

# Ischemia-Induced Changes of AMPA-Type Glutamate Receptor Subunit Expression Pattern in the Rat Retina: A Real-Time Quantitative PCR Study

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**PURPOSE.** To investigate whether the previously observed decrease in immunoreactivity of the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor subunits GluR1, -2, -3, and -4 after ischemia-reperfusion in the rat retina is associated with changes at the mRNA expression level. Furthermore, to study possible changes in the ratios of alternative splice variants of GluR2 and -4 and possible changes in the subunit composition of the receptor complex after ischemia-reperfusion. The ischemia-induced changes were related to expression levels of immediate early genes, *c-fos* and *c-jun*, and to expression levels of different cell-type-specific transcripts.

**METHODS.** A 60-minute ischemic event was induced unilaterally in the rat eye by cannulating the anterior chamber and raising the intraocular pressure. Reperfusion was allowed to occur for 2 hours up to 28 days. Total RNA was isolated from the retinas and transcript levels were assessed by real-time quantitative PCR (qPCR).

**RESULTS.** A differential decrease was observed in the expression levels of all AMPA-type GluR subunits 2 hours after ischemia-reperfusion, with a significant downregulation of GluR2 and -3 transcript levels. At the long-term (72 hours–4 weeks), expression levels for all four subunits were decreased by approximately 64%. No changes were observed, either in the expression ratio of GluR2 and -4 splice variants, or in the relative expression of the different subunits. Immediate early genes *c-fos* and *c-jun* were transiently upregulated. Expression levels of the ganglion-cell-specific transcripts Thy-1 and neurofilament and of the AII-amacrine-specific transcript parvalbumin decreased after ischemia-reperfusion, whereas the ON bipolar cell transcripts mGluR6 and PKC $\alpha$  did not show ischemia-induced changes.

**CONCLUSIONS.** Shortly after ischemia-reperfusion immunolabeling of GluR1, -2/3, and -4 is strongly decreased, whereas the corresponding mRNA levels are not affected, indicating degradation at the protein level. In contrast, the GluR2 mRNA level is reduced, whereas immunostaining is not yet affected, sug-

gesting that the GluR2 protein is relatively stable under post-ischemia conditions. The long-term decrease in mRNA levels of all AMPA-type GluR subunits suggests that ischemia affects a main component of the excitatory retinal neurotransmission. It remains to be investigated whether these changes contribute to the subsequent neurodegeneration. (*Invest Ophthalmol Vis Sci.* 2004;45:330–341) DOI:10.1167/iovs.03-0285

Retinal ischemia leads to a loss of ganglion cells and ischemic conditions have therefore been implicated in a variety of neuroretinal disorders, including glaucoma.<sup>1–3</sup> In a previous study, we have shown that induction of ischemia in the rat retina leads to rapid changes in the protein expression levels of  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type GluR subunits, as was determined by immunocytochemistry.<sup>4</sup> AMPA-type GluRs are involved in excitatory retinal signal transmission between the different types of retinal neurons in both OPL and IPL, forming an extensive and complex neuronal circuitry.<sup>5–7</sup> The AMPA-type GluR is composed of four different subunits (GluR1, -2, -3, and 4) that combine to form homomeric or heteromeric receptor complexes with a central ion channel. The relative presence of the GluR2 subunit determines the permeability to calcium and other divalent cations.<sup>6,8,9</sup>

After a 60-minute period of ischemia, we demonstrated differential changes in GluR subunit-specific immunostaining levels in the inner plexiform layer (IPL). Immunoreactivity against GluR1 was almost completely lost 2 hours after ischemia, GluR2/3 and -4 staining showed a significant reduction at 4 hours. Thereafter, levels for GluR1 and 4 recovered, whereas the staining for GluR2/3 was persistently reduced up to 7 days after ischemia.<sup>4</sup> GluR2 subunit specific immunoreactivity was not significantly affected by ischemia before the onset of inner plexiform thinning. How these differential changes in GluR subunits may take place is not well understood, but downregulation of the gene expression of the GluR1–4 subunits may be the underlying mechanism. The main purpose of the present study was to assess the evolution of GluR1–4 transcript levels.

For this purpose the technique of real-time quantitative PCR (qPCR), a sensitive, reliable, and flexible method to assess gene expression levels, was applied.<sup>10–12</sup> There are several other reasons why an investigation of the transcript levels of AMPA-type GluR subunits is of interest in relation to the immunocytochemical study already performed. Real-time qPCR assay is suited for the detection and quantification of known splice variants.<sup>13,14</sup> For GluR2 and -4 subunits, alternative splicing has been described in the C-terminal region.<sup>15–17</sup> This is of particular relevance, because the antibodies used in our previous study were raised against the C-terminal sequence of the most abundant variants of GluR2 and -4 but did not detect the other variants. In addition, the short (i.e., GluR2-short, -3, and -4-short), but not the long (i.e., GluR1, -2-long, and -4-long) variants of the GluR subunits contain a PDZ-binding domain at the extreme C terminus. Among the PDZ-containing proteins that interact with (short) AMPA subunits are PKC $\alpha$ -binding protein (PICK1), glutamate receptor binding protein (GRIP), and

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AMPA receptor-binding protein (ABP).<sup>17,18</sup> All these proteins are involved in receptor targeting into and removal from the postsynaptic membrane. A potential alteration in the relative expression of the different splice variants after ischemia could therefore underlie a loss of the antibody epitope sequence explaining the decrease in immunoreactivity, and indicating a change in the functional interaction with PDZ proteins.<sup>19</sup> Furthermore, a quantification of the transcript levels of the AMPA-type GluR subunits would also provide information on a possible change in permeability properties of the expressed receptor complex, due to a shift in the subunit composition.<sup>20–25</sup>

The use of real-time qPCR is a new approach in the retinal ischemia-reperfusion model and we tested gene expression levels in response to ischemia of several genes that may serve as reference gene(s). Furthermore, transcript levels of genes with a known response to an ischemic insult were determined: (1) *c-fos* and *c-jun*, proto-oncogenes with a recognized role in apoptosis and a fast transient upregulation after ischemia,<sup>26–28</sup> and (2) cell-specific transcripts, including two retinal ganglion cell-specific transcripts, Thy-1, and neurofilament (NF),<sup>29–32</sup> one amacrine cell transcript, parvalbumin (PV),<sup>33,34</sup> and two bipolar-cell-specific transcripts, PKC $\alpha$ , and mGluR6.<sup>35,36</sup> The ganglion and AII cell transcripts are expected to be downregulated after ischemia as a result of cell loss, whereas the bipolar cell transcripts are expected to be relatively resistant to an ischemic insult.<sup>29,31</sup>

## MATERIALS AND METHODS

### Ischemia-Reperfusion Model

Animal handling and experimental procedures were reviewed and approved by the ethics committee for animal care and use of the Royal Netherlands Academy for Sciences, acting in accordance with the European Community Council directive of 24 November 1986 (86/609/EEC) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All efforts were made to minimize suffering and the number of animals used in the study.

The ischemia-reperfusion procedure has been described in detail.<sup>4,29,30,37</sup> In short, adult male Wistar rats (Harlan, The Netherlands) weighing 200 to 300 g were anesthetized and mounted onto a stereotaxic frame. A steel 30-gauge infusion needle connected to a saline reservoir was placed in the middle of the anterior chamber of the left eye. The reservoir was opened and lifted to 1.70 m. After 60 minutes of ischemia, the reservoir was lowered, and the pressure in the eye was allowed to equilibrate. Reperfusion started immediately. After the reperfusion time, each animal was given an overdose of pentobarbital sodium (0.8 mL; 60 mg/mL), intraperitoneally.

Sham operations, in which a needle was inserted into the anterior chamber without elevating IOP, were performed ( $n = 30$ ) to evaluate the possibility that the process of cannulation itself would induce differences between ischemic and control eyes ( $n = 21$ ). Because there was no statistically significant difference between the expression data of control and sham retinas, it was decided to pool these groups into a single control group ( $n = 51$ ). The ischemic retinas (in total  $n = 50$ ) were grouped according to the reperfusion time: 2 ( $n = 8$ ), 6 ( $n = 5$ ), 12 ( $n = 6$ ), 24 ( $n = 6$ ), 48 ( $n = 5$ ), and 72 ( $n = 5$ ) hours and 7 ( $n = 4$ ), 14 ( $n = 5$ ), and 28 ( $n = 5$ ) days.

