 90% of striatal neurons were medium-sized spiny neurons, at least a part of mGLUR4-immunonegative terminals in the neostriatum were likely to originate from medium-sized spiny neurons.

In the present study, we tried to reveal the origins of the mGLUR4-immunoreactive axon terminals by using single- and double-immunoelectron microscopy, neuronal depletion with kainic acid, and double-immunofluorescence microscopy. We further investigated the main targets of the mGLUR4-immunoreactive terminals by double-immunofluorescence and double-immunoelectron microscopies.

### Materials and Methods

The present experiments were conducted in accordance with the rules of animal care by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. In the experiments, we used adult male Wistar rats (200–350 g body weight; Japan SLC, Shizuoka, Japan) and tried to minimize the number of animals used and animal pain and distress.

#### Injection of kainic acid and Sindbis virus and fixation

We used kainic acid solution (2 mg/ml in 0.1 M sodium phosphate, pH 7.4) for chemical depletion of intrastriatal neurons and replication-deficient recombinant Sindbis virus vector for axon labeling of striatal medium-sized spiny neurons. The recombinant Sindbis virus vector contains a gene encoding enhanced green fluorescent protein (GFP) tagged with the N-terminal palmitoylation signal sequence of growth-associated protein-43 (Moriyoshi et al., 1996) that is driven by a subgenomic promoter of the virus for expression in mammalian cells (Furuta et al., 2001). Rats were anesthetized by intraperitoneal injection of chloral hydrate (350 mg/kg body weight) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The kainic acid solution (0.2–0.5 µl) and virus solution (0.5–1 µl, 2 × 10^4 infectious U/ml) were injected into the neostriatum of four and eight rats, respectively, by pressure through a glass micropipette attached to Picospritzer II (General Valve, Fairfield, NJ). The coordinates for the injection site were 0.2 mm anterior to the bregma, 2.5 mm lateral to the midline, and 4.0 mm deep to the brain surface. The rats survived for 1 week after injection of the kainic acid solution and for 18 hours after injection of the virus solution.

For light and fluorescence microscopy, four kainic acid-injected rats, four virus-injected rats, and eight intact rats were deeply anesthetized with an overdose of chloral hydrate (500 mg/kg body weight) and perfused transcardially with 100–150 ml of 5 mM phosphate-buffered 0.9% (w/v) saline (PBS; pH 7.4), followed by 250–300 ml of 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4). About 30 minutes after the perfusion, the rat brains were removed and cut into several blocks. The blocks were postfixed in the same fixative at 4°C for 4 hours, cryoprotected with 30% (w/w) sucrose in PBS, and cut into 35-µm-thick coronal sections on a freezing microtome. At least five sections through the neostriatum from each rat were used in immunostaining for light and fluorescence microscopy, unless otherwise stated. For immunoelectron microscopy, the other four virus-injected rats and four intact rats were perfused with 4% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 0.1 M PB. The brain blocks were postfixed in the same fixative at 4°C for 4 hours and cut into 50-µm-thick coronal sections on a vibratome (Microslicer DTK-1000; Dosaka, Kyoto, Japan). At least three vibratome sections from each rat were used in immunostaining for electron microscopy.

