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Multiplicity of Glutamate Receptor Subunits in Single Striatal Neurons: An RNA Amplification Study

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SUMMARY

The RNA amplification technique was used to examine the pattern of coexpression of mRNAs encoding 16 subtypes/subunits of the glutamate receptor (GluR) in acutely dissociated neurons from adult rat striata. The signal intensity for each mRNA varied within single neurons, but the general pattern of low versus high expression signals was similar among neurons, except for the GluR4 subunit of the (±)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. The mRNAs for GluR1–3 subunits of the AMPA receptor were present in all cells, with the signal intensity of GluR1 mRNAs usually the lowest. The kainate receptor subunit mRNAs (GluR5–7) were present in most neurons, and the signal intensity for GluR6 mRNA was the highest. The signals for *N*-methyl-D-aspartate (NMDA)R1 and NMDAR2B mRNAs were high in most neurons; however, NMDAR2A and NMDAR2C mRNAs gave low or undetectable signals. For mRNAs encoding metabotropic GluRs (mGluRs), signals for mGluR1, mGluR2, and mGluR3 mRNAs were low or undetectable, whereas mGluR4 and mGluR5 mRNA signals were high in most neurons. In most cases (12 of 16 mRNAs), the results agreed with data from *in situ* hybridization experiments in which individual mRNAs were examined. All neurons expressed subtypes/subunits mRNAs for all four types of GluRs; however, there were differences in the relative intensity of the mRNA signals detected in individual cells, suggesting that these receptors could exist in various combinations within individual neurons and thus confer synapse-specific function for information processing in the striatum.

The basal ganglia play an important role in the regulation of motor behaviors and the pathophysiology of movement disorders, including Parkinson's and Huntington's diseases. Behavioral, anatomic, and pharmacological investigations indicate that proper excitatory neurotransmission is crucial for the normal function of the basal ganglia (1). The striatum, the main input nucleus of the basal ganglia, receives a major excitatory innervation from the cerebral cortex and, to a lesser extent, from the thalamus. These inputs form synapses with medium-sized spiny neurons, which constitute ~95% of striatal neurons and are the efferent component of striatum (2). Anatomic as well as electrophysiological evidence suggests that these excitatory afferents to the striatum use glutamate as a neurotransmitter (2).

Glutamate exerts its physiological function through two major families of receptors. The ionotropic receptor family is comprised of ligand-gated ion channels that mediate the fast excitatory neurotransmission in the central nervous system and include AMPA, kainate, and NMDA receptor classes (3). The metabotropic receptor family is coupled by G proteins to intracellular second messenger systems and mediates biological effects on a slower time scale. So far, eight subtypes of metabotropic receptors (mGluR1–8) have been identified based on pharmacological and molecular characteristics (3). At least 23 GluR subtypes/subunits have been identified through molecular cloning (3). The multiplicity and diversity of the GluR subtypes/subunits suggest that native GluRs can exist as a combination of diverse subunits and subtypes. Indeed, *in situ* hybridization and immunohistochemical studies have indicated an extensive and heterogeneous distribution of multiple receptor subtypes/subunits with often-overlapping domains in the striatum (4–8). Furthermore, receptor autoradiography and electrophysiological studies have confirmed the presence of the four GluR classes in the

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ABBREVIATIONS: AMPA, (±)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, *N*-methyl-D-aspartate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SSC, sodium chloride/citric acid buffer; SDS, sodium dodecyl sulfate; RT-PCR, reverse transcription-polymerase chain reaction; GFAP, glial fibrillary acidic protein; NFL, low molecular weight neurofilament; GluR, glutamate receptor; mGluR, metabotropic glutamate receptor; aRNA, antisense RNA; bp, base pair(s); BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; dNTP, 2′-deoxynucleoside 5′-triphosphate.

striatum (9, 10). These receptors play a major role in short and long term potentiation (10, 11), long term depression (10), motor control, and modulation of neuronal responses (12). They may also play a role in neuronal damage occurring in the striatum in neurodegenerative diseases (13).

Although the distribution of GluRs and their mRNAs in the striatum have been investigated at the tissue section level, the composition of GluRs in single striatal neurons is still not clear. This information is of central importance in understanding the mechanisms of actions of excitatory neurotransmission in normal striatal function and in pathophysiology. Localization of more than two receptor subtype/subunit proteins or mRNAs at the single-cell level is limited with the immunohistochemical and *in situ* hybridization techniques currently available. Therefore, we used the single-cell RNA amplification technique, which allows for the screening of many mRNAs at the single-cell level, to study the expression of 16 GluR subtype/subunit mRNAs in single neurons of the striatum.

Materials and Methods

Acute dissociation procedure. Striatal neurons from adult (>4 weeks old) male Sprague-Dawley rats (Harlan, Indianapolis, IN) were acutely dissociated using procedures similar to those previously described (14). Rats were anesthetized with methoxyflurane and decapitated; the brains were quickly removed, cooled on ice, and blocked for slicing. Blocks of tissue were cut into 400-μm slices with a Vibroslice (Campden Instruments, London, UK) while being bathed in a low Ca^{2+} (100 μM), HEPES-buffered saline solution containing 140 mM Na isothionate, 2 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 23 mM glucose, and 15 mM HEPES, pH 7.4, 300–305 mOsm/liter. Slices were then incubated for 1–6 hr at room temperature (20–22°) in a $NaHCO_3$-buffered saline oxygenated with 95% O_2/5% CO_2 and containing 126 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1 mM pyruvic acid, and 10 mM glucose, pH 7.4 with NaOH, 300–305 mOsm/liter. In later experiments, reduced glutathione (100 μM) or ascorbic acid (100 μM) was added to the holding solutions to minimize oxidation damage. No difference in the results were noticed other than the neurons retained better morphology after dissociation. Slices were then placed in the low Ca^{2+} buffer, and, with the aid of a dissecting microscope, regions of the dorsal neostriatum were dissected and placed in an oxygenated cell-stir chamber (Wheaton, Millville, NJ) containing Pronase (1–3 mg/ml) in HEPES-buffered Hanks' balanced salt solution (Sigma Chemical Co., St. Louis, MO) at 35°. Dissections were limited to tissue rostral to the anterior commissure to reduce the possibility of contamination from the pallidum. After 20–30 min of enzymatic digestion, the tissue was rinsed three times in the low Ca^{2+}, HEPES-buffered saline and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension (2 ml) was then plated into a 35-mm Lux Petri dish mounted on the stage of an inverted microscope containing 1 ml of low Ca^{2+}, HEPES-buffered saline. After allowing the cells to settle, the solution bathing the cells was changed to the normal recording external solution.