### Real-Time Quantitative PCR

**Isolation of Total RNA from Rat Retinas.** After enucleation, retinas were isolated and either stored at  $-80^{\circ}\text{C}$  or processed immediately. Retinal tissue was homogenized, and total RNA was isolated by a single-step method, based on guanidine thiocyanate extraction, according to the manufacturer's instructions (Ultraspec; Biotech Laboratories, Inc., Houston, TX). Isolated RNA was dissolved in 8  $\mu\text{L}$  diethylpyrocarbonate (DEPC)-treated water. In a series of prelimi-

nary trials, the concentration and quality of total retinal RNA were determined (2100 Bioanalyser; Agilent Technologies Netherlands BV, Amstelveen, The Netherlands) and found to be around 10  $\mu\text{g}/\text{retina}$  with sharp ribosomal RNA bands.

**Reverse Transcription.** Total RNA (dissolved in 8  $\mu\text{L}$  DEPC-treated  $\text{H}_2\text{O}$ ) was DNase I-treated (1 unit DNase I, Amplification Grade; Invitrogen BV, Breda, The Netherlands) to degrade possible genomic DNA contamination. Of this mix, 4  $\mu\text{L}$  was reverse transcribed into first-strand cDNA with 50 U/ $\mu\text{L}$  of RNase H<sup>-</sup> reverse transcriptase (1  $\mu\text{L}$ ; Superscript II Plus; Invitrogen) and 50 ng/ $\mu\text{L}$  random hexamer primers, during 50 minutes at  $42^{\circ}\text{C}$ . To the resultant cDNA sample, 15  $\mu\text{L}$  10 mM Tris and 1 mM EDTA were added, bringing the volume to a total of 35  $\mu\text{L}$ . From all samples a 1:20 dilution was made to be used for qPCR analysis. All samples were stored at  $-20^{\circ}\text{C}$  until analysis.

To check the cDNA for any genomic contamination, a conventional end point PCR for  $\beta$ -actin was performed using intron-spanning primers, under the following conditions: annealing at  $60^{\circ}\text{C}$ , elongation at  $74^{\circ}\text{C}$ , denaturing at  $94^{\circ}\text{C}$ , 90 seconds each step for 30 cycles, with  $\text{Mg}^{2+}$  concentration at 1.5 mM and 0.75 U *Taq* DNA polymerase (Qiagen, Westburg, The Netherlands). The resultant PCR products were analyzed by 1% agarose gel electrophoresis, and single bands of the anticipated size were found. For control purposes, nontemplate controls were subjected to PCR amplification, but they never yielded PCR products.

**qPCR Primer Design.** qPCR primer pairs were designed on computer (PrimerExpress V 2.0 software; PE Applied Biosystems, Warrington, UK). The length of the amplicons was kept as close as possible to 80 to 100 bp, and the melting temperature of the primers was set at  $58^{\circ}\text{C}$  to  $60^{\circ}\text{C}$ .<sup>14</sup> Details of the primers and the GenBank Accession Numbers are given in Table 1. Specificity of the primers was confirmed by a BLAST search.

**Real-Time Quantitative PCR.** Real-time qPCR is based on the real-time monitoring of fluorescent SYBR Green I by a sequence detection system (Prism 5700; Applied Biosystems Inc, Nieuwekerk a/d IJssel, The Netherlands). SYBR Green I dye is fluorescent only when bound to double-stranded (ds)DNA and can be used as a parameter for the amount of DNA specifically amplified during the PCR reaction.<sup>10,38</sup> A passive reference dye (ROX) is included in the PCR buffer, providing an internal reference to which the SYBR Green dsDNA complex signal is normalized. This allows for a correction for fluorescent fluctuations caused by variations in concentration or volume. The amount of product resulting in detectable fluorescence at any given cycle within the exponential phase of the PCR is proportional to the initial number of template copies. The number of PCR cycles needed to pass a set threshold of SYBR Green fluorescence (cycle threshold,  $C_t$ ) reflects, therefore, the template concentration in the original cDNA sample.

The PCR conditions were as follows:  $1\times$  SYBR Green PCR buffer; 3 mM  $\text{MgCl}_2$ ; 200  $\mu\text{M}$  dATP, dGTP, and dCTP and 400  $\mu\text{M}$  dUTP; 0.5 U *Taq* polymerase (Ampli $Taq$  Gold; Applied Biosystems); 0.2 U uracil-N-glycosylase (UNG; AmpErase; Applied Biosystems), 2 pmol primers; and 2  $\mu\text{L}$  of the 1:20 dilution of cDNA in a total volume of 20  $\mu\text{L}$ . This amount of cDNA corresponds to approximately 14 ng total RNA. An initial step of  $50^{\circ}\text{C}$  for 2 minutes was used for UNG incubation, followed by 10 minutes at  $95^{\circ}\text{C}$  to inactivate UNG and to activate the *Taq* polymerase. Cycling conditions were a melting step at  $95^{\circ}\text{C}$  for 15 seconds and annealing-elongation at  $60^{\circ}\text{C}$  for 1 minute, with 40 cycles. The real-time detection of double stranded DNA allows construction of a dissociation curve at the end of the PCR run by ramping the temperature of the sample from  $60^{\circ}\text{C}$  to  $95^{\circ}\text{C}$ , while continuously collecting fluorescence data. The curves of the melting profiles showed a single product and did not reveal accumulation of primer-dimers as was confirmed by electrophoresis (data not shown).

Nontemplate controls were included for each primer pair to check for any significant levels of contaminants. These samples always resulted in a difference of at least eight cycles of the  $C_t$  values compared to the template containing samples.

TABLE 1. Primer Sequences, GenBank Accession Codes, and Anticipated Size of the Amplified Product for the Different Genes Studied

Gene	Forward Primer	Reverse Primer	bp	GenBank
HPRT	ATGGGAGGCCATCACATTGT	ATGTAATCCAGCAGGTCAGCAA	77	M31642
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGA	87	M33197
$\beta$ -actin	GCTCCTCTGAGCGCAAG	CATCTGCTGGAAGGTGGACA	75	NM_031144
GluR1	CGAGTTCTGCTACAAATCCCG	TGTCCGTATGGCTTCATTGATG	91	M38060
GluR2-short	TTGAGTTCTGTTACAAGTCAAGGGC	AGGAAGATGGGTTAATATTCTGTGGA	81	M38061
GluR2-long	GCCTTGGTTTGGCAATGC	GACATCACTCAAGGTCATCTTCATTC	92	NM_017261
GluR3	GGGCAGAGTCCAAACGCAT	TGTAGCGTAATCTGAGTGTGGTG	86	M38062
GluR4-short	TGATAGAGTTCTGTTACAAGTCCAGGG	CGAGGAAGTTGGGTTAAAAGTCTGT	86	S94371
GluR4-long	CCAGGGCAGAGGCGAAG	CGTTTTCTCCCACACTCCCA	93	NM_017263
c-fos	CCAAGCGGAGACAGATCAACTT	TCCAGTTTTCTCTCTTTCAGTAGAT	87	X06769
c-jun	CGACCCCACTCAGTCTTGT	GCAGCGTATTTCTGGCTATGCA	108	X17163
Thy-1	ACAAGCTCCAATAAACTATCAATGTGAT	GGAAGTGTTTTGAACCAGCAGG	84	NM_012673
NF	TCGCCGCATATAGGAACTACTG	GAGGGCTGTCCGGTGTGTGTAC	97	NM_017029
PV	AGCCGATAGGAGCCTTTACTG	ACATCATCCGCACCTTTTTCTTC	91	NM_022499
PKC $\alpha$	TCCAATGGGCTTTCCGAT	TGGATCGGATGGTTTTGGTT	92	X07286
mGluR6	TTGCGCCTGTTTGGGATAC	GAGGCACCACTCGGGAGAA	97	D13963

**PCR Amplification Efficiency.** During PCR amplification, the number of molecules synthesized ( $X_n$ ) depends on the number of molecules present at the start of the reaction ( $X_0$ ), the reaction efficiency ( $E$ ; ideally equals 2), and the number of amplification rounds ( $n$ ):  $X_n = X_0 \cdot E^n$  (or  $X_n = C \cdot E^{-C}$ ). For presentation reasons, we have set  $C$  at  $10^{10}$ . Preliminary experiments were performed to establish the amplification efficiency for each of the primer pairs, allowing a comparison of the expression levels of different target genes and transforming the observed changes in  $C_t$  to the linear range.<sup>10,39</sup> Part of the cDNA of control and sham retinas was pooled and used to make two independent dilution ranges (1:20–1:40–1:80–1:160) in DEPC-treated water. The pooled cDNA dilution ranges were subjected to qPCR. The resultant  $C_t$  values are related to the logarithm of the dilution factor, and the slope of the best-fit line is a measure for the reaction efficiency  $E = 10^{-(1/\text{slope})}$ , according to the instructions of the manufacturer (User Bulletin #2; Applied Biosystems). The  $C_t$  values correlated highly with the dilution factor ( $R^2 > 0.99$ ). The reaction efficiency  $E$  had a value close to 2 for all primer combinations, and for all our calculations  $E = 2$  was used. These preliminary experiments were also performed to determine the optimal dilution of the cDNA to position the  $C_t$  between 15 and 30 cycles, as recommended by the manufacturer. A dilution of 1:20 was found to be optimal for all primer sets, and all presented data were derived from the same aliquot of diluted cDNA. The actual analysis of the samples was performed by preparing a solution containing all PCR components including the primers. The wells of a 96-well PCR plate were filled with 18  $\mu$ L of this solution and 2  $\mu$ L of each cDNA sample to be analyzed was added. In this way, methodological variation was minimized.