#### Antibodies

In the present study, we used the following 14 primary antibodies. 1) Guinea pig antibody raised against the C-terminal tricosapeptide (23 amino acid residues) of rat mGLUR4 (anti-mGLUR4[890–912]) antibody; Ki-
noshita et al., 1996) recognized a protein band of 100 kDa (molecular weight of mGluR4), which band was abolished by preincubation of the antibody with the antigen, on Western blot analysis of membrane preparation from rat cerebellum (Kinoshita et al., 1996) and hippocampus (Shigemoto et al., 1997). 2) We also used, as reference, rabbit antibody raised against the fusion protein between C-terminal 79 amino acid residues of rat mGluR4 and the maltose binding protein (anti-mGluR4 [854–912] antibody; Corti et al., 2002). This anti-mGluR4 rabbit antibody recognized a protein band of 100 kDa (molecular weight of mGluR4), which band was abolished by preincubation of the antibody with the antigen, on Western blot analysis of membrane preparation from various rat brain areas, including the striatum (Corti et al., 2002). Furthermore, no signal was seen when the anti-mGluR4 [834–912] antibody was used to stain brain tissue from a mGluR4 knockout mouse (Corti et al., 2002). 3) Rabbit antibody raised against the C-terminal 19 amino acids (residues 542–560) of rat vesicular glutamate transporter (VGluT1) (Hioki et al., 2003) and 4) rabbit antibody raised against the C-terminal 29 amino acids (residues 554–582) of rat VGluT2 (Hioki et al., 2003) recognized protein bands of 58 kDa (molecular weight of VGluT1) and 62 kDa (molecular weight of VGluT2), respectively, on Western blot analysis of rat brain extracts. Furthermore, VGluT1 and VGluT2 immunoreactivities were completely abolished by preincubation of these antibodies with an excess amount of the antigenic peptides on the membrane and in the brain tissue (Hioki et al., 2003). 5) Mouse monoclonal antibody raised against recombinant glutamic acid decarboxylase 67 (GAD67) protein (MAB5406; Chemicon, Temecula, CA) stained a protein band of 67 kDa (molecular weight of GAD67) on Western blot analysis of the rat brain lysate (Fong et al., 2005; manufacturer’s data sheet). 6) Rabbit antibody raised against the C-terminal 17 amino acids (residues 509–525) of rat vesicular GABA transporter (VGAT; VGAT11-A; Alpha Diagnostic International, Inc., San Antonio, TX) recognized a protein band of 57 kDa (molecular weight of VGAT) on Western blot analysis of the rat testis and brain homogenates (Redecker et al., 2003). 7) Goat antibody raised against human placental choline acetyltransferase (ChAT; AB144P; Chemicon) recognized two bands of approximately 72 kDa (molecular weight of VGAT) on Western blot analysis of rat brain extracts. Furthermore, VGluT1 and VGluT2 immunoreactivities were completely abolished by preincubation of these antibodies with an excess amount of the antigenic peptides on the membrane and in the brain tissue (Hioki et al., 2003). 8) Mouse monoclonal antibody raised against purified frog muscle parvalbumin (ChAT; AB144P; Chemicon) and 10) sheep serum raised against the C-terminal 21 amino acids (residues 1409–1429) of rat neuronal nitric oxide synthase (nNOS; AB1529; Chemicon) have been used in recent many studies (Arai et al., 1999; Bubser et al., 2000; Drake and Milner 2002) and in (Li et al., 2001; Cauli et al., 2004), respectively. These immunostainings were identical to those of well-characterized anti-calretinin antibodies (Jacobowitz and Winsky, 1991; Bennett and Bolam, 1993; Figueiredo-Cardenas et al., 1996) or of a well-characterized anti-nNOS antibody (Dawson et al., 1991). 11) Rabbit serum raised against synthetic substance P coupled to human γ-globulin with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Hatanaka and Amano, 1981) and 12) rabbit antibody raised against the C-terminal 19 amino acids (residues 251–269) of rat preproenkephalin (Lee et al., 1997a) specifically recognized antigens in the dot blotting test. Moreover, when these antibodies were preincubated with an excess amount of antigens, the immunoreactivities of these antibodies were abolished on the membrane and in the tissue (anti-substance P antiserum; Sugimoto and Mizuno, 1987; anti-preproenkephalin antibody; Lee et al., 1997a). 13) Rabbit serum raised against the C-terminal 23 amino acids (residues 385–407) of rat substance P receptor (NK1 receptor; AB5060; Chemicon). The immunostaining was abolished when the antibody was preabsorbed with an excess amount of C-terminal peptide [393–407] of NK-1 receptor (Piggins et al., 2001). Furthermore, the immunostaining was the same as that of a well-characterized rabbit antibody against the C-terminal 59 amino acid residues of rat NK1 receptor in the rat brain (Shigemoto et al., 1993; Nakaya et al., 1994). 14) Rabbit antibody raised against GFP (Tamamaki et al., 2000) reacted with GFP-expressing cell lines but did not react with non-GFP-expressing cell lines. No signal was seen when brain sections from normal rats were stained with the anti-GFP antibody (data not shown). When the primary antibodies or antisera were omitted or replaced with normal IgG for control experiments, no immunoreactivities for the omitted or replaced antibodies were detected in any of the following immunostainings for light, fluorescence, and electron microscopies.

**Immunoperoxidase staining**

The sections obtained from four intact rats and the four kainic acid-injected rats were incubated overnight with one of the following antibodies: 1) 1 μg/ml anti-mGluR4 [890–912] guinea pig antibody; 2) 2 μg/ml anti-mGluR4 [834–912] rabbit antibody; 1 μg/ml anti-VGluT1 rabbit antibody; or 1 μg/ml anti-VGluT2 rabbit antibody in PBS containing 0.3% (v/v) Triton X-100, 0.25% (w/v) λ-carrageenan, and 1% (v/v) normal donkey serum (PBS-XCD).

After several washes in PBS containing 0.3% (v/v) Triton X-100 (PBS-X), these sections were incubated for 6 hours with biotin-conjugated anti-[rabbit IgG] or anti-[guinea pig IgG] donkey antibody (10 μg/ml; AP182B or AP193B, respectively; Chemicon) in PBS-XCD, followed by a rinse with PBS-X. The sections were further incubated for 1 hour with avidin-biotinylated peroxidase complex (1:100, ABC-Elite; Vector Laboratories, Burlingame, CA) in PBS-X. After a rinse with PBS, bound peroxidase was visualized by incubation of the sections with 0.02% (w/v) 3,3′-diaminobenzidine-4HCl (DAB; Dojindo, Kumamoto, Japan) and 0.003% (v/v) hydrogen peroxide in 50 mM Tris-HCl, pH 7.6, for 20–40 minutes. The sections were thoroughly washed in PBS, mounted on gelatin-coated glass slides, air dried, dehydrated in an ethanol
ORIGIN AND TARGET OF mGluR4-POSITIVE GABAergic TERMINALS

The sections obtained from four intact rats were incubated overnight with a mixture of 1 μg/ml anti-mGluR4[890–912] guinea pig antibody and one of the following antibodies: 2 μg/ml anti-GAD67 mouse antibody, 2 μg/ml anti-VGAT rabbit antibody, 1/500-diluted anti-ChAT goat antibody, 1/4,000-diluted anti-parvalbumin mouse antibody, 1/500-diluted anti-calretinin goat serum, 1/500-diluted anti-nNOS sheep serum, 2 μg/ml anti-VGlut1 rabbit antibody, 2 μg/ml anti-VGlut2 rabbit antibody, 1/5,000-diluted anti-substance P rabbit serum, 1 μg/ml anti-preproenkephalin rabbit antibody, or 1/500-diluted anti-NK1 receptor rabbit serum in PBS-XCD.

The sections obtained from the four virus-injected rats were incubated with the CHAT antibody or the parvalbumin antibody. The sections obtained from intact rats were incubated with one of the secondary antibodies. In the presence of the normal serum, the sections were further incubated for 4 hours with either Cy5-conjugated anti-[guinea pig IgG] donkey antibody (10 μg/ml; AP193S; Chemicon) for confocal laser scanning microscopy or Alexa Fluor 594-conjugated anti-[guinea pig IgG] goat antibody (10 μg/ml; A11034; Invitrogen) for epifluorescence microscopy. After several washes in PBS-X, the sections were further incubated for 1 hour with 1/100-diluted ABC-Elite in PBS-X, and the bound peroxidase was developed by incubation with fluorescein-conjugated tyramide (1:50; NEL701A, Tyramide Signal Amplification-fluorescein systems; PerkinElmer Life Science, Boston, MA) for 3–5 minutes.