RNA amplification. Whole-cell configuration of patch-clamp technique was used to record and access the cytoplasm of the individual cells (14). Electrodes were pulled from Corning 7052 glass and fire-polished before use. Electrode resistance was typically 3–6 MΩ in the bath. Recordings were obtained with an Axon Instruments 200 patch-clamp amplifier and controlled and monitored with a 486 PC running pCLAMP (version 5.0–5.5) with a 125-KHz interface (Axon Instruments, Foster city, CA). The electrode internal solution consisted of 180 mM *N*-methyl-D-glucamine, 40 mM HEPES, 4 mM $MgCl_2$, 0.1 mM BAPTA, 12 mM phosphocreatine, 2 mM Na_2ATP, 0.2 mM Na_3GTP, and 0.1 mM leupeptin, pH 7.2–7.3 with H_3PO_4 or CH_3SO_3H, 265–270 mOsm/liter. The external solution consisted of 135 mM NaCl, 20 mMCsCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.001 mM tetrodotoxin, 2–10 mM $CaCl_2$ or $BaCl_2$, and 10 mM glucose, pH 7.3 with NaOH, 300–305 mOsm/liter.

The dissociated cell was approached with positive pressure maintained on the electrode to avoid entry of cellular debris. After rupture of the membrane patch, the electrode and attached cell were lifted into a stream of control salt solution to minimize the risk of contamination from debris. In addition to internal solution, electrodes contained an RT mixture of 2 mM concentration of dNTPs, 1 ng/μl oligo$(dT)_{27}$-T7 primer, and 1 unit/μl avian myeloblastosis virus reverse transcriptase (Seikagaku America, Rockville, MD). RNA amplification does not use sequence-specific primers for amplification of individual mRNAs. The solution diffuses into the cell during the recording time and allows for the RT and synthesis of the first-strand cDNAs from the cellular mRNAs, starting at the poly$(A)^+$ tail. Therefore, each of the single-stranded cDNAs contains a T7 RNA polymerase promoter sequence. For control purposes, some cells were aspirated in a control salt solution without RT, and conversely, salt solution (without cell) was aspirated into the normal RT solution. These controls were consistently negative.

After 5–30 min of recording, the cell was aspirated into the recording pipette. The completion of first-strand cDNA synthesis and amplification proceeded as previously described (15). Briefly, the content of each electrode was expelled into an Eppendorf tube (Sigma-Aldrich Techware, St. Louis, MO), and first-strand cDNA synthesis was allowed to proceed for 60–90 min at 37°. The cDNA was extracted, precipitated, and, with T4 DNA polymerase at 14° for 5 hr, made into double-stranded template for *in vitro* transcription. Synthesis of amplified aRNA involved the use of T7 RNA polymerase (1000 units/μl) (Epicentre Technologies, Madison, WI) at 37° for 3–4 hr. The synthesized aRNA was reverse-transcribed into single-stranded cDNA, which subsequently was made into double-stranded cDNA template for a second round of *in vitro* transcription. The amplified aRNA was labeled with [^{32}P]CTP and used in screening of the Southern blots. The amplified aRNA samples were analyzed through denaturing agarose gel electrophoresis, and RNA sizes as large as 3 kb were detected. The double-stranded cDNA can be kept at −20° and used as template for amplification in multiple experiments. Amplification of water controls consistently yields negative data. Data in the present study were obtained from three sets of striatal cells that were prepared separately. The success rate with dissociated cells is very high, on the order of 80–90%.

Reverse Northern hybridization and analysis. Each of the cDNA plasmids used on Southern blots was first linearized with the proper restriction enzyme, and an aliquot was used for electrophoresis through a 1% agarose gel, stained with ethidium bromide, and visualized to ensure that the insert was the proper size. Subsequently, a 2-μg aliquot of each linearized cDNA was blotted onto nitrocellulose membrane and immobilized by baking in a vacuum oven at 80° for a minimum of 2 hr. All blots were preincubated with hybridization solution consisting of 50% formamide, 6× SSC (1× = 0.15 M NaCl and 0.015 M sodium citrate), 5× Denhardt's reagent, 100 μg/ml sheared, denatured salmon sperm DNA, and 0.5% SDS at 42° for 2 hr. The radiolabeled aRNA was added directly to this solution and incubated for 48 hr at 42°. After incubation, the blots were successively washed in 2× SSC/0.5% SDS for 5 min at room temperature, 2× SSC/0.1% SDS for 15 min at room temperature, and 0.1× SSC/0.5% SDS for 30 min at 37°, followed by a quick rinse with 0.1× SSC at room temperature. The blots were not allowed to dry and were sealed in heat-sealable plastic bags and exposed to X-ray film with intensifying screen at −70°. After autoradiograms were obtained, the blots were washed in 0.1× SSC/0.5× SDS at 51–52° for 90 min and reexposed to X-ray film with intensifying screen at −70°. The intensity of signals on blots were quantified with a laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA). An arbitrary scale was used to assign intensity levels to the signals. For each blot, the signal intensity for each mRNA was compared with that of

the background signal for the plasmid vector and of NFL mRNA. Signal/background of <2 was considered undetectable; signal/NFL of ≤30% was considered low; signal/NFL of >30% and ≤75% was considered medium, and signal/NFL of >75% was considered high. To compare the levels of mRNA signals within one neuron, the signal intensity for mRNAs in each neuron was ranked, with the highest rank assigned to the lowest (including undetectable) signal and the lowest rank (a value of 1) to the most intense signal.