**Normalization.** As outlined, the amount of total RNA in the samples was not determined. To correct for differences in cDNA load between the different samples, the target PCR has to be normalized to a reference PCR, usually an endogenous reference gene. From the obtained  $C_t$  values of individual retinas,  $E^{-C_t}$  was calculated for target and reference genes. When the PCR reached  $C_t$ , the number of amplified molecules for the target PCR and reference PCR are equal:  $X_{C_t, \text{target}} = X_{C_t, \text{reference}} = X_0, \text{reference} \cdot E^{-C_t, \text{reference}}$ . From this, it follows that the ratio of the number of cDNA target molecules over the number of cDNA reference molecules at starting conditions:  $X_{0, \text{target}}/X_{0, \text{reference}} = E^{-C_t, \text{target}}/E^{-C_t, \text{reference}}$ .<sup>38,39</sup> The selection of which gene is to be used as a reference gene in a quantitative PCR approach is a matter of ongoing debate.<sup>10,14,40–42</sup> Fundamental to a reference gene is that its expression is abundant and essential for basic maintenance of cells and typically a housekeeping gene is selected. However, the fact that a gene is a housekeeping gene does not necessarily mean that it will behave as a reference gene in a particular paradigm, because its multifunctional nature suggests versatility in the mechanisms regulating its expression.<sup>40</sup> Three housekeeping genes that are often used as a reference gene were studied: hypoxanthine phosphoribosyl transferase (*HPRT*),<sup>42</sup> glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*),<sup>12,14</sup> and  $\beta$ -actin.<sup>14,39</sup>

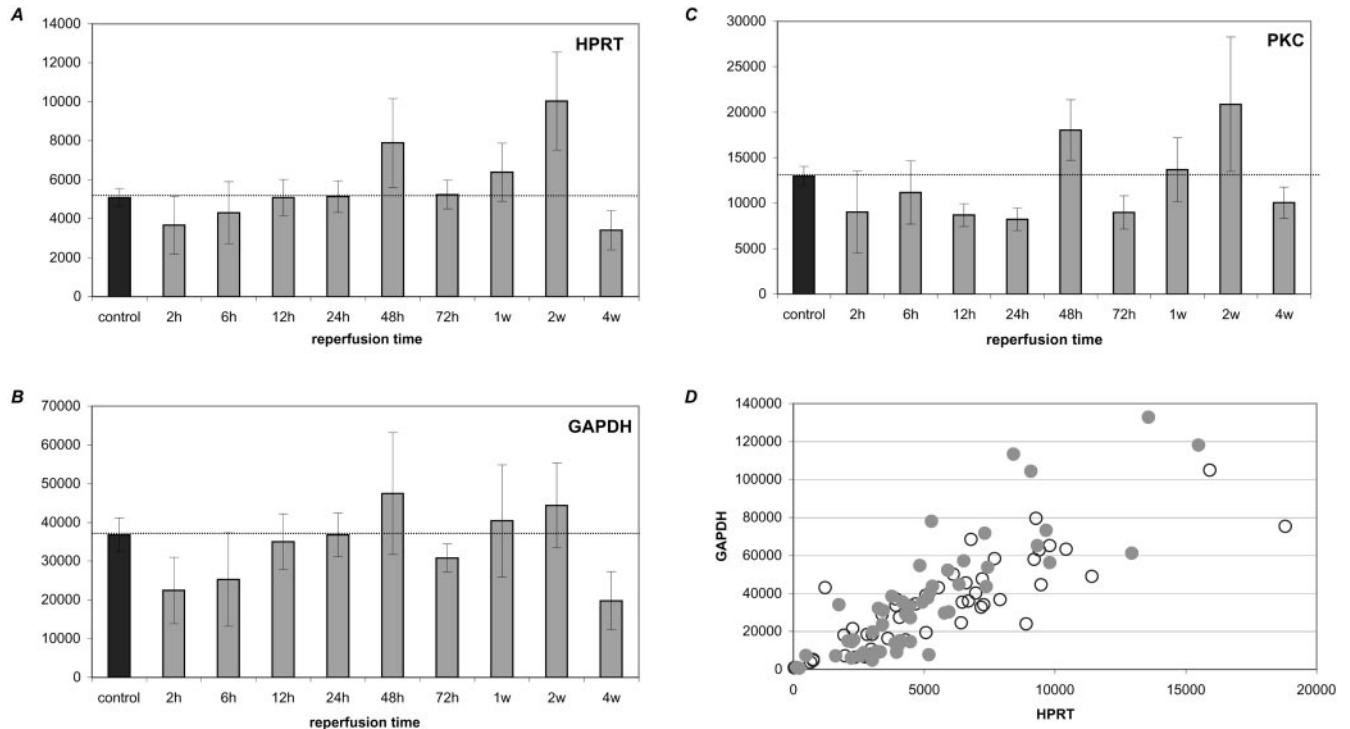
The expression ratio of two ideal internal reference genes is identical in all samples, regardless of the experimental condition. Variation in the expression ratios of two genes reflects the fact that one or both of the genes are not constantly expressed. Using the data from all 88 cDNA samples and from all 15 studied genes, for each gene the pair-wise variation in relation to all other genes was determined as the standard deviation of the logarithmically transformed expression ratios. The internal gene stability measure  $M$  was defined as the average pair-wise variation with all other control genes. Stepwise exclusion of the gene with the highest  $M$  value identified the combination of two constitutively expressed genes that have the most stable expression in the tested samples. This analysis is facilitated by the use of the virtual basic applet GeNorm developed by Vandesompele et al.<sup>43</sup> In our model, *HPRT* and *GAPDH* were identified as the most stable genes. Indeed, statistical analysis with ANOVA confirmed that these genes are not significantly altered by ischemia. To measure expression levels accurately, normalization to multiple reference genes is preferred. A normalization factor based on the expression levels of *HPRT* and *GAPDH* was calculated by using the geometric mean of the  $C_t$ . In addition, normalization factors were calculated for *HPRT-GAPDH-PKC*, *HPRT-GAPDH-PKC $\alpha$ -Thy-1*, and so forth. The pair-wise variation ( $V$ ) was calculated between these normalization factors; a large  $V$  value means that the added gene has a significant effect and should be included for the final normalization factor.  $V_{(\text{HPRT-GAPDH})} = 0.65$ ;  $V_{(\text{HPRT-GAPDH-PKC}\alpha)} = 0.15$ ;  $V_{(\text{HPRT-GAPDH-PKC}\alpha\text{-Thy-1})} = 0.09$ . After the suggested cutoff value of 0.15 by Vandesompele et al.,<sup>43</sup> we decided to use the geometric mean of the  $C_t$  of *HPRT-GAPDH-PKC $\alpha$*  for final normalization.

#### Quantitative Assessment of Target Gene Expression.

The qPCR  $C_t$  values were converted to absolute amounts of cDNA present ( $E^{-C_t}$ ), and presented as  $C \cdot E^{-C_t}$  with  $C = 10^{10}$ . For the target genes, the absolute amount was normalized against the absolute amount derived from the normalization  $C_t$ .

#### Immunocytochemistry

Ischemia was evoked as described earlier. The animals were killed at different reperfusion times (controls [ $n = 14$ ]), 2 [ $n = 2$ ], 4 [ $n = 2$ ], 6 [ $n = 4$ ], 12 [ $n = 4$ ], 24 [ $n = 4$ ], and 72 [ $n = 3$ ] hours and 7 days [ $n = 3$ ]) with an overdose of pentobarbital sodium. Immunocytochemical procedures have been described before.<sup>4</sup> Primary antibodies used were: *c-fos* rabbit polyclonal (1:20,000; Santa Cruz Biotechnology, Heerhugowaard, The Netherlands) and *c-jun* mouse monoclonal (1:5000; Santa Cruz Biotechnologies). The antibodies were visualized with either rabbit-anti-goat Cy3 or goat-anti-mouse Cy3 (1:600; Jackson ImmunoResearch, Brunswick, Chemie BV, Amsterdam, The Netherlands). In addition, a DNA-binding dye (Hoechst 33258 used at 1:2000; Molecular Probes Europe BV, Leiden, The Netherlands) was added to visualize all nuclei in the retina.