The sections obtained from the four virus-injected rats were incubated with a mixture of 1 μg/ml anti-mGluR4[890–912] guinea pig antibody and 1 μg/ml anti-GFP rabbit antibody in PBS-XCD. After several washes in PBS-X, the sections were incubated for 4 hours with Alexa Fluor 488-conjugated anti-[rabbit IgG] goat antibody (10 μg/ml; A11034; Invitrogen) in PBS-XCD, rinsed with PBS-X, and then placed for 30 minutes in PBS-XCD containing 10% (v/v) normal rabbit, mouse, sheep, or goat serum to block cross-reactions of the secondary antibodies. In the presence of the normal serum, the sections were further incubated for 4 hours with either Cy5-conjugated anti-[guinea pig IgG] donkey antibody (10 μg/ml; AP193S; Chemicon) for confocal laser scanning microscopy or Alexa Fluor 594-conjugated anti-[guinea pig IgG] goat antibody (10 μg/ml; A11076; Invitrogen) for epifluorescence microscopy. After several washes in PBS-X, the sections were further incubated for 1 hour with 1/100-diluted ABC-Elite in PBS-X, and the bound peroxidase was developed by incubation with fluorescein-conjugated tyramide (1:50; NEL701A, Tyramide Signal Amplification-fluorescein systems; PerkinElmer Life Science, Boston, MA) for 3–5 minutes.

The sections obtained from the four virus-injected rats were incubated with a mixture of 1 μg/ml anti-mGluR4[890–912] guinea pig antibody and 1 μg/ml anti-GFP rabbit antibody in PBS-XCD. After several washes in PBS-X, the sections were incubated for 4 hours with Alexa Fluor 488-conjugated anti-[rabbit IgG] goat antibody (10 μg/ml; A11034; Invitrogen) in PBS-XCD, rinsed with PBS-X, and then placed for 30 minutes in PBS-XCD containing 10% (v/v) normal rabbit serum. In the presence of the normal rabbit serum, the sections were further incubated for 4 hours with 10 μg/ml Cy5-conjugated anti-[guinea pig IgG] donkey antibody.

All the sections were washed thoroughly in PBS, mounted onto gelatin-coated glass slides, air dried, and coverslipped with 90% (v/v) glycerol and 2.5% (w/v) triethylenediamine (antifading agent) in 20 mM Tris-HCl, pH 7.6. The sections were observed under an epifluorescence microscope Axiohot 2 (Carl Zeiss, Oberkochen, Germany) with the appropriate filter sets for fluorescein and Alexa Fluor 488 (excitation 450–490 nm, emission 514–565 nm) and for Alexa Fluor 594 (excitation 530–585 nm, emission, ≥615 nm), or under confocal laser scanning microscopes by illuminating the specimen with a single laser beam and collecting the image using an appropriate emission filters for fluorescein and Alexa Fluor 488 (excitation 488 nm, emission 505–530 nm), for Alexa Fluor 594 (excitation 543 nm or 594 nm, emission ≥630 nm), and for Cy5 (excitation 633 nm, emission ≥650 nm). At each wavelength, eight confocal images (1,024 × 1,024 pixels in size with an eight-bit pixel depth) were obtained and averaged to reduce noise. For the analysis of mGluR4 immunoreactivity on chemical marker-positive varicosities (Fig. 3a–j) and on varicosities of GFP-expressing medium-sized spiny neurons (Fig. 3n–s), z-stack digital images were captured on an AOBS-TCS SP2 confocal laser scanning microscope (Leica, Heidelberg, Germany) with a ×63 oil-immersion objective lens (HCX PL APO, numerical aperture 1.4; Leica) with pinhole set at 1.0 Airy disc (optical slice thickness of less than 0.6 μm), and with a zoom factor of 10, which resulted in a pixel size of 23.3 nm × 23.3 nm. Up to 30 images with a z-interval of 81.4 nm were acquired per stack, and deconvolved in the software Huygens Essential (Scientific Volume Imaging, Hilversum, The Netherlands). All the other confocal digital images were acquired with an LSM 5 PASCAL (Carl Zeiss) confocal laser scanning microscope with a ×63 water-immersion objective lens (C-Apochromat, numerical aperture 1.2; Carl Zeiss) with pinhole set at 1.0 Airy disc (optical slice thickness of less than 1.0 μm) and with a zoom factor of 1.0–10 for high-magnification images (Figs. 3k,l, 4b,c,e,f, 5b,d,f) or a ×40 objective lens (Plan-NEOFLUAR, numerical aperture 0.75; Carl Zeiss) for low-magnification confocal images (Figs. 4a–a’–d–f’, 5a,c,e). All digital images were arranged and modified (<30% contrast enhancement) in the software Canvas 9 (ACD Systems of America, Inc., Miami, FL) and saved as TIFF files.