RT-PCR for metabotropic receptor mRNAs in striatal slices. Adult male Sprague-Dawley rats were killed by decapitation, and the brains were immediately removed, cooled, and blocked for slicing on a vibratome. Four coronal slices (400 μm thick) were prepared from each brain, starting at the level of +1.7 mm anterior to bregma (41). The same low Ca^{2+} buffer and incubation buffer used in tissue preparation for RNA amplification were used for these experiments (see above). Three experimental paradigms were used to prepare striatal tissue. Tissue from each rat (four slices) were used for only one paradigm. Dorsal striatum was separated from each slice before freezing. Dorsal striatum from one brain was immediately frozen after slicing (control). Tissue from a different rat was incubated in oxygenated incubation buffer immediately after slicing for 3 hr, and then the dorsal striatum was dissected out and frozen (incubated). Tissue from another rat was incubated in oxygenated incubation buffer containing 100 μg/ml of transcription inhibitor actinomycin D (Life technologies, Gaithersburg, MD) for 3 hr, and the dorsal striatum was dissected and frozen (incubated plus actinomycin D). Striatal tissue RNA was extracted from these tissue samples with the guanidine monothiocyanate/LiCl procedure (16) and kept at −70°.

Striatal RNA was treated with 2 units of RQ1 RNase-free DNase (Promega, Madison, WI) for 20 min at 37°. The RNA was then extracted with phenol and chloroform and precipitated with ethanol and glycogen. First-strand cDNA was synthesized with the use of 200 ng of hexanucleotide random primer (Boehringer Mannheim, Indianapolis, IN) and 200 units of Superscript II reverse transcriptase (Life Technologies) according to the manufacturer's protocol. PCR primers used for detection of the mGluR3 mRNA were 5′-CGACAACAATGTGCATCTCC-3′ (bases 2540–2559) and 5′-ACAATCACAGAGATGAGGTGG-3′ (bases 2765–2785). The size of the amplified mGluR3 cDNA is 246 bp (GenBank accession No. M92076). Primers for the mGluR4 mRNA were 5′-TCTGAGGATG-GCACATTGG-3′ (bases 2847–2865) and 5′-AACAGACAACACATG-CAGAGC-3′ (bases 3195–3215). The size of the amplified mGluR4 cDNA is 369 bp (GenBank accession No. M92077). Primers for the mGluR5 mRNA were 5′-TCCAATCTGCTCCTCCTACC-3′ (bases 3432–3451) and 5′-CAACGATGAAGAACTCTGCG-3′ (bases 3761–3780). The size of the amplified mGluR5 cDNA is 349 bp (GenBank accession No. D10891). PCR experiments used 2.5 μl of the first-strand cDNA. Each PCR tube contained 1× PCR buffer (1× = 10 mM Tris·HCl, pH 8.3 at 25°, 50 mM KCl, and 0.001% gelatin), 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT), 1 μM concentration of each primer, 0.2 mM concentration of dNTPs, and 2.5 mM $MgCl_2$ in a final volume of 50 μl. The PCR conditions were 95° for 3.5 min for one cycle (initial denaturation step), after which $MgCl_2$ was added (Hot Start PCR), and then 94° for 1.5 min, 55° for 2 min, and 72° for 2 min, for a total of 35 cycles. Samples also underwent a final extension time of 10 min at 72°. A 25-μl aliquot of the PCR products was analyzed through electrophoresis on a 2% agarose gel containing ethidium bromide (0.5 μg/ml) and visualized with UV light.

RT-PCR for metabotropic receptor mRNAs in single striatal neurons. Preparation of the striatal single cells, recording, extraction of cellular RNA, and synthesis of the single-stranded cDNA followed the same procedures as described for RNA amplification technique. PCR experiment included 2 μl of the single-stranded cDNA template, 0.2 mM concentration of dNTPs, 1 μM concentrations of each primer, 2.5 units of AmpliTaq DNA polymerase, 1× PCR buffer, and 2.5 mM $MgCl_2$ in a final volume of 50 μl. The PCR conditions were 95° for 5 min for one cycle, and then 94° for 1 min, 56° for 1 min, and 72° for 1.5 min, for a total of 45 cycles. A final extension time of 10 min at 72° was also included. The products were analyzed as described above.

cDNA sources. The cDNAs for AMPA/kainate receptor subunits (GluR1–7) were obtained from the Salk Institute, Molecular Biology Laboratory (San Diego, CA). The GluR1–7 cDNAs were 2992, 3505 (FLOP), 3083 (FLOP), 2971 (FLIP), 3250, 4559, and 3593 bp, respectively, and they all contained both 3′- and 5′-untranslated regions. The sequence homology between the GluR1–7 cDNAs ranges from 39% (GluR1, GluR4, and GluR7) to 81% (GluR6 and GluR7) (17). The cDNAs designated as FLIP or FLOP isoforms are capable of hybridizing to both forms of the receptor mRNA. The NMDAR1 cDNA was 4213 bp (18) and has ~25–29% homology with the GluR1–7 cDNAs (3). NMDAR2 (A–C) cDNAs were 4717, 4560, and 3289 bp (Dr. D. Pritchett, Department of Pharmacology, University of Pennsylvania, Philadelphia, PA), and each shows ~15% homology to NMDAR1 and ~50% homology to each other (19). The mGluR cDNAs (mGluR1–5) were 4.64, 3.34, 3.24, 3.94, and 8.54 kb, respectively (3, 20). The sequence homology between these cDNAs ranges from 39% (mGluR1 and mGluR4) to 67% (mGluR2 and mGluR3) (21). The rat GFAP and NFL cDNAs were 2.5- and 1.6-kb sequences, respectively (22, 23). The Kv3.1 cDNA was 1793 bases, corresponding to nucleotides 1162 to 2955 of the rat cDNA, which contains sequences shared by Kv3.1 α and β isoforms (24). The Kv4.2 cDNA was a 2.7-kb sequence from rat hippocampus (25).