**FIGURE 1.** Absolute, non-normalized, amounts of reference genes calculated as  $X_0 = C \cdot E^{-G}$ , with  $C$  at  $10^{10}$ . Data represent the average per reperfusion group  $\pm$  SEM (A) *HPRT*, (B) *GAPDH*, and (C) rod ON-bipolar specific transcript *PKC $\alpha$*  levels do not differ statistically significant between the groups (*HPRT*:  $F = 1.91$ ,  $P = 0.06$ ; *GAPDH*:  $F = 0.86$ ,  $P = 0.56$ ; *PKC $\alpha$* :  $F = 1.61$ ,  $P = 0.12$ ; ANOVA). (D) Absolute amounts of *HPRT* plotted against the absolute amounts of *GAPDH*, illustrating that over a wide range, the amounts of these two genes are highly correlated in both control (●) and ischemic (○) samples.  $R^2$  is 0.73 for the control retinas and 0.72 for the ischemic retinas. There are no significant differences in the ratio of *HPRT-GAPDH* between control ( $0.20 \pm 0.01$ ) and ischemic ( $0.20 \pm 0.02$ ) samples ( $P = 0.98$ , Student's *t*-test).

The number of labeled cells in the ganglion cell layer (GCL) and the inner nuclear layer (INL) were counted in a 250- $\mu$ m-wide column in two to four different sections of each animal. Data are presented as the percentage of labeled cells in the total number of cells of that layer.

### Statistical Analysis

To demonstrate whether statistically significant differences existed among the different groups, single-factor ANOVA was used. If the ANOVA indicated such differences, Student's *t*-tests were performed comparing the different ischemic groups with the control group. For the reference genes, this approach was followed using the absolute amounts of cDNA; for all target genes the statistical analysis was performed on the normalized values. The amount of transcript in the control group was set at 100%; ischemia groups were compared with the control group. Most changes showed a time course in which the levels became constant after 72 hours of reperfusion. The data from the animals of the 72-hour and 7-, 14-, and 28-day groups ( $n = 20$ ) were taken together to acquire a more precise quantification of the long-term changes; this group is referred to as long-term. On the control and the long-term group a power analysis was performed, resulting in values over 0.99 for all the target primer sets, except in the case of *Glur2-long* (0.24) and *Glur4-long* (0.93), and *NF* (0.94). For *c-fos*, the power analysis was performed on the control group versus the 2-hour group (0.87), for *c-jun* on the control versus the 6- and 12-hour groups (0.84), and for  $\beta$ -actin on the control versus the 12-, 24-, 48-hour groups (0.95).

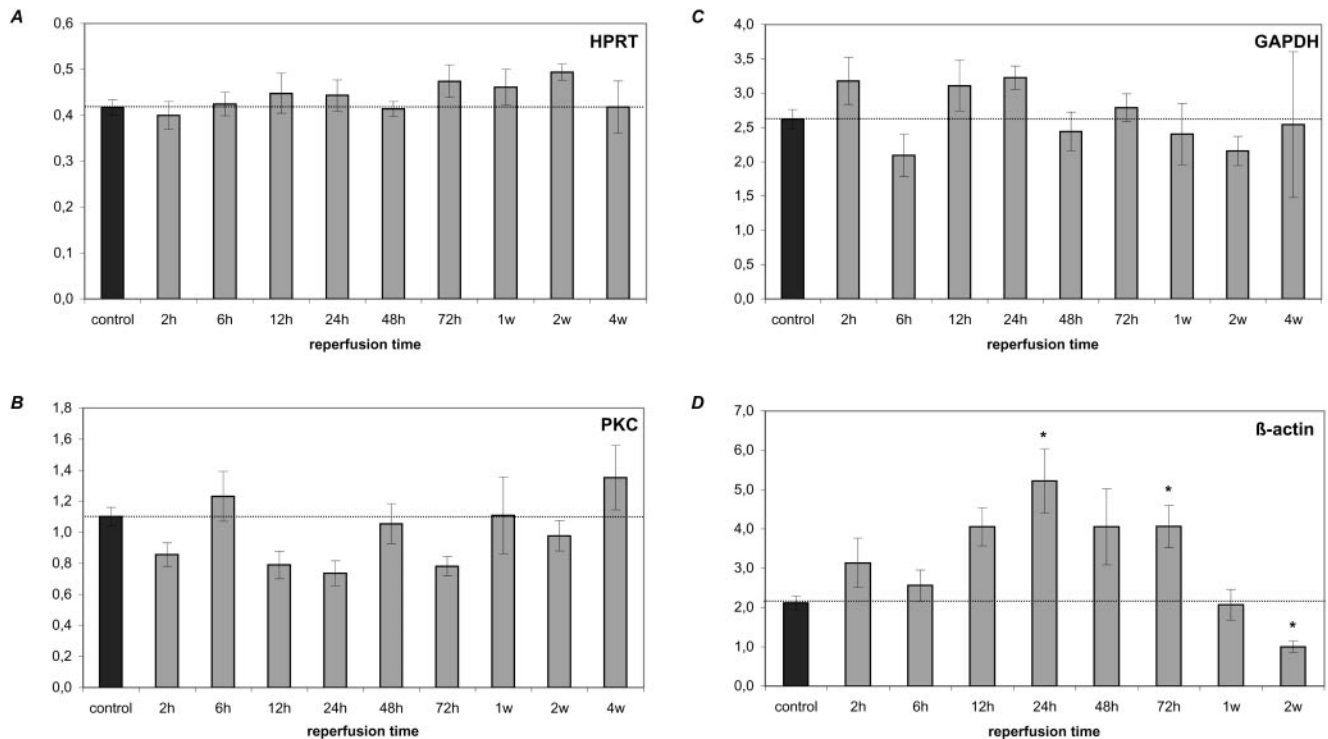
## RESULTS

### Reference Genes

The absolute, non-normalized, amounts of the housekeeping genes *HPRT* and *GAPDH* showed no statistical significant

ischemia-induced changes (ANOVA and Student's *t*-test). In contrast,  $\beta$ -actin showed a significant ischemia-induced upregulation and was, therefore, not applicable as a reference gene in this paradigm. In addition to these housekeeping genes, the rod bipolar cell transcript *PKC $\alpha$*  also showed no significant regulation. Figures 1A-C show the absolute amounts of the three reference genes selected to normalize the target genes: *HPRT*, *GAPDH*, and *PKC $\alpha$* , respectively.<sup>43</sup> There was a high correlation between the detected amounts of these genes in individual samples of both the control and ischemia group. The correlation coefficient for *HPRT-GAPDH* in control retinas and ischemic retinas was 0.73 and 0.72, respectively. The ratio of these genes was  $0.20 \pm 0.01$  (mean  $\pm$  SEM) in control and  $0.20 \pm 0.02$  in ischemic retinas. For *HPRT-PKC $\alpha$*  the correlation coefficient in control and ischemic retinas was 0.30 and 0.42, with ratios of  $0.45 \pm 0.04$  and  $0.51 \pm 0.03$ , respectively. For *GAPDH-PKC $\alpha$*  the correlation coefficient for control and ischemic retinas was 0.40 and 0.48, with ratios of  $3.05 \pm 0.30$ , and  $3.38 \pm 0.28$ . Figure 1D illustrates the relation between the amounts of the two most correlated genes, *HPRT* and *GAPDH*.

Figures 2A-C show the expression levels of the reference genes after the normalization procedure, based on the expression level of the combination *HPRT-GAPDH-PKC $\alpha$* . *HPRT* expression was stable at all reperfusion times. *GAPDH* and *PKC $\alpha$*  expression showed more variability between the various groups, but showed no clear trend and was not significant. In contrast, the relative expression of  $\beta$ -actin clearly showed development over time (Fig. 2D). After an ischemic insult, levels of  $\beta$ -actin mRNA first increased by 145% at 24 hours of reperfusion, followed by a gradual decrease until back at the control level again at 7 days.



**FIGURE 2.** Expression levels after normalization. Data are presented as mean value per group  $\pm$  SEM. The levels of (A) *HPRT*, (B) *GAPDH*, and (C) *PKC $\alpha$*  did not show significant differences among the different groups (*HPRT*:  $F = 0.55$ ,  $P = 0.83$ ; *GAPDH*:  $F = 0.95$ ,  $P = 0.48$ ; *PKC $\alpha$* :  $F = 0.91$ ,  $P = 0.06$ ; ANOVA). (D) In contrast, the expression of  $\beta$ -actin was significantly different between the groups ( $F = 7.14$ ,  $P = 5.23 \cdot 10^{-7}$ ). Levels reached a maximum at 24 hours of reperfusion with a 145% increase compared with control levels. Thereafter, levels decreased again. \* $P < 0.05$ ; Student's *t*-test against the control group.