Immunoelectron microscopy

The vibratome sections obtained from four intact rats and the four virus-injected rats were placed in PBS containing 30% (w/v) sucrose for 1 hour for cryoprotection and were freeze-thawed three times using liquid nitrogen to enhance penetration of antibodies. The sections were thoroughly washed and preincubated in PBS containing 20% (v/v) normal donkey serum for 1 hour at room temperature to block nonspecific binding of antibodies. The sections obtained from intact rats were incubated overnight at 4°C in PBS containing 2% (v/v) normal donkey serum and 0.05% (v/v) Photo-Flo (Kodak, Rochester, NY) with 2 μg/ml anti-mGluR4[890–912] guinea pig antibody for single labeling or with a mixture of 2 μg/ml anti-mGluR4[890–912] guinea pig antibody and either 1/500-diluted anti-ChAT goat antibody or 1/500-diluted anti-NK1 receptor rabbit serum for double labeling. The sections obtained from the virus-injected rats were incubated in PBS containing 2% (v/v) normal donkey serum and 0.2% (v/v) Photo-Flo with a mixture of 2 μg/ml anti-mGluR4[890–912] guinea pig antibody and 1 μg/ml anti-GFP rabbit antibody for double labeling.

After several washes in PBS, the sections were further incubated overnight at 4°C with secondary antibodies. For single immunogold-silver labeling, the sections were incubated with 1.4-nm-gold-conjugated anti-[guinea pig IgG] goat IgG Fab’ fragment (1:50; catalog No. 2055; Nanoprobes, Stony Brook, NY) in PBS containing 2% (v/v) normal goat serum. These sections were washed in PBS.
and postfixed in 1% (v/v) glutaraldehyde in 0.1 M PB for 15 minutes at room temperature. After washes in distilled water, the gold particles were silver-intensified with an HQ silver kit for 7–10 minutes. For single immunoperoxidase labeling, the sections were incubated with 10 μg/ml biotin-conjugated anti-[guinea pig IgG] donkey antibody in PBS containing 2% (v/v) normal donkey serum, and, after several washes in PBS, further incubated at room temperature with 1/50-diluted ABC-Elite in PBS for 4 hours. After several rinses in PBS, bound peroxidase was developed with 0.02% (w/v) DAB and 0.003% (v/v) hydrogen peroxide in 0.1 M Tris-HCl, pH 7.6, for 20–40 minutes.

For double labeling of mGluR4 and either ChAT or NK1 receptor, the sections were placed in a mixture of 10 μg/ml biotin-conjugated anti-[guinea pig IgG] donkey antibody and either 1.4-nm-gold-conjugated anti-[goat IgG] rabbit IgG Fab' fragment (1:50; catalog No. 2006; Nanoprobes) in PBS containing 2% (v/v) normal donkey and rabbit serum or anti-[rabbit IgG] goat IgG Fab' fragment (1:50; catalog No. 2004; Nanoprobes) in PBS containing 2% (v/v) normal donkey and goat serum. After several rinses in PBS, ChAT or NK1 receptor immunoreactivity was first visualized by the silver enhancement technique, and then mGluR4 immunoreactivity was developed by the ABC method and DAB reaction as described above. For double labeling of mGluR4 and GFP, the sections were placed in a mixture of 10 μg/ml biotin-conjugated anti-[rabbit IgG] donkey antibody and 1/50-diluted 1.4-nm-gold-conjugated anti-[guinea pig IgG] goat IgG Fab' fragment in PBS containing 2% (v/v) normal donkey and goat serum and 0.2% (v/v) Photo-Flo. After several rinses in PBS, mGluR4 and GFP immunoreactivities were visualized as described above.

Finally, the immunostained sections were washed thoroughly in 0.1 M PB, placed in 1% (w/v) osmium tetroxide in 0.1 M PB (pH 7.4) for 40 minutes at room temperature, counterstained for 30 minutes with 1% (w/v) uranyl acetate, dehydrated, and flat-embedded in epoxy resin (Luveak 812; Nacalai Tesque, Kyoto, Japan). Once the resin was polymerized, the tissue samples were cut into ultrathin sections on an ultramicrotome, Reichert-Nissei Ulturscut S (Leica, Vienna, Austria). The ultrathin sections were mounted on mesh grids and examined with an electron microscope (H-7100; Hitachi, Tokyo, Japan).

**RESULTS**

**mGluR4 immunoreactivity in the rat neostriatum**

The immunoreactivities of the two antibodies raised against C-terminal portions of the rat mGluR4—1) guinea pig antibody to mGluR4(residues 890–912) (Kinoshita et al., 1996; Fig. 1a,b) and 2) rabbit antibody to mGluR4(843–912) (Corti et al., 2002; data not shown)—looked very similar in the rat neostriatum. Furthermore, both the immunoreactivities were completely abolished by preincubation with an excess amount of C-terminal tricosapeptide of mGluR4, which corresponded to residues 890–912 (anti-mGluR4[890–912] guinea pig antibody, Fig. 1c; anti-mGluR4[843–912] rabbit antibody, data not shown). This result indicates that both the antibodies recognized a similar site in the C-terminal tricosapeptide, although the rabbit antibody was raised against much longer C-terminal peptide. In all of the other immunostainings, anti-mGluR4[890–912] guinea pig antibody was used.

Two types of mGluR4 reaction products were observed in the rat neostriatum (Fig. 1a,b) as reported previously (Corti et al., 2002). Many small punctate reaction products with weak mGluR4 immunoreactivity were randomly distributed throughout the neostriatum (type 1), whereas larger deposits with more intense immunoreactivity were aligned in a linear fashion (type 2). The type 2 terminals were also observed throughout the neostriatum, with stronger immunoreactivity at the medial portion than the lateral portion of the neostriatum. In the electron-microscopic analysis, type 2 mGluR4-immunopositive varicosities with DAB reaction products often made symmetric synapses on aspiny dendritic shafts (Fig. 1d,e). With the silver enhancement method of subcellular resolution, mGluR4 immunoreactivity was localized on or just inside of presynaptic membranes (Fig. 1f). Type 1 mGluR4-immunoreactive varicosities were, in contrast, observed mostly in presynaptic profiles forming asymmetric synapses with dendritic spines (Fig. 1g). These results suggested that type 1 axon varicosities were excitatory and presumably glutamatergic axon terminals and that type 2 varicosities were inhibitory and probably GABAergic terminals.