Results

A total of 24 acutely dissociated adult striatal cells were processed for mRNA amplification in this study. After whole-cell recording was established, the cellular cytoplasmic content of these cells was harvested into the recording electrode and processed for mRNA amplification. The phenotype of these cells was established by examining the presence of mRNAs encoding the NFL protein, a neuron-specific marker, and GFAP, an astrocyte-specific marker. All 24 cells studied expressed the NFL mRNA, whereas none demonstrated the presence of GFAP mRNA (Fig. 1A). The possibility of a long

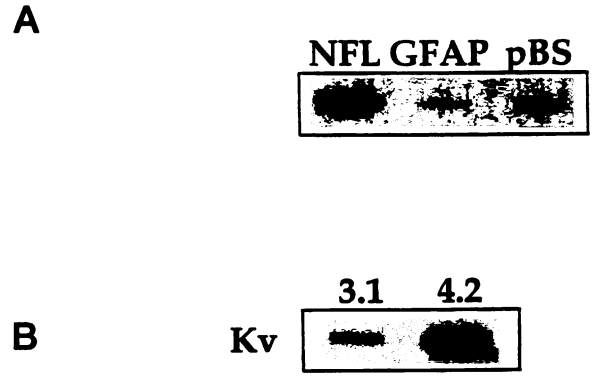

Fig. 1. Representative examples of signals for phenotypical markers of striatal efferent neurons examined with RNA amplification in acutely dissociated neurons of the adult rat striatum. Cellular mRNA was accessed through the whole-cell patch-clamp technique, the cellular RNA was amplified as described in Materials and Methods, and the radiolabeled amplified RNA hybridized to cDNAs was immobilized on nitrocellulose membranes. A, All striatal cells examined showed a strong signal for the neuron-specific filament (*NFL*), but none showed any detectable levels of glial fibrillary acidic protein (*GFAP*) mRNA. The nonspecific background hybridization was determined with the hybridization signal of pBluescript SK(−) (*pBS*), which was taken into account for quantifying the signal intensities. B, Strong signals for the mRNA encoding the Shal-like potassium channel Kv4.2 were seen in most neurons; however, the shaw-like Kv3.1 potassium channel mRNA gave very low signals in these neurons.

term general transcriptional activity in these neurons due to tissue preparation procedure was examined by investigating the expression of the immediate early gene c-*fos* and c-*jun* mRNAs. Both c-*fos* and c-*jun* mRNAs were undetectable in all neurons examined (n = 16) (data not shown). The pattern of mRNA hybridization signals for individual neurons did not change after the second wash of higher stringency (51–52° for 90 min), which confirms the high degree of hybridization specificity under our experimental conditions. Hybridization signal for the plasmid vector pBluescript SK$^{(-)}$, an indicator of nonspecific hybridization, was very low or undetectable (Fig. 1A) and was used to determine the level of nonspecific background for each blot.

We also examined the mRNA expression pattern of two potassium ion channels, the shaw-like Kv3.1, which in the striatum is only detected through *in situ* hybridization histochemistry in a subpopulation of interneurons (26), and the shal-like Kv4.2, which is expressed by the majority of striatal efferent neurons (27). The Kv3.1 mRNA signal intensity was very low in the 10 striatal neurons examined for this mRNA. In contrast, the signal intensity for Kv4.2 mRNA was medium to high in 87% and low in 13% of the striatal neurons examined (Fig. 1B).

Expression of AMPA/kainate receptor subunits in striatal neurons. All striatal neurons examined (24 neurons) expressed a strong signal for mRNAs encoding AMPA receptor subunits GluR1–3, and most neurons also expressed GluR4 mRNA (Fig. 2A). The intensity of the mRNA signals for the AMPA receptor subunits was measured in a subset of 16 striatal neurons using laser scanning densitometry. The cDNA probes used for GluR1–4 can hybridize to both FLIP and FLOP isoforms of the mRNA transcripts (28). Therefore, the signal intensity for AMPA receptor subunits reflects the sum of both mRNA isoforms. Ranking of the mRNA signal intensities revealed heterogeneity in the relative level of mRNA expression within individual striatal neurons (Table 1). GluR1 mRNA signal was lower than that of GluR2 and GluR3 in 87% of striatal neurons. GluR2 mRNA signal was the most intense in half of the neurons, whereas in the other half, GluR3 mRNA signal was equal to or more intense than GluR2 mRNA. In 69% of neurons, GluR4 mRNA signal was the lowest of the AMPA receptor subunits. It was noticeable, however, that in contrast to the other AMPA receptor mRNAs, the intensity of the GluR4 mRNA signal markedly differed among neurons. Although the signal for GluR4 mRNA was lower than that for the other AMPA subunit mRNAs in all except one neuron, compared with NFL, it was high in 44%, medium to low in 31%, and undetectable in 25% of the neurons examined.

