Coexpression Patterns of mGLuR mRNAs in Rat Retinal Ganglion Cells: A Single-Cell RT-PCR Study

Arman Tebrani, Thomas H. Wheeler–Schilling, and Elke Guenther

PURPOSE. Eight different subunits of metabotropic glutamate receptors (mGluRs) are known to date. mGluRs have been linked to an extensive list of neuromodulatory effects, depending on which intracellular or membrane-bound effector system is activated. Activation of mGluRs can influence neuronal activity and can result in changes of intracellular Ca\textsuperscript{2+}, thus leading to changes in factors that are known to be crucial for cellular differentiation and cell death. Because mGluRs are known in modulating both intracellular and intercellular activities, this study was designed to determine which types of mGluRs are coexpressed in a neuron and whether distinct coexpression patterns can be found that reflect the different physiological requirements of a neuron at different stages of development and to learn whether neuronal injury results in adaptive changes of mGluR expression.

METHODS. Juvenile and adult rat retinal ganglion cells (RGCs) and adult RGCs after axotomy were analyzed for their gene expression pattern of mGluRs by single-cell reverse transcription–polymerase chain reaction (RT-PCR).

RESULTS. Adult RGCs predominantly expressed one or two different mGluR mRNAs, whereas juvenile RGCs coexpressed two and more. mGluR3, -5, and -7 mRNAs were found more frequently in juvenile than in adult RGCs, whereas mGluR6 was detected in juvenile RGCs in low abundance but never in adult RGCs. However, mGluR6 was expressed in adult RGCs after axotomy. mGluR1 and -7 were also found more frequently in axotomized RGCs than in the adult control group.

CONCLUSIONS. All types of mGluR mRNAs are expressed in RGCs. This is in contrast with previous reports, suggesting that in situ hybridization and immunohistochemical studies in which expression of mGluR3, -5, and -6 was not reported. The expression of some mGluR mRNAs seems to be developmental, although no distinct copatterns were found. (Invest Ophthalmol Vis Sci. 2000;41:314–319)

G glutamate is the major excitatory neurotransmitter in the central nervous system and exerts its action through ionotropic (iGluRs) and metabotropic receptors (mGluRs). Recent molecular cloning studies have identified eight different subtypes of mGluRs, all of which are coupled to guanosine triphosphate (GTP)–binding proteins (G-proteins) and possess seven putative membrane-spanning domains. Although structurally related, mGluR subtypes are highly heterogeneous in their agonist selectivity, signal transduction mechanisms, and distribution in the brain. Because mGluRs interact with iGluRs, other ion channels, and mem- brane enzymes, they can modulate cellular activities and are involved in processes of differentiation and degeneration. mGluRs have been divided into three subclasses according to the second-messenger pathways activated and their pharmacologic properties. Activation of group I receptors (mGluR1 and -5) results in an increase in intracellular calcium through a phospholipase C-inositoltriphosphate pathway and inhibits potassium currents. mGluR1 also stimulates cyclic adenosine monophosphate (cAMP) formation and arachidonic acid release, whereas mGluR5 does not. Different agonists selectively activate mGluRs of group II (mGluR2 and -3) and group III (mGluR4, -6, -7, and -8), resulting in a decreased formation of cAMP or cyclic guanosine monophosphate (cGMP) through inhibition of adenylyl cyclase or phosphodiesterase, respectively. Different types of calcium channels are inhibited by mGluRs of groups II and III, probably resulting in a decreased intracellular calcium concentration and antagonizing group I mGluR action when coexpressed within the same cell.

The heterogeneity of mGluRs indicates that they have diverse functions in the brain, although the signal transduction mechanisms studied in heterologous expression systems may differ from those of the native receptors expressed in situ. The diversity of mGluR functions is supported by the finding that some types of mGluRs (mGluR2, -3, -5, and -7) are widely expressed throughout the brain, whereas others (mGluR1 and -8) have a more restricted expression. For example, mGluR6 is mainly restricted to the retina and seems to play an important role in retinal synaptic transmission within the on pathway (see References 17 and 18, but see Reference 20).