**Kainic acid injection into the neostriatum**

It is known that the neostriatum receives glutamatergic afferent terminals from the cerebral cortex and thalamic nuclei and that most GABAergic terminals originate from the inside of the neostriatum, except those from the globus pallidus (for review see Gerfen and Wilson, 1996) and substantia nigra (Rodriguez and González-Hernández, 1999). Thus, type 1 and type 2 mGluR4-immunoreactive terminals were supposed to be of extrastriatal and intrastral origins, respectively. We first tried to investigate the origins of type 1 and type 2 mGluR4-immunoreactive terminals by depletion of striatal neurons with kainic acid lesion.

When a small injection with kainic acid was made in the neostriatum (Fig. 2a–c), most type 2 mGluR4-immunoreactive varicosities disappeared from the region depleted of striatal neurons, whereas type 1 varicosities remained resistant to the chemical damage (Fig. 2b,c). It has been reported that injection of kainic acid causes a selective degeneration of neurons, of which cell bodies are located in the area of injection, but relatively spares axon terminals arising from neuronal cell bodies outside the damaged region (Schwarz and Coyle, 1977; Coyle et al., 1978; Olney and de Gubareff, 1978). In the present study, immunoreactivities for VGluT1 and VGluT2, which were located in corticostrial and thalamostriatal axon terminals, respectively (Fujiyama et al., 2004), actually remained in the damaged region (VGluT1, Fig. 2b,c; VGluT2, data not shown). This indicated that most afferents to the neostriatum were preserved in the damaged region, although some changes would have occurred in the afferent terminals (Wuerthele et al., 1978; Krammer et al., 1979). Thus, the present findings suggested that type 1 mGluR4-positive excitatory terminals were of cortical or thalamic origin. On the other hand, most type 2 inhib-
itory terminals were considered to be of intrastriatal origin.

Chemical characterization of type 2 mGluR4-immunopositive varicosities

Because most type 2 mGluR4-immunoreactive varicosities were derived from intrastriatal neurons, we then investigated whether or not the varicosities showed immunoreactivity for GABAergic or cholinergic marker by using a confocal laser scanning microscope (Fig. 3). Many type 2 varicosities displayed immunoreactivity for GABAergic markers, GAD67 (arrowheads in Fig. 3a) and VGAT (arrowheads in Fig. 3b), but not for a cholinergic marker, ChAT (Fig. 3e). Immunoreactivity

Fig. 1. mGluR4 immunoreactivity in the rat neostriatum. Two types of mGluR4 immunoreactivity were observed in the neostriatum (a,b): small, weakly immunopositive varicosities that were randomly distributed (type 1) and larger, intensely immunopositive varicosities that were aligned linearly (type 2). Boxed areas are shown at higher magnification. When the anti-mGluR4 antibody was preincubated with an excess amount of C-terminal tricosapeptide of mGluR4, no immunoreactivity was detected (c). In the electron-microscopic analysis, type 2 varicosities formed symmetric synapses with shafts of aspiny dendrites (d-f), whereas type 1 varicosities with weak immunoreactivity made asymmetric contacts with dendritic spines (g). Arrowheads in e–g indicate postsynaptic membranes. a–e were stained by the immunoperoxidase method, and f,g were labeled by the immunogold particle and silver enhancement method. D, dendritic profile; M, mitochondrion; S, dendritic spine; T, axon terminal. Scale bars = 100 μm in a; 10 μm in b (applies to b,c); 1 μm in d; 0.3 μm in g (applies to e–g).
Fig. 2. Effect of neuronal depletion on mGluR4 immunoreactivity in the neostriatum. When a small amount of kainic acid was injected into the neostriatum, a striatal region encircled by a dashed line (a) was damaged to lose neuronal cell bodies (b). Boxed areas are shown at higher magnification. In the damaged region, type 2 mGluR4-immunopositive varicosities were markedly decreased (b'), whereas type 1 varicosities remained positive (b'). VGluT1-immunoreactive varicosities did not display a difference between damaged (b'') and intact regions (c''). Sections including b, b', and b'' (or c, c', and c'') were consecutive ones and were stained for Nissl, mGluR4, and VGluT1, respectively. Each number in b–c'' indicates the same axon bundles of the internal capsule. Scale bars = 1 mm in a; 100 μm in c' (applies to b–c'').
for GAD67 was detected in 195 (56%) of 348 type 2 mGluR4-immunoreactive varicosities. These results support the inhibitory nature of type 2 axon terminals. However, little immunoreactivity of chemical markers for GABAergic interneurons, such as parvalbumin, calretinin, or nNOS, was detected in type 2 mGluR4-immunoreactive varicosities (about 1%; Fig. 3f–h). Thus, medium-sized spiny neurons (MSNs), well known to be GABAergic projection neurons, were candidates for the major source of type 2 mGluR4-immunopositive axon terminals in the neostriatum.