### GluR1-4 Subunit Gene Expression

All AMPAR-subunit encoding transcripts were significantly changed after ischemia. The development of the alterations over the different reperfusion times is presented in Figures 3A-F, together with the outcome of the statistical analysis. Compared with the levels in the control group, all subunits were found to decrease over time after ischemia with an average reduction ranging between 55% (GluR1) and 69% (GluR4-short) determined in the animals studied at and after 72 hours of reperfusion (hereafter referred to as the long-term reperfusion-group;  $n = 20$ ).

**GluR1.** After ischemia-reperfusion, a gradual decrease of GluR1 was observed over the first 24 hours of reperfusion (Fig. 3A). Levels of GluR1 stabilized in the long-term group at an average level 55% lower compared with control levels. The decrease was significant at 12 hours and in all the following reperfusion times.

**GluR2.** For GluR2 expression two splice variants were studied. Levels of GluR2-short decreased rapidly after ischemia. In the long-term reperfusion group, levels stabilized at a level 63% lower than the control level (Fig. 3B). The decrease was significant at 2 hours (44%) and remained significantly lower than control levels thereafter. The GluR2-long expression levels showed a similar trend as the short splice variant, although due to the higher variability in expression level in the control group, this decrease was not statistically significant. However, expression stabilized in the long-term group at levels 56% lower than control levels (Fig. 3C). When both splice variants were summed, the decrease was significant at 2 hours or more and resulted in the long-term group in levels 66% lower than control levels ( $F = 2.79$ ,  $P = 0.007$ ; ANOVA).

The short splice variant was more abundant than the long splice variant in all studied samples. The short variant repre-

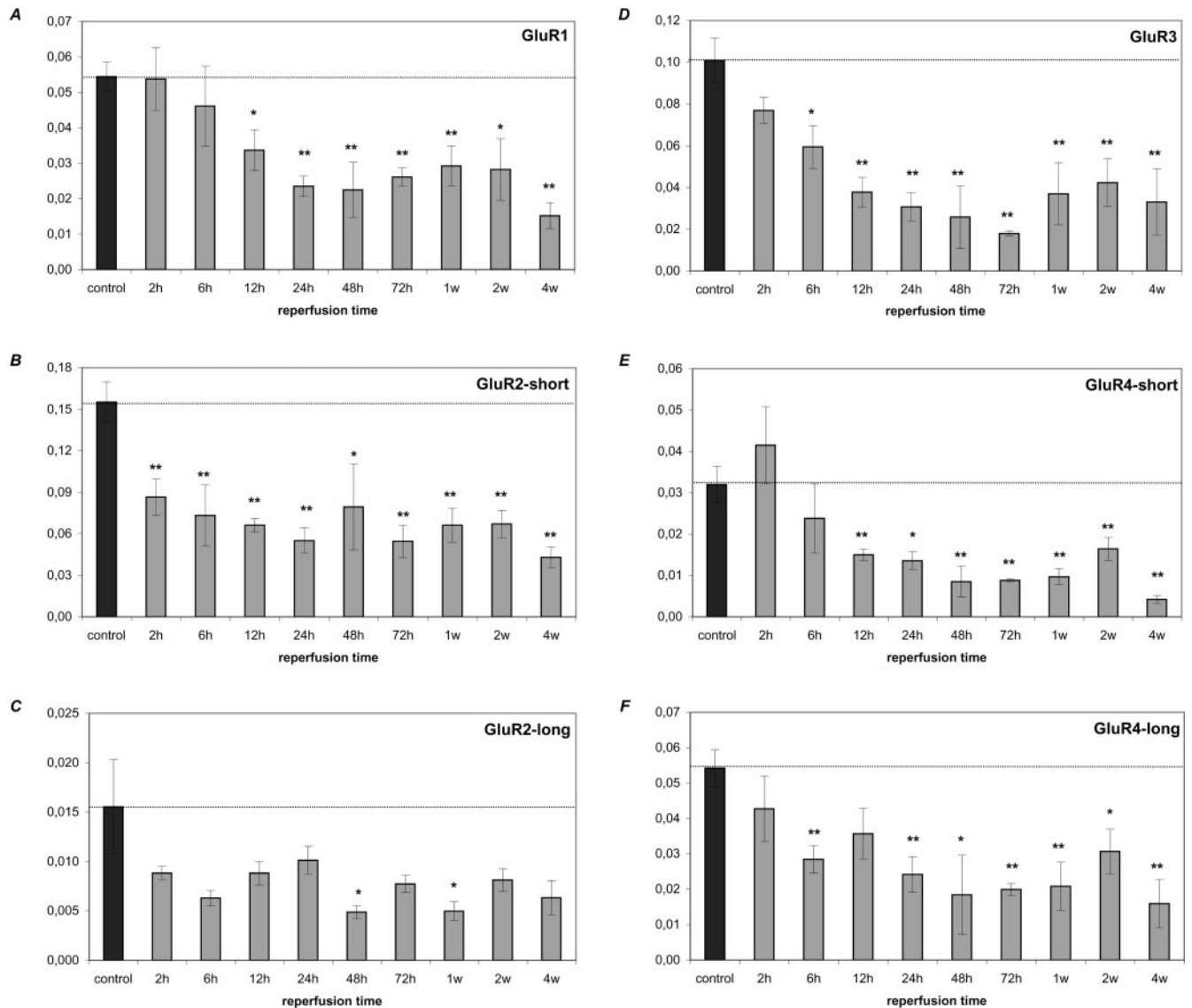
sented 89% (mean  $\pm$  SEM) of the total GluR2 transcripts. No significant changes were observed in this ratio after ischemia-reperfusion ( $F = 0.64$ ,  $P = 0.76$ ; ANOVA).

**GluR3.** Levels of GluR3 decreased gradually after ischemia-reperfusion (Fig. 3D). The reduction was significant at 6 hours with 41% lost. Compared to control levels, GluR3 decreased significantly with an average loss of 68% during long-term reperfusion times. However, at 48 and 72 hours, the mean levels of GluR3 were 74% and 82% lower than control levels, whereas in the last 3 weeks it was only around 63% lower than control levels, indicating that some recovery took place.

**GluR4.** Of the GluR4 subunit, two C-terminal splice variants exist. GluR4-short (Fig. 3E) had increased with 30% at 2 hours. However, at 6 hours, levels had decreased with 26% compared with control levels. The decrease then progressed gradually and was significant at 12 hours and thereafter. Levels had decreased with 69% during long-term reperfusion times. Levels of the GluR4-long splice variant (Fig. 3F) were reduced faster. Long-term levels were 60% lower than control levels. When the short and long splice variants were summed, the decrease in the long-term group was 64% ( $F = 3.31$ ,  $P = 0.002$ ; ANOVA).

In control retinas, the short variant made up  $36\% \pm 2\%$  (mean  $\pm$  SEM) of the total GluR4 subunit transcripts. As with the GluR2 splice variants, the ratio between the GluR4 splice variants was not significantly different between the control and various ischemic groups ( $F = 1.37$ ,  $P = 0.21$ ; ANOVA).

**AMPA-Type Glutamate Receptor Complex.** To obtain an indication of possible changes in the subunit composition of the receptor complex, calculations were made on the contribution that each of the subunits and splice variants made to the total AMPA-receptor encoding transcript population (Table 2). In the control retinas, the GluR1 subunit contributed approximately 15%. The GluR2 subunit (short plus long splice vari-



**FIGURE 3.** Normalized expression levels of GluR1-4. Data are presented as the mean level per group  $\pm$  SEM. (A) Expression of GluR1 decreased gradually over time ( $F = 3.34$ ,  $P = 0.001$ ; ANOVA). Levels reached a minimum at 24 hours and stabilized thereafter. The mean decrease over the 72-hour and 7-, 14-, and 28-day groups (long-term) was 55% compared with control values. (B) Levels of GluR2-short were significantly downregulated as soon as 2 hours after ischemia ( $F = 2.98$ ,  $P = 0.004$ ). Levels decreased 63% compared with control values in the long-term. (C) GluR2-long was not significantly regulated by ischemia ( $F = 0.50$ ,  $P = 0.87$ ), although the average decrease was 56% in the long-term. (D) Levels of GluR3 decreased gradually and reached a minimum at 72 hours ( $F = 3.26$ ,  $P = 0.002$ ). In the long-term, the decrease was 68% compared with control levels. (E) Levels of the GluR4-short splice variant increased at 2 hours and decreased thereafter up to 69% in the long-term ( $F = 2.27$ ,  $P = 0.03$ ). (F) The mean decrease of GluR4-long was 60% in the long-term ( $F = 2.73$ ,  $P = 0.007$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; Student's *t*-test against the control group.

ants) encompassed the largest proportion of the receptor complex with 39%. The GluR3 and GluR4 (short plus long variants) represented 25% and 22%, respectively.