In view of the different functions that different subtypes of mGluRs may have in modulating cellular and intracellular activities, we were interested in finding which types of mGluRs...
are coexpressed in a single neuron and whether different coexpression patterns can be found under different physiological conditions—for example, at distinct stages of neuronal development or as a result of adaptive changes due to neuronal injuries. To clarify this issue, a detailed single-cell reverse transcription–polymerase chain reaction (RT-PCR) examination of mGluR1 through -8 mRNAs was performed in rat retinal ganglion cells (RGCs) at different ages of development and after optic nerve axotomy. Part of the present study has been published in abstract form.21

**METHODS**

**Animals**

This study was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the European Communities Council Directive (86/609/EEC). Pigmented rats (Brown Norway) at postnatal day (P)7 to P14 and adult rats (>P60) were killed by decapitation. Each eye was dissected immediately after enucleation at room temperature by hemisection along the ora serrata and lifting off the anterior segment. The retinas were gently removed from the underlying tissue. The same procedure was used for adult axotomized rats (>P60, axotomized). Axotomy was performed by an intrabulbar cut of the optic nerve in one eye, as described previously.22 After surgery, rats were allowed to recover for 4 days. To identify RGCs, all animals were neonatally labeled by injection of 2 μl of the fluorescent dye DiI (25 mg/500 μl; Molecular Probes, Goettingen, Germany) into the superior colliculus.

**Single-Cell RT-PCR for mGluR1 through -8**

The retinas were kept in extracellular solution (ec1) containing 130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 10 mM HEPES, and 20 mM glucose (pH 7.4) continuously bubbled with oxygen. Retinal slices of 200 μm were cut transversely in ice-cold saline. Finally, the slices were incubated in ec1, continuously bubbled with oxygen, and stored at room temperature.23 After a 1-hour incubation, retina slices were transferred into a chamber on a microscope (Axioskop, Zeiss, Oberkochen, Germany) and superfused with oxygenated ec1. A glass pipette with an opening diameter of 1 μm, filled with 8 μl intracellular solution (ic) containing 90 mM CsAc, 40 mM CsCl₂, 10 mM EGTA, 1 mM MgCl₂, 0.2 mM CaCl₂, 10 mM HEPES (pH 7.2), was lowered to the membrane of an RGC, identified by its fluorescence. After a whole-cell configuration had been established, the RGC content was harvested under visual control into the glass pipette by applying negative pressure.

**cDNA Synthesis**

The content of each patch pipette was expelled completely into a 0.2-ml tube (MicroAmp; Perkin Elmer, Weiterstadt, Germany) on ice, by using positive pressure. The tubes contained 1 μl hexamer random primers (2 μg/μl; Boehringer–Mannheim, Mannheim, Germany), 1 μl dNTPs (10 mM; MBI Fermentas, St. Leon-Rot, Germany), 1 μl MgCl₂ (25 mM; Perkin Elmer), 1 μl 10X reaction buffer containing 100 mM Tris-HCl (pH 8.3) and 500 mM KCl (Perkin Elmer), 0.5 μl RNase inhibitor (50 U/μl; Rnasin; Promega, Madison, WI), and 0.5 μl MuLV reverse transcriptase (50 U/μl; Perkin Elmer). The reaction was incubated at 25°C for 10 minutes and then at 42°C for 60 minutes. PCR amplification was performed immediately, or the tubes were transferred onto dry ice and stored at −80°C. Samples with only ic solution were used as negative controls.

**First PCR Amplification**

For the first amplification, the entire product of the RT reaction was used. Four microliters 10X reaction buffer, 1 μl 25 mM MgCl₂, 1 μl of each outer primer (10 μM), and 18.75 μl H₂O was added to the RT reaction. The sequences of all primers used in the PCR-reactions are listed in Table 1. A hot-start PCR was performed by incubating the reaction at 94°C for 4 minutes and adding 0.25 μl DNA polymerase (5 U/μl; AmpliTaq; Perkin Elmer). Reaction conditions were: denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 1 minute (with an autoextension of 10 sec/cyc) for 5 cycles, followed by 25 cycles with an increased annealing temperature of 60°C, with a final extension step at 72°C for 10 minutes. The optimal number of cycles for both the first and second amplification was determined experimentally by analyzing the exponential phase of the amplification reaction (data not shown). The PCR amplifications were performed on a thermal cycler (GeneAmp PCR System 2400; Perkin Elmer).