To investigate mGluR4 immunoreactivity in axon collaterals of MSNs, we injected Sindbis viral vector designed to express membrane-targeted GFP into the neostriatum (Furuta et al., 2001). In Figure 3k,l, an MSN infected with the virus showed intense green fluorescence throughout its dendrites and axon collaterals. In the confocal laser scanning microscopic analysis of total of 40 sections (contain GFP-expressing MSN axon varicosities) from the four virus-injected rats, 34 (3.7%) of 922 GFP-positive axon varicosities showed immunoreactivity for mGluR4 (Fig. 3n–s). In the electron-microscopic analysis, furthermore, mGluR4 immunoreactivity was located on or just beneath the presynaptic membrane of GFP-positive terminal of axon collateral of the neostriatum. Thus, it might be concluded that at least some type 2 varicosities were derived from MSNs. Because striatondigral/striato-entopeduncular and striatopallidal MSNs are known to produce substance P and enkephalin, respectively (for review see Lee et al., 1997a), it was expected that type 2 varicosities would show at least either substance P or preproenkephalin immunoreactivity. As shown in Figure 3c, 42 (11.6%) of 362 type 2 mGluR4-positive varicosities displayed substance P immunoreactivity, whereas much fewer mGluR4-positive varicosities showed preproenkephalin immunoreactivity (about 1%; Fig. 3d). Since substance P and preproenkephalin immunoreactivities in striatal neuropil were much weaker than those in neuropil of the substantia nigra and globus pallidus, we might have underestimated the number of substance P-positive or preproenkephalin-positive axon varicosities in the neostriatum.

A few type 1 mGluR4-immunopositive varicosities showed VGluT1 immunoreactivity (arrowheads in Fig. 3i), which is a marker of corticostriatal terminals (Fujiyama et al., 2004). Because many layer V cortical neurons express mRNA for mGluR4 (Ohishi et al., 1995), this result may suggest a low sensitivity of the present double-immunofluorescence staining method, resulting in an underestimation of mGluR4 immunoreactivity in VGluT1-positive varicosities. Otherwise, only a small proportion of corticostriatal axon terminals might be loaded with mGluR4, as revealed for mGluR7 in corticocortical terminals (Dalezios et al., 2002). A very few varicosities with VGluT2 immunoreactivity, a marker of thalamostriatal terminals (Fujiyama et al., 2004), showed intense immunoreactivity for mGluR4 (arrowhead in Fig. 3j). However, those VGluT2 and mGluR4 double-positive, large varicosities were not clustered or aligned linearly as were type 2 varicosities, but were distributed as individual boutons within the neostriatum.

### Targets of type 2 mGluR4-immunopositive terminals

Type 2 mGluR4-immunopositive varicosities were not randomly distributed but aligned rather linearly, suggesting that some dendrites were selectively associated with type 2 varicosities. Furthermore, in the electron-microscopic analysis, the targets of type 2 terminals were dendritic shafts without dendritic spines (Fig. 1d). Thus, the candidate targets were aspiny striatal neurons, which were all known to be interneurons, including cholinergic, parvalbumin-containing GABAergic, calretinin-containing GABAergic, and somatostatin/neuropeptide Y/nNOS-producing GABAergic neurons (for review see Kawaguchi et al., 1995; Kawaguchi, 1997; Tepper and Bolam, 2004). In Figures 4 and 5, double-immunofluorescence staining for mGluR4 and any one of the four interneuron markers revealed that almost all ChAT-positive dendrites were frequently, and a few nNOS-positive dendrites were much less frequently, associated with type 2 mGluR4-positive varicosities (Figs. 4a–c, 5a). However, almost no calretinin- or parvalbumin-positive dendrites appeared to be innervated by type 2 mGluR4-immunoreactive varicosities (Fig. 5c–f). Electron-microscopic analysis further revealed that type 2 mGluR4-positive axon terminals formed symmetric synapses with ChAT-immunoreactive dendrites (Fig. 6a).

Some type 2 mGluR4-positive varicosities did not seem to be associated with ChAT-positive dendrites in the neostriatum (Fig. 4a–a’). However, this could be caused by incomplete labeling of dendrites of cholinergic neurons with the anti-ChAT antibody. To check this possibility, rat striatal sections were doubly immunostained for mGluR4 and NK1 receptor. In the rat neostriatum, NK1 receptor is selectively expressed by two kinds of striatal interneurons, cholinergic and nNOS-producing interneurons (Kaneko et al., 1993; Li et al., 2001), and NK1 receptor immunoreactivity is distributed up to the tip of dendrites of those interneurons (Shigemoto et al., 1993), being different from ChAT or nNOS immunoreactivity. Almost all type 2 varicosities were, as expected, densely associated with NK1 receptor-positive dendrites (Fig. 4d–f). In addition, type 2 mGluR4-positive axon terminals often made symmetric synapses with NK1 receptor-positive dendrites (Fig. 6b).

Finally, in an attempt to quantify the appositions, we randomly sampled 100 dendrites (100-μm-long or longer) immunopositive for each interneuron marker on the double-stained neostriatum from four intact rats and counted the number of mGluR4-positive varicosities apposed to the dendrites (Fig. 7). Ninety-nine percent of ChAT-positive dendrites received 16 or more mGluR4-positive varicosities per 100-μm dendrite length, whereas none of the nNOS-positive, parvalbumin-positive or calretinin-positive dendrites received 16 or more varicosities per 100-μm length. On average, 41.3 ± 14.3 (mean ± SD), 3.5 ± 1.6, 2.5 ± 2.3, and 2.0 ± 1.5 appositions/100-μm length were found on dendrites with ChAT, nNOS, parvalbumin, and calretinin immunoreactivities, respectively. NK1 receptor-positive dendrites showed two peaks; 41 dendrites with NK1 receptor immunoreactivity admitted 16 or more varicosities, and 59 dendrites received fewer than 16 varicosities/100-μm length, suggesting that NK1 receptor-positive dendrites were composed of two different populations, probably dendrites of cholinergic neurons and those of nNOS-producing neu-
Figure 3
DISCUSSION

Two types of mGluR4-immunoreactive axon terminals were observed in the neostriatum; type 1 terminals were small, weakly immunoreactive, and distributed randomly, whereas type 2 were large, intensely immunoreactive, and mostly aligned in a linear way. The present results indicated that at least a part of type 2 mGluR4-positive GABAergic axon terminals were derived from substance P-producing, striatonigral/striato-entopeduncular medium-sized spiny striatal neurons and that no other major candidate for the source of type 2 terminals was found in the neostriatum. It was further revealed that type 2 mGluR4-positive terminals made synapses almost selectively with cholinergic striatal interneurons.