After ischemia-reperfusion, the contribution of GluR1 to the AMPA-R complex was not significantly altered. The contribution of GluR2 (short plus long splice variants) decreased from 39% in control retinas to 28% at 2 hours of reperfusion. After that, the contribution increased again. The GluR3 contribution significantly decreased gradually from 25% in the control group to 13% at 48 hours. At 14 days, the contribution had returned to control levels (23%). The contribution of GluR4 (short plus long) initially increased from 23% to 32% at 2 hours, followed by a gradual decrease to control levels.

### Immediate Early Genes

***c-Fos*.** Transcript levels of *c-fos* were readily detectable under normal conditions (Fig. 4A). An ischemic insult caused a rapid and significant increase of the *c-fos* transcript levels of 434% at 2 hours of reperfusion. Thereafter, mRNA levels gradually returned to control levels within 48 hours.

The immunocytochemical data were in agreement with these findings, showing several *c-fos* immunoreactive somata in the control retina. After ischemia-reperfusion, a strong increase in immunoreactivity was observed. At 2 hours, 28% of the total number of cells in the INL and 49% of the total number of cells in the GCL were immunopositive (Figs. 4B, 4C). Thereafter, the number of labeled cells progressively decreased and had returned to the control level at 7 days. No

TABLE 2. Relative Subunit Contributions to the Total AMPA-Type GluR Subunit Level

	GluR1 (%)	GluR2 (%)	GluR3 (%)	GluR4 (%)
Controls	15 ± 1	39 ± 2	25 ± 1	22 ± 1
2 hours	16 ± 3	28 ± 2	23 ± 1	32 ± 3
6 hours	19 ± 5	32 ± 6	22 ± 2	27 ± 9
12 hours	17 ± 2	40 ± 4	19 ± 2*	25 ± 2
24 hours	15 ± 1	41 ± 3	19 ± 2	24 ± 1
48 hours	18 ± 6	53 ± 12	13 ± 4	17 ± 4
72 hours	20 ± 3	44 ± 6	14 ± 1†	22 ± 2
7 days	18 ± 1	45 ± 3	14 ± 2*	22 ± 2
14 days	15 ± 3	38 ± 2	23 ± 4	24 ± 2
28 days	16 ± 2	43 ± 12	20 ± 8	21 ± 2
ANOVA	F = 0.61, P = 0.78	F = 0.58, P = 0.81	F = 1.81, P = 0.08	F = 1.01, P = 0.43

The relative contribution of the GluR1-4 subunits to the receptor complex does not differ among reperfusion groups. The GluR3 contribution is significantly affected; decreases gradually until at 7 days it is 44% lower than control levels but is at control level again at 14 days. No difference in the ratio of the short and long variants of GluR2 and -4 was found ( $F = 0.64$ ,  $P = 0.76$  and  $1.37$ ,  $P = 0.21$ ; ANOVA, respectively).

\*  $P < 0.05$ ; †  $P < 0.01$ ; Student's *t*-test against the control group.

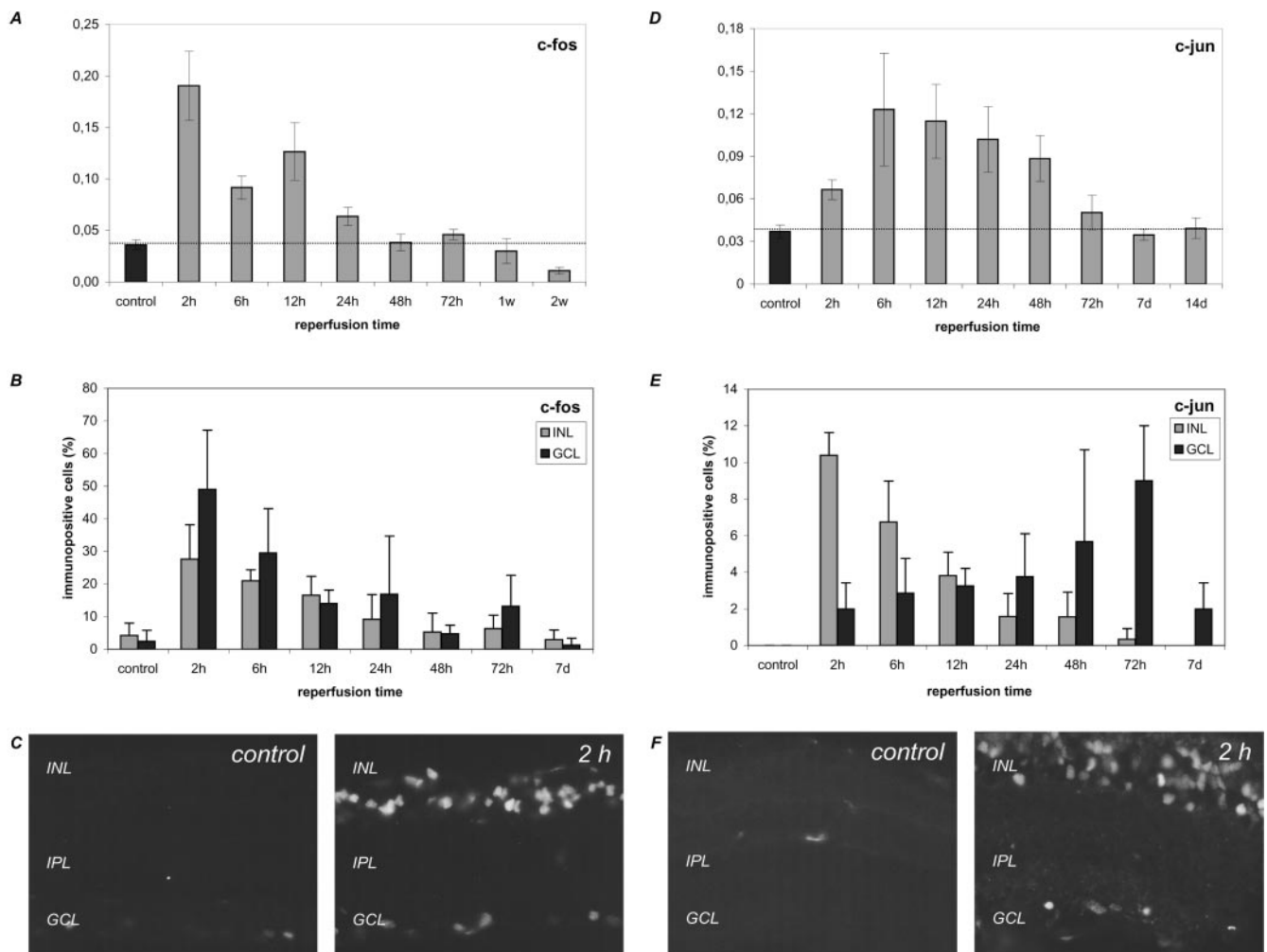
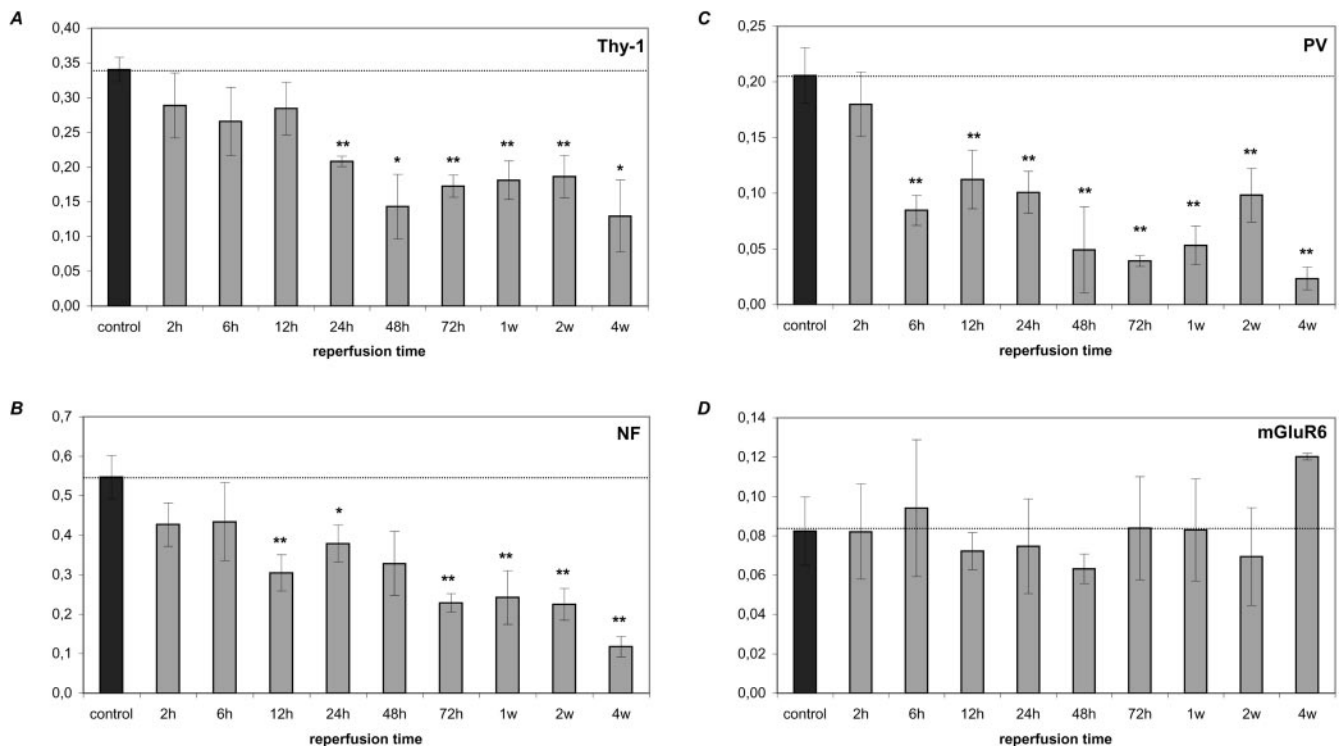