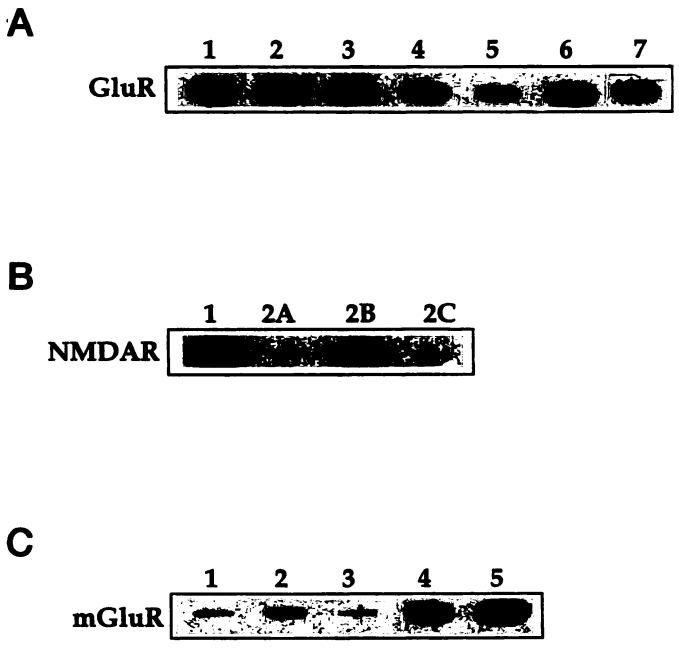

Fig. 2. Representative blots for GluR subunit/subtypes mRNAs in single striatal neurons. A, The mRNAs for all subunits of AMPA and kainate receptors (GluR1–7) were expressed in the majority of striatal neurons examined. However, the relative abundance of the subunits varied in individual neurons. B, NMDAR1 and NMDAR2B subunit mRNAs were expressed at high levels in all neurons. However, the NMDAR2A and NMDAR2C subunit mRNAs were low to undetectable in all neurons. C, Expression of the metabotropic receptor mRNAs was heterogeneous in individual neurons. This example shows a cell with a low signal for mGluR2, whereas mGluR1 and mGluR3 signals were undistinguishable from the vector control in this blot. In contrast, signals for mGluR4 and mGluR5 mRNAs were higher in this cell, as in all of the neurons examined.

Among the kainate receptor subunits (GluR5–7), GluR6 mRNA signal was the most intense in all 16 striatal neurons analyzed quantitatively (Fig. 2A and Table 1). GluR7 mRNA signal was detected in all neurons examined, but GluR5 mRNA signal was undetectable in one of the neurons. GluR5 mRNA signal was the least intense of the kainate receptor subunits (Table 1). In a majority of neurons examined, the mRNA signal intensity for kainate receptor subunits were lower than that for AMPA receptor subunits.

Expression of mRNAs encoding NMDA receptor subunits in striatal neurons. The NMDAR1 cDNA used on Southern blots can hybridize all splice variants of the NMDAR1 mRNA transcript (29). Therefore, the NMDAR1 mRNA signal detected is the sum of all mRNA isoforms present in these striatal neurons. NMDAR1 receptor mRNA was present in all 24 striatal neurons examined (Fig. 2B). The intensity of the mRNA signals was measured in a subset of 16 striatal neurons. NMDAR1 mRNA signal had the highest intensity in 81% of these neurons (Table 1). For the NMDAR2 subunits, NMDAR2B mRNA signal was detected in all neurons, whereas NMDAR2A and NMDAR2C mRNA signals were very low or undetectable in all 16 neurons (Fig. 2B and Table 1).

Expression of metabotropic receptors mRNAs in striatal neurons. The expression of the mGluR mRNAs was examined in 15 striatal neurons. The mRNA signal intensity for these receptors was heterogeneous between neurons, with varying mRNA signals within individual neurons (Fig. 2C). mGluR1 receptor mRNA signal was undetectable in 73% and mGluR2 receptor mRNA signal was undetectable in 27% of the neurons examined. In the remaining neurons, although detectable, the signal was very low. However, signal for mGluR2 mRNA was always higher than that for mGluR1 mRNA (Table 1). In the 15 striatal neurons examined, signal for mGluR3 receptor mRNA was undetectable or undistinguishable from the nonspecific signal given by the Bluescript plasmid on the same blot. In contrast, signals for mGluR4 and mGluR5 mRNAs were detected in all neurons examined and were always higher than for mGluR1 and mGluR2 (Table 1). The level of mGluR4, however, varied between neurons, from high (80%) to low (7%), when compared with NFL. In 60% of the neurons, mGluR5 mRNA

TABLE 1

The expression pattern of AMPA, kainate, NMDA, and metabotropic receptor subunits/subtypes in single striatal neurons

The hybridization signals for receptor subunits/subtypes were rank-ordered in each neuron according to the signal intensity as measured with scanning laser densitometry. The most intense hybridization signal was assigned a 1; the least intense signal, a 4 (AMPA and NMDA), 3 (kainate), or 5 (metabotropic). In case of a tie in ordering, the ranks were summed and then divided by the number of signals.

Neuron	AMPA/kainate (GluR)							NMDA				mGluR				
	1	2	3	4	5	6	7	1	2A	2B	2C	1	2	3	4	5
1	3	1.5	1.5	4	3	1	2	1	3.5	2	3.5	4.5	3	4.5	1	2
2	3	1.5	1.5	4	3	1	2	1	3.5	2	3.5	4.5	3	4.5	2	1
3	2.5	1	2.5	4	3	1	2	1	3.5	2	3.5	4	4	4	2	1
4	3.5	2	1	3.5	3	1	2	1	3.5	2	3.5	4.5	3	4.5	2	1
5	2.5	1	2.5	4	3	1	2	2	3.5	1	3.5	4	4	4	1.5	1.5
6	3	1	2	4	3	1	2	1	3.5	2	3.5	4	4	4	2	1
7	3	1.5	1.5	4	3	1	2	1	3	2	4	4.5	3	4.5	1.5	1.5
8	3	1.5	1.5	4	3	1	2	1	3	2	4	4.5	3	4.5	2	1
9	3	1	2	4	3	1	2	1	3.5	2	3.5	4	4	4	2	1
10	3	1	2	4	2.5	1	2.5	1	3.5	2	3.5	4	4	4	2	1
11	4	3	1.5	1.5	3	1	2	1.5	3.5	1.5	3.5	4.5	3	4.5	1	2
12	4	3	1	2	3	1	2	1	3.5	2	3.5	ND	ND	ND	ND	ND
13	4	1	2.5	2.5	3	1	2	1	3.5	2	3.5	4.5	3	4.5	1	2
14	4	2.5	1	2.5	3	1	2	1.5	3.5	1.5	3.5	4.5	3	4.5	1	2
15	3	1	2	4	3	1	2	1	3.5	2	3.5	4.5	3	4.5	1	2
16	3	1	2	4	3	1	2	1	3.5	2	3.5	4.5	3	4.5	1	2