**Table 1. Metabotropic Glutamate Receptor Primer Sets Used for the PCR Amplification of cDNAs**

<table>
<thead>
<tr>
<th>Upstream Oligonucleotide [5’ → 3’]</th>
<th>Downstream Oligonucleotide [5’ → 3’]</th>
</tr>
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<tbody>
<tr>
<td>R1 outer</td>
<td>CCC ATT GAT GCC AGG AAG</td>
</tr>
<tr>
<td>R1 nested</td>
<td>TTT GTC GGA GTG TCT GGA GAG</td>
</tr>
<tr>
<td>R2 outer</td>
<td>CGG CCT GTA CAA AGA CTT CG</td>
</tr>
<tr>
<td>R2 nested</td>
<td>GCT CGT TGA TCC AAT GAA CAT C</td>
</tr>
<tr>
<td>R3 outer</td>
<td>GCT CGT TGA TCC AAT GAA CAT C</td>
</tr>
<tr>
<td>R3 nested</td>
<td>GCC GCT TGA TTA GAT CAT G</td>
</tr>
<tr>
<td>R4 outer</td>
<td>CCA GCT GCT TAA GTA CAT GAG</td>
</tr>
<tr>
<td>R4 nested</td>
<td>TAC CAA CGT CCG AAT GGC</td>
</tr>
<tr>
<td>R5 outer</td>
<td>GTO ATG CGA TGA AGG CAA TT</td>
</tr>
<tr>
<td>R5 nested</td>
<td>GCC GCT TGA TTA GAT CAT G</td>
</tr>
<tr>
<td>R6 outer</td>
<td>GCC ACC CAT GGA GCC TAC T</td>
</tr>
<tr>
<td>R6 nested</td>
<td>AGC ACA GAT GCC TGC ACA A</td>
</tr>
<tr>
<td>R7 outer</td>
<td>TCC GTA TAT GCT GGA ACC</td>
</tr>
<tr>
<td>R7 nested</td>
<td>TAC ATG GTC TCA TTG GCC AG</td>
</tr>
<tr>
<td>R8 outer</td>
<td>AGC ATG CTC TCG GAC GCT</td>
</tr>
<tr>
<td>R8 nested</td>
<td>GCC TAC TGA TCC TCG GAC</td>
</tr>
</tbody>
</table>

The content of each patch pipette was expelled completely into a 0.2-ml tube (MicroAmp; Perkin Elmer, Weiterstadt, Germany) on ice, by using positive pressure. The tubes contained 1 μl hexamer random primers (2 μg/μl; Boehringer–Mannheim, Mannheim, Germany), 1 μl dNTPs (10 mM; MBI Fermentas, St. Leon-Rot, Germany), 1 μl MgCl₂ (25 mM; Perkin Elmer), 1 μl 10X reaction buffer containing 100 mM Tris-HCl (pH 8.3) and 500 mM KCl (Perkin Elmer), 0.5 μl RNase inhibitor (50 U/μl; Rnasin; Promega, Madison, WI), and 0.5 μl MuLV reverse transcriptase (50 U/μl; Perkin Elmer).
PCR products were cleaned up (PCR Purification Kit; Qiagen, Hilden, Germany), eluted in 30 µl 5 mM Tris-HCl (pH 8.5), and immediately used for the second PCR amplification.

**Second PCR Amplification**

For each first PCR, eight second PCR reactions were set up to identify which mGluRs are coexpressed in a single RGC. All reactions had the composition: 3 µl 10× reaction buffer, 1.8 µl 25 mM MgCl₂, 0.6 µl dNTPs (10 mM each), 22.05 µl H₂O, 0.15 µl DNA polymerase (5 U/µl), and 1.2 µl of the first PCR product. The amplification conditions were identical as described, with the exception that nested primer pairs were used (0.6 µl [10 µM] of each corresponding forward and reverse primer per reaction; Table 1). The cycling parameters were: 4 minutes at 94°C, followed by 30 cycles of 45-seconds at 94°C, 45 seconds at 60°C, and 1 minute at 72°C, with a 10-second autoextension per cycle. A final annealing step of 10 minutes at 72°C followed. Amplification products were analyzed by gel electrophoresis (Fig. 1). The identity of the PCR products was determined by direct fluorescent DNA sequencing on a genetic analyzer (ABI PRISM 310; Perkin Elmer).

**RESULTS**

The gene expression of mRNAs encoding eight mGluRs was studied in rat RGCs at two different developmental stages (juvenile, P7–14; adult, P60). In addition, mGluR mRNA expression was analyzed 4 days after optic nerve transection (adult, axotomized) to look for adaptive changes in glutamate receptor properties after neuronal injury. Three hundred seventy-five identified RGCs were analyzed. Only RGCs that showed at least one clear positive amplification signal for mGluR1 through -8 mRNAs were included in further analysis (juvenile: n = 98; adult: n = 76; axotomized: n = 73). All mGluR mRNAs were detected in RGCs. Figure 1 shows representative agarose gels of each of the amplified mGluR cDNA fragments.