Immunoreactivity of mGluR4 in the rat neostriatum

The two anti-mGluR4 antibodies used in the present study recognized the same or very similar structures in the neostriatum, and both the immunoreactivities were absorbed with the C-terminal tricosapeptide (23 amino acid residues) of rat mGluR4. This indicates that the immunoreactivity observed in the present study was highly specific to mGluR4 protein. It was, however, surprising that both the immunoreactivities were absorbed by preincubation with an excess amount of C-terminal tricosapeptide of mGluR4, although these antibodies were, respectively, raised against 23 and 79 C-terminal amino acid residues. This may indicate that anti-mGluR4[834–912] rabbit antibody raised against the longer C-terminal peptide recognized a very limited portion of the long C-terminal sequences. It is known that proteins generally have a few main immunogenic regions in their amino acid sequences. When monoclonal antibodies were produced to a protein molecule, only a few restricted portions of the molecule were recognized by many lines of monoclonal antibodies (Tzartos et al., 1981; Crawford et al., 1982; Kaneko et al., 1988). Thus, if the main immunogenic region of mGluR4 C-terminal was restrictively located in the C-terminal tricosapeptide, both the antibodies could bind only to the same main immunogenic region.

Another possibility of the restricted antigenicity was a limited accessibility of the antibodies to the antigen sites. Even though a polyclonal antibody contained several IgG clones that recognized many sites of its antigen protein, only a limited number of clones could bind to the protein if most antigen sites were inaccessible by the steric hindrance caused by the structure of the antigen protein or by the presence of certain binding proteins in the tissue. In the case of mGluR4, it was thus possible that only the C-terminal tricosapeptide portion might be freely accessible to the antibodies under the conditions of immunostaining in the tissue.

Origin of mGluR4-immunoreactive axon terminals

Most type 1 mGluR4-immunoreactive axon terminals made asymmetric synapses with dendritic spines, indicating their excitatory nature. Excitatory, glutamatergic axon terminals in the neostriatum are known to be mostly of extrastriatal origin (for review see Gerfen and Wilson, 1996) and only partially derived from cholinergic interneurons (Gras et al., 2002; Schäfer et al., 2002; Fujiyama et al., 2004). Because no cholinergic interneurons expressed mGluR4 mRNA as shown by single-cell reverse transcriptase PCR (Bell et al., 2002), the type 1 mGluR4-immunoreactive axon terminals were considered to be derived from the cerebral cortex and thalamic nuclei, which displayed moderate to intense signals for mGluR4 mRNA in in situ hybridization histochemistry (Testa et al., 1994; Ohishi et al., 1995). In the present study, only a few type 1 mGluR4-immunoreactive varicosities showed immunoreactivities for VGluT1 and VGluT2, which were loaded in corticostriatal and thalamostriatal axon terminals, respectively (Fujiyama et al., 2004). This may suggest a low sensitivity of the present immunofluorescence method for mGluR4. However, because group III mGluRs are known to be located in a target cell-specific manner (Shigemoto et al., 1996, 1997; Dalezios et al., 2002; Somogyi et al., 2003; Ferraguti et al., 2005), it is more likely that only a part of the corticostriatal and thalamostriatal terminals are positive for mGluR4.

In contrast to type 1 terminals, type 2 mGluR4-positive terminals made symmetric synapses on dendritic shafts and showed immunoreactivity for GABAergic markers. These indicate that type 2 mGluR4-positive varicosities in the neostriatum are originated from GABAergic inhibitory neurons. The neostriatum is known to receive GABAergic inputs from the globus pallidus (Staines et al., 1981) and...
substantia nigra (Rodríguez and González-Hernández, 1999), but neurons in the globus pallidus and substantia nigra do not express mRNA for mGluR4 (Testa et al., 1994; Ohishi et al., 1995). Moreover, type 2 varicosities almost disappeared from the neostriatal region, where neurons were depleted by kainic acid injection (Fig. 2).
Thus, the sources of mGluR4-immunopositive GABAergic terminals in the neostriatum are likely to be striatal neurons, including MSNs and three kinds of inhibitory interneurons producing parvalbumin, calretinin, and nNOS. Since little parvalbumin, calretinin, or nNOS immunoreactivity was observed in type 2 mGluR4-immunoreactive varicosities, only MSNs, which are well known to express mGluR4 mRNA (Testa et al., 1994; Ohishi et al., 1995), are candidates for the major source of type 2 varicosities. Actually, in the present study, some varicosities (3.7%) of local axon collaterals of MSNs were revealed to be immunopositive for mGluR4.

It is well known that most MSNs are divided into two types; one is substance P-producing striatonigral/striato-entopeduncular MSNs, and the other is enkephalin-producing striatopallidal MSNs (for review see Lee et al., 1997a). Some type 2 mGluR4-immunopositive varicosities showed substance P immunoreactivity, but much fewer type 2 varicosities showed preproenkephalin immunoreactivity. Although the positivity rate was low (11.6% of type 2 varicosities), substance P-producing MSNs were considered to be the major source of type 2 mGluR4-immunoreactive axon varicosities because of the absence of the other intrastriatal candidates. It is known that only a part of the axon terminals of mGluR7-mRNA expressing cortical or hippocampal neurons are positive for mGluR7a protein and that the mGluR7a-positive axon terminals selectively form synapses in a target neuron-specific manner in the rat hippocampus (Shigemoto et al., 1996, 1997; Somogyi et al., 2003) and in the rat somatosensory cortex (Dalezios et al., 2002). In addition, it was revealed recently that mGluR8-positive axon terminals also selectively form synapses in a target neuron-specific manner in the rat hippocampus (Ferraguti et al., 2005). These suggest that type 2 mGluR4-immunopositive axon terminals of MSNs are also targeted to a certain kind of postsynaptic elements.