ND = Data not available.

signal was either the most intense or at the same level as the mGluR4 mRNA (Table 1). All 15 striatal neurons studied showed colocalization of mGluR4 and mGluR5 mRNAs with mGluR1 and mGluR2 mRNAs when they were detectable. Specifically, mGluR1, mGluR2, mGluR4, and mGluR5 were colocalized in 27% and mGluR2, mGluR4, and mGluR5 were colocalized in 47% of the neurons.

RT-PCR analysis of metabotropic receptor mRNAs. Dorsal striatal tissue of adult rats was prepared and treated according to the same protocol as for RNA amplification experiments and was used to examine the presence of metabotropic receptor mRNAs (mGluR3–5). These receptor mRNAs were chosen because the expression patterns of mGluR3 and mGluR4 were in contrast to, and mGluR5 mRNA pattern was in agreement with, *in situ* hybridization data (8, 30, 31). mGluR3 and mGluR5 mRNAs were present in the dorsal striatum before and after incubation and were also present in the tissue incubated in the presence of actinomycin D (100 μg/ml). In contrast, PCR did not detect the presence of mGluR4 mRNA in any of the striatal tissue prepared (Fig. 3). The presence of enkephalin mRNA in these striatal tissue was examined as a positive control for the quality of the RNA and the status of the PCR experiment. All three striatal samples showed the presence of enkephalin mRNA (data not shown). Treatment of the tissue RNA with DNase was performed to ensure that the source of the cDNA templates for the PCR amplification was the cellular RNA and that there was no contamination from the genomic DNA. RT-PCR experiments for metabotropic receptors were also performed on single striatal neurons. Two neurons were examined for the presence of mGluR3–5 mRNA. mGluR3 was not detected in these two neurons, whereas one showed the presence of mGluR4, and the other neuron showed the presence of mGluR5 (Fig. 4). The negative control samples, which did not have the RT step, did not show any amplified bands (Fig. 4).

Colocalization of GluR subtypes/subunits in single striatal neurons. An examination of the GluR expression pattern in striatal neurons revealed that the striatal neurons

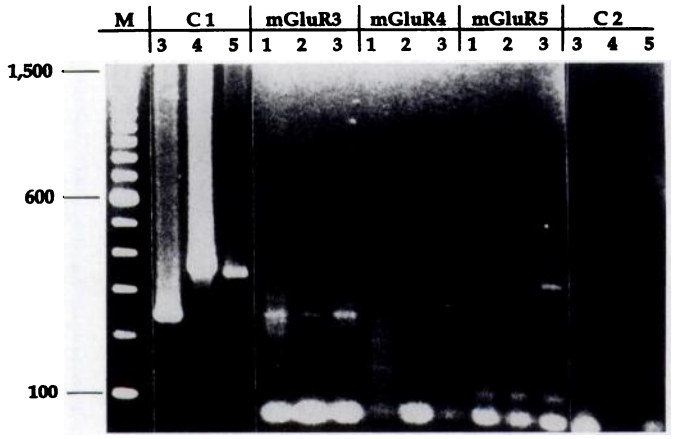

Fig. 3. RT-PCR analysis of the metabotropic receptor mRNA in striatum. The cDNA templates were obtained from RT of total striatal RNA. mGluR3–5 amplified fragments are 246, 369, and 349 bp, respectively. The C1 samples, positive controls, are control reactions that used metabotropic receptor cDNA plasmids as cDNA templates and show the appropriate amplified fragments. The C2 samples, negative controls, are control reactions that did not include cDNA templates and do not show any amplified products. *C1 and C2, 3–5*, mGluR3–5. *mGluR3–5*, striatal samples that were analyzed for metabotropic receptor mRNAs. *1*, Striatal slice that was immediately frozen; *2*, striatal slice that was incubated in oxygenated physiological buffer for 3 hr and then frozen; *3*, striatal slice that was incubated in oxygenated physiological buffer in the presence of actinomycin D (100 μg/ml) for 3 hr and then frozen. *mGluR3*, all three striatal samples show the appropriate amplified fragment. *mGluR4*, PCR amplification did not detect any amplified fragment for the samples. *mGluR5*, all three samples show the appropriate amplified fragment. The fragment in *sample 2* is present, but the band intensity was very low, which may make the band difficult to see in this figure. *Bottom of most lanes*, residual primer bands. *M*, molecular weight ladder.

examined coexpressed mRNAs for the four major GluR classes and that individual striatal neurons colocalize at least 11–13 GluR subtypes/subunits (Table 2).