Clear differences were found in the occurrence of distinct types of mGluRs in RGCs during development and after neuronal injury (Fig. 2A). Whereas mGluR3 mRNA was expressed in 43% of the juvenile RGCs (n = 44), it was found in only 20% of the adult RGCs (n = 26). A reduction to less than half the number of RGCs was also observed for mGluR5 and -7 mRNAs (Fig. 2A, arrows). No significant alterations in the occurrence of expression between young and old RGCs was found for mGluR1, -2, -4, and -8 mRNAs.

In the adult axotomized retina, mGluR1 and -7 mRNAs were expressed in a higher number of RGCs than in the age-matched control group, whereas mGluR4 mRNA was found in a lower percentage of RGCs after axotomy. Interestingly, mGluR6 mRNA, which was previously reported to be expressed only in bipolar cells,18,19 was detected in very low abundance in juvenile RGCs (n = 2) and in adult RCS after axotomy (n = 4). However, it was never observed in adult RGCs of the control group.

The coexpression of different types of mGluR mRNAs in RGCs is shown in Figure 2B. Only one to two types of mGluRs were coexpressed in the majority of adult RGCs (75%, n = 57), whereas the majority of juvenile RGCs (79%, n = 77) expressed two or more mGluRs. No unequivocal changes in the number of subtypes was observed in adult RGCs after axotomy, although coexpression of more than one mGluR subunit seemed to increase (see also Fig. 2D).

Alterations in the physiological state of an RGC may be accompanied by changes in the intracellular pathways due to activation of different groups of mGluRs. We therefore not only analyzed the numbers of mGluR subunits coexpressed in RGCs under different physiological conditions but also their distribu-
tion within the different mGluR groups (Fig. 2C). An RGC was assigned to one group when the mRNA of at least one mGluR subtype of this group was expressed. No clear developmental alterations in favor of one group were observed, and axotomy did not result in preferential expression of one distinct group of mRNAs in RGCs. However, group I and II mGluR mRNAs were found more frequently in juvenile and adult RGCs after axotomy.

Analysis of the coexpression profiles of different groups of mGluRs in a single ganglion cell (Fig. 2D) also showed that mGluRs of all three groups were much more frequently found in juvenile and adult axotomized RGCs than in adult RGCs (Fig. 2D, arrow). The mGluR mRNA expressed in 55% of the adult RGCs belonged to groups I, II, or III compared with values of only 31% in juvenile and 42% in adult RGCs after axotomy. Thus, the variety of intracellular pathways triggered by the activation of mGluRs was greater during early periods of neuronal development and after neuronal injury.

**DISCUSSION**

mGluRs have been linked to an extensive list of neuromodulatory effects, depending on which intracellular or membrane-bound effector system is activated. A variety of studies have reported bidirectional modulation of cellular processes—for example, glutamate release or calcium influx by activation of different groups of mGluRs within the same type of neuron. Moreover, neurotoxic effects seem to depend on which groups of mGluRs are expressed in a neuronal population, because antagonists of group I mGluRs can reduce the amount of cell loss during excitotoxicity, whereas agonists of group II and II mGluRs exert a neuroprotective action.

The purpose of the present study was to analyze the coexpression patterns of mGluRs in a single neuron to understand better the dual effects mGluRs may have and to answer the question of whether the different physiological conditions of a neuron at different periods of development are reflected in the expression of distinct types of mGluRs. Moreover, we were interested in whether neuronal injury results in adaptive changes of mGluR expression.

All studies were performed on RGCs of the rat retina, because these neurons can be identified at birth and afterward by retrograde labeling through an injection of fluorescent dye into their target tissue, the superior colliculus. Moreover, neuronal injuries can easily be created by cutting the optic nerve that is formed by RGC axons. To our knowledge, this study yielded the first systematic determination of coexpression patterns of mGluRs on the single-cell level under different physiological conditions of an identified neuron.