**Fig. 5.** Double-immunofluorescence labeling for mGluR4 and any one of neuronal nitric oxide synthase (nNOS; a,b), calretinin (CR; c,d), or parvalbumin (PV; e,f) in the neostriatum. A few mGluR4-positive varicosities closely associated with an nNOS-positive dendrite (b; arrowheads). Almost no CR- or PV-positive dendrites were tightly associated with type 2 mGluR4-positive varicosities. b,d,f are confocal laser scanning microscopic images, and a,c,e are composed images of four, three, and six confocal images, respectively. Boxed areas are shown at higher magnification. Scale bars = 50 μm in e (applies to a,c,e); 5 μm in f (applies to b,d,f).

**Targets and functions of type 2 mGluR4-immunopositive axon terminals**

The present results clearly indicate that type 2 mGluR4-immunoreactive terminals were almost selectively targeted to cholinergic interneurons in the rat neostriatum. Substance P-producing striatonigral/striato-entopeduncular MSNs, which were the only candidate for the major source of type 2 axon terminals, are known to be GABAergic neurons, and thus their axon collaterals do not release glutamate. Therefore, in order to activate mGluR4 on type 2 axon terminals, glutamate has to come from neighboring excitatory terminals, such as corticostriatal afferents, thalamostriatal afferents, and cholinergic/glutamatergic intrinsic terminals (Gras et al., 2002; Schafer et al., 2002; Fujiyama et al., 2004). mGluR4 can be activated with a low concentration of glutamate, which is spilled over from those excitatory terminals, because mGluR4 has relatively high affinity for glutamate (EC$_{50}$ = 3–5 μM) compared with the other group III mGluRs (EC$_{50}$ = 1–1.3 mM for mGluR7a; for review see Schoepf et al., 1999).

Since cholinergic/glutamatergic interneurons send much fewer axon collaterals to NK1 receptor-expressing cholinergic neurons (Lee et al., 1997b), the most likely candidates for the source of the glutamate are corticostriatal and thalamostriatal afferents. Actually, cholinergic neurons are known to receive many corticostriatal and thalamostriatal afferent terminals (Wilson et al., 1990; Lapper and Bolam, 1992; Thomas et al., 2000). Thus, the glutamate that is released from the striatopetal afferents might heterosynaptically act on mGluR4 of MSN axon collaterals, suppress the synaptic release of GABA from the collaterals (Trombley and Westbrook, 1992), and thereby enhance the excitatory effect of the glutamatergic afferents on the cholinergic neurons.

To our knowledge, there is no publication revealing the GABAergic effect of MSN axon collateral inputs to cholin-
ergic neurons. However, it has been reported that GABA transmission by MSN axon collaterals has little influence (Jaeger et al., 1994) or only a weak inhibitory effect on postsynaptic MSNs (Tunstall et al., 2002; Koós et al., 2004). Since it is unlikely that some terminals of axon collaterals of an MSN release much GABA but the others give off very little GABA, it might be postulated that MSN axon collaterals have very limited GABAergic effect on cholinergic neurons. Thus, the function of mGluR4 on MSN axon collateral inputs to cholinergic neurons might not be so relevant to GABAergic transmission. On the other hand, substance P is well known to be produced and released by striatonigral/striato-entopeduncular MSNs, and the released substance P is considered to work strongly on cholinergic and nNOS-producing interneurons, because these interneurons intensely express NK1 receptor, the receptor for substance P (Kaneko et al., 1993; Li et al., 2001). Actually, it has been reported that substance P produces depolarization of cholinergic interneurons in in vitro whole-cell patch-clamp recording (Aosaki and Kawaguchi, 1996). Moreover, it has been revealed that the substance P-containing terminals of MSN axon collaterals frequently form synapses with

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**Fig. 6.** Electron microscopic double immunolabeling for mGluR4 and either choline acetyltransferase (ChAT) or NK1 receptor (NK1) in the neostriatum. mGluR4-positive large varicosities with DAB reaction products formed symmetric synaptic contacts (arrowheads) with dendrites immunopositive for ChAT (a) or NK1 receptor (b), which were labeled with silver grains. D, dendritic profile; M, mitochondrion; T, axon terminal. Scale bar = 0.3 μm.

**Fig. 7.** Association of mGluR4-immunopositive varicosities with dendrites of neostriatal interneurons. In the double immunostained sections as shown in Figures 4 and 5, 100 interneuronal dendrites of more than 100-μm length were randomly selected under a fluorescence microscope, and the number of mGluR4-positive varicosities in close apposition to the dendrites was counted. ChAT, choline acetyltransferase; nNOS, neuronal nitric oxide synthase.
cholinergic interneurons in the rat striatum (Bolam et al., 1986; Martone et al., 1992; Lee et al., 1997b). Given the fact that mGluR4 is often located on the terminals of axon collaterals of substance P-producing MSNs rather than enkephalin-producing MSNs, the function of mGluR4 might be relevant to substance P transmission. Thus, the glutamate released from the striatopetal afferents is likely to act on mGluR4 of MSN axon collaterals, inactivates the synapses releasing substance P, and suppresses the overexcitation of cholinergic neurons. This scenario might appear suitable for the target neuron-specific localization of mGluR4; if mGluR4 on MSN axon collaterals served as a general presynaptic inhibitory mechanism of GABAergic transmission, mGluR4 would then be located not only in the synapses on cholinergic neurons but also in the other synapses on non-cholinergic neurons.

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