Discussion

The widespread distribution of the mRNAs for the four classes of GluRs observed in this study is in agreement with

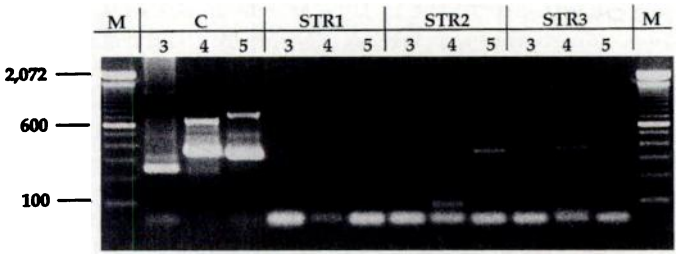

Fig. 4. RT-PCR analysis of the metabotropic receptor mRNAs in single striatal neurons. *C*, positive controls, are control reactions that used total RNA from rat cortex. *3–5*, mGluR3–5. The cortical samples show the presence of the three metabotropic receptors (246, 369, and 349 bp, respectively). *STR1*, RNA from single striatal neurons that was not reverse-transcribed before PCR for mGluR3–4. *STR2* and *STR3*, RNA from single striatal neurons that was reverse-transcribed before PCR. *STR1*, without RT reaction, does not show any amplified DNA for any of the metabotropic receptor mRNAs. *STR2* and *STR3*, with RT reaction, show one amplified fragment for mGluR4 or mGluR5, respectively. The other extraneous bands in *C* and *STR2* (mGluR4) lanes are nonspecific by-products of PCR amplication. *Bottom of most lanes*, residual primer bands. *M*, molecular weight ladder.

TABLE 2

The composition of the glutamate receptor subtypes and subunits in single striatal neurons

The numbers in parentheses indicate the number of subunits that were examined, and the numbers in the columns indicate the number of subunits that were present in each of the striatal neurons. The sum of all the subunits present in each striatal neurons is indicated in the last column.

Neuron	AMPA (4)	Kainate (3)	NMDA (4)	mGluR (5)	Total
1	4	3	2	3	12
2	4	3	2	3	12
3	4	3	2	2	11
4	4	3	2	3	12
5	3	3	3	2	11
6	3	3	4	3	13
7	4	3	3	2	12
8	4	3	3	3	13
9	4	3	2	2	11
10	4	3	2	4	13
11	4	3	2	4	13
12	4	3	2	ND	9
13	4	3	2	3	12
14	4	3	2	3	12
15	3	3	2	4	12
16	3	2	2	4	11

ND = Data not available.

the compelling biochemical, physiological, and pharmacological evidence for extensive glutamatergic neurotransmission in the striatum. The results provide the first comprehensive view of the expression and colocalization of GluR classes in single neurons of the adult rat striatum.

RNA amplification technique: validity and specificity of the results. The validity of the RNA amplification results has been previously discussed (14) and has been confirmed in this study by the negative data obtained with all negative and RT-negative controls (see Materials and Methods), the absence of glial contamination, the absence of immediate early gene mRNA lasting induction, the very low-to-undetectable hybridization to the plasmid vector, and the consistent amplification of some mRNA species over others. The specificity of the hybridization data is shown by the preservation of the mRNA signal patterns after the more stringent wash at 51–52° compared with the first wash at 37°. The significant differences in hybridization signal intensity between multiple GluR subunits (GluR5–7) belonging to the same class with 74–81% sequence homology further indicates that, under our hybridization and wash conditions, the hybridization signals are specific and that minimal, if any, cross-hybridization occurs between homologous subunit cDNAs.

AMPA/kainate receptor subunits in striatal neurons. In agreement with data from *in situ* hybridization experiments (6, 32, 33), our results indicate that all four AMPA receptor subunit mRNAs (GluR1–4) are expressed and colocalized in a majority of adult striatal neurons, with a heterogeneous signal intensity within individual neurons. The relative signal intensity of these subunit mRNAs is also in agreement with *in situ* hybridization data indicating that GluR2 and GluR4 mRNAs have the highest and lowest level of expression, respectively, and GluR1 and GluR3 mRNAs have intermediate levels of expression (6, 33, 34). However, the signal intensity for GluR4 was higher than expected after RNA amplification in 44% of the neurons examined. These cells were not different from the other neurons analyzed with regard to their pattern of expression of the other subtype/subunit mRNAs examined or the level of expression of the potassium channel Kv4.2. It is unclear whether they correspond to a distinct subpopulation of striatal neurons. Immunohistochemical investigations with specific antibodies against AMPA receptor subunits have indicated that in dorsal striatum, medium-sized spiny neurons are stained with moderate intensity with antibodies for GluR1 and GluR2–3 and with much lighter intensity with antibodies for GluR4 (4, 5). Thus, protein localization studies support RNA amplification results on the expression pattern of these subunit mRNAs, except for those cells with high signal for GluR4 mRNA. It should be noted that the differences in level of expression of mGluR4 mRNAs were the most pronounced among the 16 mRNAs examined.

Kainate receptor subunit mRNAs (GluR5–7) were also expressed in all striatal neurons examined, with the exception of one neuron that did not express a detectable level of GluR5 mRNA. *In situ* hybridization studies have detected GluR5–7 expression in striatum, with GluR6 and GluR5 mRNAs being high and low to undetectable, respectively (17, 34). Although a specific antibody against a common epitope of GluR5–7 has been used to study the distribution of these receptors in the brain, immunohistochemical data are not available for the striatum. However, immunohistochemical observations in the cerebral cortex of Macaque monkeys have shown an extensive colocalization of the AMPA (GluR2–3) and kainate receptor subunits (GluR5–7) (35). Our observations confirm the notion of extensive colocalization of the different classes of GluRs in neurons and extend this observation to the striatum.

NMDA receptor subunits in striatal neurons. The signal detected for the NMDAR1 mRNA is the summed expression of all isoforms present in striatum. The RNA amplification data were in agreement with *in situ* hybridization and immunohistochemical studies that revealed a high level of expression NMDAR1 receptor mRNA and protein in all medium-sized neurons of the striatum (7, 18, 36). Also in agreement with *in situ* hybridization data (7), our results indicate a high signal intensity for NMDAR2B mRNA but very low-to-undetectable signal level for NMDAR2A and NMDAR2C mRNAs (Fig. 3B).