Our main findings were: all mGluR mRNAs are expressed in RGCs; there is a developmental downregulation of distinct subtypes of mGluR mRNA; there is a developmental decrease in the number of mGluR subtypes and mGluR groups coexpressed in a single RGC; there is a higher number of RGCs expressing group I and II mGluRs in juvenile stages and after axotomy; and there is an upregulation of mGluR1 and -7 mRNAs after axotomy.
In a previous in situ hybridization study, the expression of six mGluR mRNAs (mGluR1 and -2 and -4 through -7) but not mGluR3 was shown in the retina of adult albino rats. The authors reported labeling of mGluR1, -2, -4, and -7 mRNAs in cells of the ganglion cell layer, whereas mGluR5 was found only in horizontal and bipolar cells and mGluR6 only in bipolar cells. mGluR1 and -4 were expressed in a much higher number of cells in the ganglion cell layer than mGluR2 and -7. No discrimination was made between RGCs and displaced amacrine cells. However, a comparison between percentages of discrimination was made between RGCs and displaced amacrine cells. mGluR1 and -4 were expressed in a much higher number of cells than mGluR6 only in bipolar cells of the ganglion cell layer, whereas mGluR5 was found almost perfectly matches the number of RGCs expressing mGluR7 mRNA. This match is a clear indication of the reliability of our single-cell RT-PCR method in determining expression rates of neurotransmitter receptors on the single-cell level.

In contrast to previous studies, we found an expression of mGluR5 and -6 mRNAs in RGCs and were able to demonstrate the existence of labeled RGCs mRNA in the retina at all developmental stages. The occurrence of mGluR3 mRNA in juvenile RGCs, however, was significantly higher than in adult RGCs. A comparable preference for expression in younger RGCs was also found for mGluR5 and -7 mRNAs. Because these mGluR subtypes belong to different mGluR groups, their developmental downregulation did not result in preferential expression of a distinct mGluR group. We found no indications of specific mGluR coexpression patterns that might reflect the different physiological conditions of RGCs during differentiation. However, group I and II mGluR mRNAs were observed more often in juvenile RGCs than in the adult stages. This is explained partly by the fact that adult RGCs rarely expressed all three types of mGluR groups but rather group III mGluRs either alone or together with group I or II mGluRs, respectively. Thus, the variety of intracellular pathways triggered by the activation of mGluRs seems to be higher in earlier developmental stages; however, no specific pathways are related to the different developmental stages.

The differences of mGluR expression in RGCs found by former studies and ours indicate that the RT-PCR method may be more sensitive than in situ hybridization in detecting mGluR mRNAs when these are expressed on a low level and in a small number of cells. An alternate explanation is that the albinos rats used in other studies are known to exhibit structural and functional abnormalities in the retina and in other respects.

Immunocytochemical and electron microscopic studies have demonstrated a distinct distribution of mGluRs in the retina. With the exception of mGluR7, which has also been found presynaptically in bipolar cell terminals, mGluRs are mainly localized postsynaptically. An occasional postsynaptic expression on RGC dendrites was reported for mGluR2, -4, and -7. No indications of mGluR5 expression were found, and the expression of mGluR1 is still unclear. Physiological experiments have indicated that RGCs express mGluRs. However, no detailed functional analysis of different mGluR subtypes exists in RGCs in situ. Based on the assumption that expression of mGluR mRNAs results in expression of the functional receptors, in our study all types of mGluRs were expressed in RGCs. Some of the mGluRs may thus be expressed presynaptically on RGC axon terminals in the target tissues such as the superior colliculus. Because we assume that 20% of adult RGCs express mGluR7 and because this subtype was rarely found on adult RGC dendrites, it is likely that it is expressed presynaptically as an autoreceptor to modulate glutamate release in the superior colliculus. Interestingly, mGluR7 is upregulated in RGCs after axotomy; this may be an adaptive response to the loss of synaptic contacts within the target tissue.

Axotomy of adult RGC axons resulted in an increase in the number of RGCs expressing mGluR1, -6, and -7 and an increase in the number of different mGluR groups coexpressed in a single RGC.

Plastic changes of mGluR expression in response to axotomy have been described before in the peripheral nervous system and result in a downregulation of mGluR1a in motoneurons. An upregulation of mGluR1 and -2, as indicated by our studies, could result in modulations of intracellular calcium levels by blocking calcium influx through voltage-activated calcium channels and activating calcium release from intracellular stores.

However, as long as it is still unclear whether the signal transduction mechanisms in a neuron in situ are the same as for the heterologous expression systems in which they were studied, to hypothesize about the functional significance of the distinct alterations shown in this study is idle speculation. Further electrophysiological experiments will clarify the relation between mGluR activation and intracellular signaling pathways under the different physiological paradigms in RGCs in situ.

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References