FIGURE 4. (A) Expression levels of immediate early gene (IEG) *c-fos* increased significantly (434%) compared with control values at 2 hours and returned to control level within 48 hours ( $F = 14.66$ ,  $P = 8.62 \cdot 10^{-13}$ ; ANOVA). (B) The percentage of *c-fos* immunoreactive cells in both INL and GCL was highest at 2 hours and gradually returned to control levels over 7 days. (C) In a control retina, a small number of cells show *c-fos* immunoreactivity (arrow). An ischemic retina at 2 hours of reperfusion shows many immunopositive cells in both INL and GCL (arrows). (D) Mean levels of *c-jun* showed a maximum increase at 6 hours of 209% compared with control levels, and then gradually returned to control levels within 7 days ( $F = 5.88$ ,  $P = 7.3 \cdot 10^{-6}$ ). (E) The percentage of *c-jun* immunoreactive cells showed a maximum increase at 2 hours in the INL, but at 72 hours in the GCL. (F) *c-jun* immunoreactivity in a control retina showed no positive cells. At 2 hours of reperfusion, the maximum number of immunopositive cells was reached in the INL. In the GCL various immunopositive cells could be detected (arrow), but the maximum number of positive cells was reached only at 72 hours of reperfusion. \* $P < 0.05$ ; \*\* $P < 0.01$ ; Student's *t*-test against the control group. Error bars represent SEMs.



**FIGURE 5.** (A) The expression of ganglion-cell-specific Thy-1 gradually decreased and was 51% compared with the control group in the long-term ( $F = 5.12$ ,  $P = 1.38 \cdot 10^{-5}$ ; ANOVA). (B) The level of ganglion-cell-specific NF decreased by 53% in the long-term ( $F = 2.16$ ,  $P = 0.03$ ). (C) The level of AII-amacrine-cell-specific PV (PV) decreased rapidly after ischemia. At 6 hours, levels decreased approximately 59% compared with control levels. In the long-term, PV levels, on average, decreased by 74% ( $F = 2.75$ ,  $P = 0.007$ ). (D) Levels of ON-bipolar-specific mGluR6 did not show ischemia-induced regulation ( $F = 0.09$ ,  $P = 0.99$ ). \* $P < 0.05$ ; \*\* $P < 0.01$ ; Student's *t*-test against the control group. Error bars represent SEMs.

*c-fos*-positive cells were found in the outer nuclear layer (ONL) at any of the different reperfusion times of ischemic retinas.

***c-jun*.** Levels of *c-jun* mRNA in control retinas are similar to those found for *c-fos* (Fig. 4D). The highest expression levels were observed at 6 hours with levels 209% higher than in control animals. After 6 hours, over the course of a week, levels gradually decreased again.

Immunocytochemical findings (Figs. 4E, 4F) showed that in control retinas, no *c-jun*-immunoreactive cells were observed. Ischemia induced a rapid increase of *c-jun* immunoreactivity in the INL, reaching a maximum at 2 hours, with 10% of the total number of cells immunoreactive, and decreasing gradually thereafter. *c-jun* immunoreactivity in the GCL showed a different development. A gradual increase was observed, reaching a maximum only at 72 hours with approximately 9% of the total number of cells immunopositive. At 7 days, this number had decreased again to 2%. No *c-jun*-positive cells were detected in the ONL.

### Cell-Type Specific Markers

**Thy-1.** At 2 hours of reperfusion, the expression of the RGC marker Thy-1 had decreased by 15% compared with control levels (Fig. 5A). Levels of Thy-1 gradually decreased further and at long-term transcript levels were 51% lower than in control retinas. Differences were statistically significant at 24 hours of reperfusion and thereafter.

**Neurofilament.** Ischemia-reperfusion resulted in a gradual decrease of neurofilament (NF), the second RGC marker used (Fig. 5B). The decrease at 2 hours was 22% and progressed to a loss of 53% in the long-term reperfusion group. Differences were statistically significant at 12 hours of reperfusion and thereafter.

**Parvalbumin.** The mRNA levels of the AII-amacrine-cell-specific transcript PV decreased rapidly after ischemia-reperfusion, showing a reduction of 59% at 6 hours (Figs. 5C). Levels continued to decrease and reached an average decrease of 74% in the long-term group.

**mGluR6.** mGluR6 is expressed by both rod and cone ON-type bipolar cells. No significant changes in response to ischemia-reperfusion were observed between the groups in transcript levels of mGluR6 (Fig. 5D).

**PKC $\alpha$ .** The rod ON-type bipolar cell-specific transcript PKC $\alpha$  did not show any significant differences in postischemic retinas (Fig. 2C). As explained above, this transcript has been included in the final normalization factor. The findings on mGluR6 and PKC $\alpha$  indicated that none of the ON bipolar cells were vulnerable to ischemia.

### DISCUSSION

Using a real-time qPCR approach, we set out to elucidate whether a downregulation of gene expression of the AMPA-type GluR subunits 1, 2, 3, and 4 underlies the previously observed changes in immunoreactivity.<sup>4</sup> The main finding of this study is that 60 minutes of retinal ischemia resulted in a downregulation of all AMPA-type GluR subunit-encoding transcripts (Table 3). The applicability of qPCR-based analysis to study alterations in gene expression in this experimental model was confirmed by showing in the same cDNA samples: (1) a transient upregulation of immediate early genes (*c-fos* and *c-jun*) in parallel with an upregulation at the protein level, as shown by immunocytochemistry; (2) a persistent downregulation of two ganglion cell-specific transcripts (Thy-1 and NF); (3) a downregulation of the AII-amacrine-cell-specific tran-



**TABLE 3.** Summary of Changes in AMPA-Type GluR Subunits after Ischemia Reperfusion at the mRNA Level (qPCR) and at the Protein Level (immunoreactivity)

	qPCR (%)			Immunoreactivity (%)		
	2 Hours	6 Hours	12 Hours	2 Hours	6 Hours	12 Hours
GluR1	-1	-15	-38	-88	-58	-63
GluR2-short	-25	-57	-62	+22	-1	-23
GluR2-long	-42	-59	-43			
GluR2/3				-72	-66	-68
GluR3	-22	-41	-62			
GluR4-short	+34	-25	-53			
GluR4-long	-19	-47	-34	-53	+26	-20

Immunoreactive changes were assessed in the IPL and have been quantified using densitometric analysis.<sup>4</sup>

script PV; and (4) unaffected levels of the bipolar cell markers PKC $\alpha$  and mGluR6.

### AMPA-Type Glutamate Receptor Subunit Expression

When comparing gene expression levels with alterations observed at the protein level detected using immunocytochemistry, it should be pointed out that the qPCR data are derived from the total retina, giving an average of all cell types expressing a particular transcript. For the cell-type-specific transcripts (Thy-1, NF, PV, PKC $\alpha$ , and mGluR6) qPCR provides detailed information on changes of a particular cell type. However, AMPA-type GluRs are expressed by different retinal cell types, which makes the interpretation of qPCR data less straightforward. Our previous study showed that the reduction of AMPA-type GluR subunit staining was restricted to the inner retina (amacrine and ganglion cells), whereas staining of the outer plexiform layer (OPL), coming from bipolar and horizontal cells, was hardly attenuated. Because a relatively small proportion of the total GluR immunostaining is located in the OPL, it is reasonable to assume that most of the retinal GluR subunit transcript in the cDNA sample originates from amacrine and ganglion cells and that only a minor fraction is carried by horizontal and bipolar cells.<sup>44-46</sup>

Another issue that should be taken into account is that ischemia induces cell loss, which by its very nature, leads to a reduction of ganglion and amacrine cell transcript levels. This raises the question of whether the changes in GluR subunit gene expression are not simply a result of a loss of cells. TUNEL-positive staining, indicative of apoptosis, was no longer observed at 72 hours and later (long-term group).<sup>4,47,48</sup> The degree of reduction in expression of GluR subunits in the long-term was between 55% and 69%, which corresponds well to the reduction found for the ganglion and amacrine-cell-specific transcripts: Thy-1, 51%; NF, 53%; and PV, 74%. Therefore, the long-term decrease of the GluR subunit expression levels is most likely explained by the loss of amacrine and ganglion cells. In contrast, after the start of reperfusion, the very first TUNEL-positive cells appear around 4 hours with a peak in their numbers at 12 to 24 hours, leading to the conclusion that changes in gene expression in the 2- and 6-hour groups are related to the consequences of the ischemic insult and are not caused by a loss of cells.<sup>4,47-49</sup> Because the alterations in subunit immunoreactivity were also found in this time window, we will focus the discussion on these groups (Table 3).