Recent studies indicate that native NMDA receptors could

be assembled from heterogeneous combinations of receptor subunits and exhibit different physiological properties (19). However, *in situ* hybridization and RNA amplification data indicate that at the mRNA level, this heterogeneity is not observed in striatal neurons. This observation might indicate that the NMDA receptors present in striatum represent a homogeneous receptor population. Alternatively, heterogeneous expression of subunit protein may occur through translational regulation, allowing for assembly of various receptor complexes.

mGluR subtypes in striatal neurons. As with *in situ* hybridization histochemistry (8), RNA amplification results indicated that striatal neurons examined express low levels of mGluR1 mRNA signal. The mGluR1 cDNA used in this study corresponds to the mGluR1a splice variant but can also hybridize the mGluR1b and R1c isoforms (37). Electron microscopic examination of mGluR1a immunoreactivity in striatum has revealed a dense staining of presynaptic terminals, although occasional postsynaptic neurons also stain for mGluR1 protein (38). Therefore, it seems that mGluR1 receptor is, for the most part, a presynaptic receptor in the striatum with low expression in postsynaptic neurons, in agreement with the low level of mRNA detected through RNA amplification.

Our data indicate a low level of mGluR2 mRNA signal for the majority of striatal neurons examined. *In situ* hybridization has revealed that within the striatum, most neurons are not labeled for mGluR2 mRNA; however, a small number of scattered neurons (1–2% of the total population), mostly large and polygonal, are weakly to moderately labeled (8, 39). The difference between RNA amplification and *in situ* hybridization is likely to be due to the high sensitivity of the former technique, enabling it to detect a lower level of mGluR2 mRNA than *in situ* hybridization.

Signals for mGluR5 mRNA were high in the sample population examined with RNA amplification. In agreement with this observation, RT-PCR experiments showed the presence of mGluR5 mRNA in striatal tissue, indicating that this mRNA was stable during the time required for slice preparation. RNA blot analysis and *in situ* hybridization have also revealed that the striatum exhibits high levels of mGluR5 mRNA. In the dorsolateral striatum, ~75% of neurons are strongly labeled for mGluR5 mRNA (8). Furthermore, the striatum is one of the most intensely labeled brain region for mGluR5 immunoreactivity (40). Electron microscopic examination of the striatum revealed accumulation of dense immunoreaction product in dendritic profiles and in the neuronal cell bodies, frequently in the vicinity of the cell membrane (40). This suggests that striatal neurons express the receptor protein, in agreement with the presence of the corresponding mRNA in intrinsic striatal neurons.

The major discrepancy between the patterns of expression observed with *in situ* hybridization histochemistry and the RNA amplification data of the present study concerned mGluR3 and mGluR4. In contrast to the consistently negative signal observed after RNA amplification, *in situ* hybridization studies revealed the presence of mGluR3 RNA in the majority of striatal neurons (8, 31). In an attempt to resolve this discrepancy, expression of mGluR3 mRNA was examined through RT-PCR in single neurons and in striatal slices. Results in single neurons were in agreement with the RNA amplification data, and those in slices were in agreement with *in situ* hybridization. The results in the slice preparation indicate that this mRNA is stable for the duration of the tissue preparation steps. It is possible that mGluR3 mRNA detected in the slices is contained in glial cells (8); however, this does not explain the failure to detect the mRNA in single neurons because *in situ* hybridization studies showed expression in striatal neurons as well (8). Because RNA amplification and RT-PCR in single cells share the step of RT of mRNA without prior extraction, one may hypothesize that the secondary structure and/or cellular components associated with mGluR3 mRNA hinder the process of RT of this mRNA under the conditions of this study.

In contrast to mGluR3 mRNA, mGluR4 mRNA was readily detected through RNA amplification, and at least in some neurons through single-cell RT-PCR, but not through *in situ* hybridization or RT-PCR of mRNA extracted from striatal slices before or after incubation. Because negative controls for RNA amplification and single-cell RT-PCR were consistently negative, it is highly unlikely that detection of mGluR4 mRNA through these methods was artifactual. On the other hand, the lack of detection of this mRNA in the slice preparation suggests that the negative data of *in situ* hybridization were not due to lack of sensitivity of the method or subcellular compartmentation of the mRNA. Although highly unusual among the large number of mRNAs examined to date, the discrepancy in the pattern of expression of mGluR4 mRNA in striatal neurons with the use of different methods remains unexplained and stresses the need to compare the results of multiple approaches before drawing definite conclusions regarding mRNA expression in single neurons.

Conclusion. The results of this work suggest that the four classes of GluRs (AMPA, kainate, NMDA, and metabotropic) are colocalized in single medium-sized striatal neurons. This notion is supported by *in situ* hybridization and immunohistochemical observations that indicate the extensive distribution of the GluR subtypes/subunits throughout striatum, as discussed above. Functional studies also support this observation. Iontophoresis of kainate, quisqualate, and NMDA produces depolarization in postsynaptic neurons of striatum. Furthermore, activation of corticostriatal fibers produce both NMDA- and non-NMDA-mediated excitatory postsynaptic potentials in striatal neurons (10). For the large majority of the mRNAs examined in this study, the results of RNA amplification are in excellent agreement with data from other methods of mRNA and protein detection at the cellular level, namely, *in situ* hybridization histochemistry and immunohistochemistry. This strongly supports the validity of the colocalization data derived from the use of RNA amplification in acutely dissociated single cells. These results are unique in that they provide information on the profile of expression of a large number of mRNAs in individual neurons with a level of specificity that cannot always be matched at the protein level with the available antibodies.

A major finding of this study was that relatively little heterogeneity in the pattern of expression of mRNAs encoding subtypes/subunits of the GluRs was observed among the 15–24 cells examined in this study. Considering that these cells are likely to be striatal efferent neurons, this suggests that absence or presence of distinct subtypes/subunits of the GluR is not a major feature distinguishing subpopulations of striatal efferent neurons. There were, however, clear differences in the ranking of signal levels within neurons. This

raises the possibility that heterogeneous combinations of GluR subtypes/subunits exist at excitatory synapses, thus providing various modulatory but synapse-specific roles for glutamatergic neurotransmission in striatum.

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