After 60 minutes of ischemia, immunocytochemical observations revealed a near-complete loss of GluR1 immunoreactivity at 2 hours of reperfusion, followed by some recovery of immunoreactivity thereafter.<sup>4</sup> In contrast, the qPCR data show

only a minor 12% reduction of GluR1 expression levels at 2 hours. These results jointly show that the fast reduction of GluR1 immunostaining is not caused by a reduction in gene expression, and indicates the involvement of selective GluR1 protein degradation.<sup>50,51</sup>

GluR2 gene expression (short plus long splice variants) decreased with a much faster time course than did GluR1. At 2 hours a significant decrease of 44% was found. The two GluR2 splice variants were affected to a similar degree and the ratio between the abundant (90%) short and long variants was not significantly altered after ischemia-reperfusion. Up to 24 hours after reperfusion, GluR2-subunit-specific immunostaining showed no detectable change in the total amount of GluR2 (short) labeling,<sup>4</sup> which leads to the conclusion that although GluR2 gene expression declines significantly after ischemia, the reduction does not seem to affect the protein level in the retina immediately.

GluR3 transcript levels were significantly decreased by 24% at 2 hours and 41% at 6 hours. The GluR3 expression data cannot be compared directly with immunocytochemical data, because no GluR3 subunit specific antibody is available.

At 2 hours of reperfusion, GluR4 short transcript levels showed a 30% increase, and levels of GluR4 long decreased 20%. These changes were not significant. Thereafter both splice variants showed a decrease in transcript levels compared with the control levels. The immunocytochemical results,<sup>4</sup> obtained with an antibody directed against the more abundant GluR4-long splice variant showed a profound decrease in labeling at 2 hours, presenting a pattern comparable to that of GluR1, in which the decrease in protein levels preceded the decrease in expression levels.

### Balance between Subunit Expression Levels and Splice Variants

The functional characteristics of the AMPA-type glutamate receptor are principally determined by subunit composition.<sup>6,8,9</sup> The presence of GluR2 underlies the linearity of the current-voltage relation and imposes a low-calcium conductance, whereas the GluR4 subunit determines the desensitization kinetics.<sup>52</sup> For the rat hippocampal CA1 area, a subunit-specific downregulation of GluR2 mRNA levels by approximately 70% has been described at 24 hours of reperfusion after 10 minutes of global ischemia.<sup>21</sup> Our immunocytochemical study on retinal ischemia-reperfusion, did not provide support for a selective loss of the GluR2 subunit at the protein level.<sup>4</sup> The data presented herein show that the contribution of GluR2 gene expression also did not change significantly after ischemia-reperfusion (Table 2). The absence of consistent changes in the balance between levels of mRNA encoding AMPA glutamate

receptor subtypes after retinal ischemia is in agreement with other gene expression studies after global brain ischemia.<sup>25,53</sup>

The experimental design of the study also addressed whether ischemia induces alterations in the splicing routes encoding for different C-terminal variants of GluR2 and -4. Interaction of PDZ-domain-containing proteins with C-terminal sequences of the GluR subunits is thought to localize proteins to specific subcellular regions and provides an important mechanism for clustering ion channels and receptors.<sup>17,54</sup> The short splice variants of the GluR subunits (GluR 2-short, -3, and -4-short),<sup>15-17</sup> contain domains that are the sites of interaction with PDZ proteins such as PICK1, GRIP, and ABP.<sup>17,55-58</sup> The long splice variants (i.e., GluR1, -2-long, and -4-long) do not contain such domains.<sup>17</sup> In control retinas, the short splice variant of GluR2 is nine times more abundant than the long splice variant. These data concur with whole-brain data from mice.<sup>17</sup> The long splice variant of the GluR4 subunit is approximately twice as abundant as the short splice variant. In the murine brain, GluR4-short is preferentially expressed in granule neurons and Bergmann glial cells in the cerebellum, whereas GluR4-long is expressed in Bergmann glial cells and interneurons in the forebrain.<sup>16,17</sup> Our observations did not provide evidence for significant alterations in the ratio of the long and short variants in the retina, suggesting that the splicing machinery of GluR2 and -4 gene expression is not affected by ischemia-reperfusion.

### Validation of the qPCR Approach

To our knowledge this is the first report using qPCR in the retinal ischemia-reperfusion model and the potential pitfalls called for a validation of the technique.<sup>10</sup> The normalization was based on the expression level of three genes based on the procedure described by Vandesompele et al.<sup>43</sup> Furthermore, the gene expression response of some well-known genes was studied. The gene expression of the immediate early genes *c-fos* and *c-jun*, proto-oncogenes with a recognized role in apoptosis, is characterized by a fast and transient upregulation after a (patho)physiological stimulus including ischemia.<sup>26-28,59,60</sup> The *c-fos* qPCR findings are in good agreement with immunocytochemical observations on c-Fos protein expression. *c-jun* transcript levels showed a similar trend, although the peak of *c-jun* expression was observed at 6 hours instead of 2 hours. This was in partial accordance with *c-jun* immunodetection in the INL, where the peak was observed at 2 hours. Immunocytochemistry revealed that the trend in the GCL was opposite the trend in the INL. Such a phenomenon of opposite changes within the retina was not detected by qPCR outcome and because the INL trend involved many more cells, this effect dominated the qPCR. These findings again show that qPCR data have to be interpreted with caution and that parallel immunocytochemical studies provide useful additional information.

The second group of genes studied was composed of transcripts with a cell-specific expression. As expected, levels of the ganglion cell-specific transcripts Thy-1 and NF decreased gradually after an ischemic insult, stabilizing at 48 to 72 hours at approximately 50% of the control value.<sup>29-31</sup> This value is in correspondence with that reported by Osborne et al.<sup>1,31</sup> using competitive PCR and also matches the degree of cell loss observed in immunocytochemical staining.<sup>61,62</sup> PV is a cytosolic calcium-binding protein expressed by the AII amacrine cells in the INL of the retina.<sup>33,34</sup> Real-time qPCR showed that the expression of PV decreased progressively after ischemia-reperfusion reaching a loss of approximately 75% in the long-term. This is in line with a gradual ischemia-induced decrease observed in the number of detectable PV-immunopositive cells.<sup>31,63</sup> PKC $\alpha$  was studied as a rod ON bipolar cell-specific

transcript.<sup>35</sup> This cell type is resistant to the effects of an ischemic insult as shown by immunocytochemistry,<sup>4,63</sup> and our results confirmed that ischemia did not induce a significant regulation of PKC $\alpha$  expression and could even be used as one of the reference genes in this paradigm. mGluR6 is a marker for both rod and cone ON-bipolar cells<sup>36</sup> and also demonstrated little variation between the various ischemia-reperfusion groups. In conclusion, qPCR confirmed that ganglion and AII amacrine cells were affected by ischemia, whereas bipolar cells were maintained stable expression levels.

That the expression of both the immediate early genes and various cell-type-specific transcripts aligned so well with immunocytochemical findings from the literature demonstrates that real-time qPCR is a useful method to study alterations in the ischemia-reperfusion paradigm.

### CONCLUSIONS

The main purpose of this study was to investigate whether observed retinal ischemia-induced changes in GluR1, -2/3, and -4 at the protein level are the result of changes at the gene expression level. A gradual decrease in mRNA levels of all subunits was observed, but the nature of these changes at 2 and 6 hours of reperfusion indicates that the observed immunocytochemical changes were not primarily the result of altered gene expression, but rather of processes taking place at the protein level. Furthermore, the relative expression of the splice variants of GluR2 and -4 was not affected after ischemia, and therefore no direct changes were introduced in the functional interaction of GluR2 or -4 with PDZ-containing proteins. Finally, ischemia did not affect the subunit composition of the expressed receptor complex, and alterations in the calcium conductance were not anticipated.<sup>64</sup>

The changes in GluR subunits show that ischemia affects a main component of the excitatory neurotransmission, suggesting an impairment of synaptic communication at the level of the IPL. Whether these changes contribute to the subsequent neurodegeneration remains to be investigated.

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